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Discovery of a Novel Inhibitory Compound Produced by the Soil Bacterium Rhodococcus sp. MTM3W5.2

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Discovery of a Novel Inhibitory Compound Produced by the Soil Bacterium *Rhodococcus sp.* MTM3W5.2

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Introduction

Use of Antibiotics and Development of Antibiotic Resistance

Commercial antibiotic drugs have been an effective tool for physicians in the treatment of bacterial infections for over 75 years. They have decreased the prevalence of human mortality as a result of bacterial infection and have improved the quality of life for many patients with access to these commercial treatments. In 1928 Alexander Fleming discovered penicillin.[1] After his discovery, the introduction of the first commercially available antimicrobial agents—sulfonamides specifically—in 1937 has enabled the treatment of once terminal infections.[1] However, there has been a steady rise in the prevalence of antibiotic resistance in bacteria that are subjected to treatment by these agents, which has begun to complicate the treatment of bacterial infections.[1]

As bacteria evolve alongside the development of new antibiotic drugs, mechanisms of antibiotic resistance have been seen in bacteria both pathogenic and nonpathogenic to humans due to the selective pressure placed on bacteria treated with commercial antibiotics.[2] The selective pressure placed on bacteria is compounded by the overuse and misuse of commercially available antibiotics. Thirty percent to 50% of all cases of antibiotic prescriptions in the United States are incorrect in their treatment indication, choice of agent, or duration of therapy.[3]Also, 30% to 60% of all antibiotics prescribed to patients in American Intensive Care Units have been deemed unnecessary, inappropriate, or suboptimal.[3] It has been observed that the instances of antibiotic resistant bacterial infections increase in conjunction with increases in the number of antibiotics prescribed. This observation has been supported by the disparity in the abundance of antibiotic resistant infections between Northern European countries that prescribe fewer

antibiotics on average to the countries which occupy the Eastern and Southern regions of the continent which prescribe more antibiotics on average.[4]

Additionally, antibiotics are commonly used in livestock production as a preventative therapy and promote the gain of additional mass in livestock animals. In the United States, it is estimated that 80% of the antibiotics purchased are used on livestock.[3] By subjecting the livestock microflora to sustained levels of antibiotic drugs, resistant strains of bacteria are highly selected for. This promotion of resistant strains of bacteria within livestock has implications for people who consume the meat produced under these conditions. The consumer may be exposed to resistant strains of bacteria still present within the meat or to the residual antibiotics which were present in the animal upon processing. The sub-inhibitory levels of antibiotics to which the microflora of consumers are exposed can promote the development of resistance by supporting changes in gene expression, gene alteration, Horizontal Gene Transfer, and mutagenesis.[3]

As antibiotic resistant human pathogens become a more pressing issue, it has become increasingly complicated to treat infections once considered benign. Bacteria are able to survive treatment by antibiotics by employing numerous mechanisms. Bacteria may exhibit lower permeability to antibiotic compounds which can occur as the result of alteration in the number or selectivity of porin proteins within the cell wall. This has implications for a broad range of antibiotic drugs.[5] Increases in efflux pumps within the bacterial cell membrane, including multidrug resistant efflux pumps, allow some bacteria to decrease cellular concentrations of the antibiotic, thereby rendering them ineffective.[5] Some resistant species express alterations in target proteins which prevent the drug from binding.[5] Hydrolysis has proven effective, especially against β-lactam drugs rendering drugs such as penicillin ineffective within the cell.[5] Bacteria also exhibit a wide range of mechanisms that alter the chemical structure of the antibiotic

including acylation, phosphorylation, nucleotidylation, and ribitoylation which prevent the altered compound from binding to the target protein due to steric hindrance.[5] A list of antibiotics, their targets, and the modes of bacterial resistance to the drug is presented in Table 1.

Antibiotic class	Example(s)	Target	Mode(s) of resistance
β-Lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromicin	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C_1 metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C_1 metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target, efflux

Table 1: Commonly used antibiotic drugs, the target of their activity, and mechanisms of resistance which have arisen in bacteria.[1]

These mechanisms enable bacteria to resist a wide variety of antibiotic drugs and in some cases may lead to multi-drug resistant strains which are increasingly difficult to treat. As a result, there has been a significant increase in reported deaths stemming from antibiotic resistant bacterial infections in the United States and throughout the world. In fact, the CDC estimated that in 2013 over 2 million people contracted antibiotic resistant bacterial infections resulting in 23,000 reported deaths.[6] In addition to the direct consequences of antibiotic resistance, the development of pan-resistant bacterial strains may render the ability to perform surgeries, organ transplantations, chemotherapy, and premature infant care impossible without inducing bacterial infections.[7] As the number of antibiotic resistant pathogens rises it is imperative that new methods be developed to control these infections.

Combating Antibiotic Resistance

Aside from advocating for antibiotic stewardship among physicians, many areas of research are concerned with overcoming the mechanisms of antibiotic resistance which have arisen in recent years. Inhibiting the mechanism of resistance within the bacterial cell is one method which has received significant attention and produced some promising results. Preventing the action of bacterial enzymes which inactivate the drugs has led to the discovery of drugs, such as Clavulanic Acid, which inhibit β-lactamase activity and are effectively able to disrupt peptidoglycan synthesis.[8] β-lactamases with active serine residues have also been found to be inhibited by boronic acids.[9] Efflux pump inhibitors have been studied as a potential means for overcoming the ability of bacteria to drive out the intracellular antibiotics. They function by preventing the bacterial efflux pumps from functioning, thereby preventing them from decreasing cellular antibiotic levels.[10]

Bacteriophages offer another promising method for treating antibiotic resistant infections. The viral parasites which are used in phage therapy are highly specific, attacking only the target virulent bacterial species involved in the infection.[7] This decreases the likelihood of secondary infections and protects the patient's commensal flora unlike traditional antibiotics. Bacteriophages also replicate at the site of the infection, thus increasing their concentration in the areas they are needed unlike traditional antibiotics. However, the specificity of these bacteriophages makes them extremely narrow-spectrum, so this creates a need for definitive identification of the bacteria involved in the infection prior to the selection of a treatment. Making this identification has become increasingly accurate and expedient with the development of more efficient bedside molecular diagnostics; however, there are currently few countries engaging in the use of bacteriophages as a treatment for bacterial infections.[7,11]

Enzymatic inhibition of bacterial growth is also an area of research which may help in addressing antibiotic resistance. Some success has been achieved in isolating and designing enzymes which have the ability to disrupt the normal functions of the bacterial cell. These enzymes are bactericidal and function by using catalytic mechanisms to degrade bacterial cell walls. Initial studies focused on endolysins contained within bacterial genomes and function in processes such as cell division in their wild type configuration.[12] By engineering these endolysins to attack the bacteria from outside the cell, the potential for resistance is substantially decreased.[12] Recently there has been some interest in using modified human lysozyme to evade the lysozyme specific inhibitory proteins of bacterial cells, enabling them to lyse the bacteria under conditions which the natural human lysozyme is ineffective.[13] These enzymatic mechanisms for addressing antibiotic resistance are promising; however, clinically viable models have not currently been achieved.

The primary area of new antibiotic drug discovery involves synthetic tailoring of known chemical scaffolds to overcome mechanisms of resistance. Most of the clinically used antibiotics come from a relatively small set of chemical structures which have been extended by modification of their structures to improve their effectiveness.[14] The altered chemical structures can improve the ability of a drug to avoid bacterial defenses such as catalytic enzymes or efflux pumps by exploiting the substrate specificity of these defense mechanisms.[14] Because many of the chemical libraries which are popular among pharmaceutical companies are populated with compounds which are favored for human bioactivity, production of completely synthetic chemical entities which display inhibitory activity against bacterial cells is difficult due to the disparities between bacterial cells and eukaryotic cells.[11]

Though synthetic tailoring has produced generations of antibiotic drugs which are effective against resistant bacterial strains by enabling them to avoid bacterial defenses, it is often a short-term solution. These new generations of semi-synthetic antibiotics retain the same core chemical scaffold and inhibit bacterial growth via the same mechanism as the original drug.[14] The activity of these drugs is often not sustainable as bacteria develop mechanisms to overcome the synthetic alterations to the drug which are meant to restore the drug's activity.[14] Discovering new chemical scaffolds would perhaps be a more sustainable method for overcoming resistance in pathogen bacteria because it would increase the diversity of mechanisms of inhibition, and it would provide a broader range of chemical scaffolds which could be synthetically tailored.[14]

Natural Products

One promising avenue for the discovery of novel chemical scaffolds is by isolating the natural organic products of living organisms. Natural products and their semi-synthetic derivatives account for over two-thirds of all clinically-approved antibiotic drugs.[14] The term natural product is used broadly to refer to all organic products produced by a living organism to ensure its survival and increase its fitness. The organic products which are produced by living organisms are divided into three broad yet distinct groups. Products which play a central role in the metabolism and reproduction of the cell such as nucleic acids, amino acids, and sugars are primary metabolites.[15] High-molecular-weight polymeric compounds such as lignins, cellulose, and proteins, are responsible for the formation of the cellular structure.[15]

Secondary metabolites have biological effects on other cells or even other organisms.[16] These metabolites may function in regulation, signaling, and defense against other organisms.[15] The ability of an organism to produce secondary metabolites is an adaptive characteristic which

confer a biological advantage upon the organism, increasing its survival and ability to reproduce. Secondary metabolites fall into several categories including terpenoids and steroids, phenylpropanoids, alkaloids, specialized amino acids and peptides, specialized carbohydrates, and polyketides.[15] They are differentiated from primary metabolites based on their functionality rather than structure.[17] Due to the bioactivity of secondary metabolites on other organisms, they have been an area of intense interest. In fact, it is estimated that over 40% of all medicines originated from secondary metabolites.[15] Some bacterial secondary metabolites have the key function of inhibiting the growth of other bacterial species making them prime candidates for the discovery of novel antibiotic drugs.[15] It has been estimated that over 45% of medically important antibiotics have been obtained from the order *Actinomycetales*.[18]

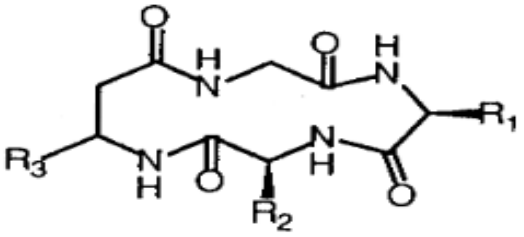
The Genus *Rhodococcus*

Rhodococcus is a genus of *Actinomycetes* which are aerobic, Gram-positive, GC-rich, nonsporulating, and non-motile bacteria which contain mycolic acids in their cellular envelope.[18] Rhodococci have chemotype IV cell walls which are composed of *meso*-diaminopimelic acid containing peptidoglycan along with arabinose and galactose as the major sugars.[17] Their cell morphology fluctuates throughout their growth cycle from the *cocci* configuration to short rods in some species and filamentous rods in other species.[19] Some rods may even begin branching out in a simple or complex manner in some *Rhodococcus* species.[19] *Rhodococcus* species can be found in soil, rocks, boreholes, groundwater, animal dung, insect guts, and in healthy or diseased plants and animals.[20]

Rhodococcus has proven useful in industry for its ability to degrade organic compounds, an ability which may be due in part to their mycolic acid containing cell walls which are proposed to allow the uptake of hydrophobic compounds.[17] Genomic sequencing of a strain of

Rhodococcus RHA1 by McLeod *et al*. found that it contained protein-coding genes which were responsible for the production of 203 oxygenases and 192 ligases. Many of these are involved in pathways predicted to degrade aromatic compounds or steroids.[21] This may contribute to the ability of RHA1 to degrade polychlorinated biphenyls (PCBs).[21] Using a wide variety of enzymes, Rhodococci, have been successfully used in industry to degrade compounds by dehydrogenation, desulfurization, dehalogenation, epoxidation, hydrolysis, hydroxylation, and oxidation.[18] The robust stress tolerance and rapid growth rates have also influenced the utility of Rhodococci in industry with applications in bioremediation and biocatalysis.[17]

The genus *Rhodococcus* is also notable for its ability to produce secondary metabolites, some of which have antimicrobial properties. The first antibiotic like compound discovered within the genus was found to inhibit the growth of fungal species *Candida albicans* and *Cryptococcus neoforms,* though it did not show antibacterial activity.[22] Chiba *et al.* discovered five cyclic tetrapeptides produced by *Rhodococcus* sp. Mer-N1033—which were isolated from soil samples collected at Mt. Hayachine, Iwate Prefecture, Japan—and named these compounds rhodopeptin C1, C2, C3, C4, and B5. The chemical structure of these compounds can be seen in Figure 1. The cyclic tetrapeptide rhodopeptins were found to be composed of three α-amino acids and one lithophilic β-amino acid.[22] They were found to be soluble in methanol, dimethyl sulfoxide, acetic acid, and water and they were insoluble in chloroform and ethyl acetate.[22]



	R_1	R_2	R_3
Rhodopeptin C1	$-(CH_2)_3NH_2$	$-CH(CH_3)-CH_3$	$-(CH_2)_6CH(CH_3)-CH_2CH_3$
Rhodopeptin C2	$-(CH_2)_3NH_2$	$-CH(CH_3)-CH_2CH_3$	$-(CH_2)_6CH(CH_3)-CH_2CH_3$
Rhodopeptin C3	$-(CH_2)_3NH_2$	$-CH(CH_3)-CH_3$	$-(CH_2)_8CH(CH_3)-CH_3$
Rhodopeptin C4	$-(CH_2)_3NH_2$	$-CH(CH_3)-CH_3$	$-(CH_2)_8CH(CH_3)-CH_2CH_3$
Rhodopeptin B5	$-(CH_2)_4NH_2$	$-CH(CH_3)-CH_3$	$-(CH_2)_9CH(CH_3)-CH_3$

Figure 1: Core Rhodopeptin Structure along with R groups which are characteristic of Rhodopeptins C1, C2, C3, C4, and B5.[22]

Another set of antimicrobial agents isolated from *Rhodococcus* were termed lariatins A and B. These compounds discovered by Iwatsuki *et al.* in 2007 and were isolated from *Rhodococcus* sp K01-B0171 which was collected in Yunnan, China. They were found to be cyclic peptides which consisted of 18 to 20 amino acid residues.[23] These residues exhibited an internal linkage between the γ-carboxyl group of the Glu8 residue and the α-amino group of the Gly1 residue.[23] The tail region of these cyclic peptides were found to pass through the ring region forming a "lasso" structure.[23] The "lasso" structures of Lariatins A and B are show in Figure 2. Lariatins A and B were found to have inhibitory activity against *Mycobacterium smegmatis* while lariatin A also exhibited activity against *Mycobacterium tuberculosis.*[23] The lariatin compounds were both found to be pale yellow solids at room temperature.[23] The molecular weight of lariatin A was found to be 2050, and the molecular weight of lariatin B was

found to be 2204.[23] These compounds were found to be soluble in water, methanol, and DMSO and insoluble in ethyl acetate and chloroform.[23]

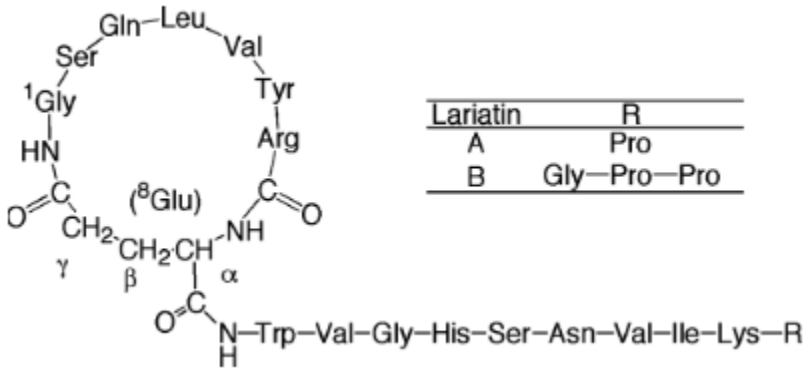


Figure 2: "Lasso" structure of lariatins A and B.[23]

Research by Kitagawa *et al.* focused on one strain of *Rhodococcus erythropolis*, identified by a larger study, which had been found to produce an inhibitory compound with a broad spectrum of activity against Gram-positive bacterial species and was able to elucidate the structure of the compound.[24] It was found that the strain, *Rhodococcus erythropolis* JCM 6824, had a quinoline structure and a molecular weight of 395.[24] The compound was termed aurachin RE.[24] This compound was found to be highly similar to aurachin C, an antibiotic isolated from *Stigmotella aurantiaca*, a Gram-negative myxobacterium.[24] The sole difference between the two compounds is the presence of a hydroxyl group on the hydrocarbon chain at the C-9' position in aurachin RE rather than a hydrogen in aurachin C. Aurachin RE exhibited considerably stronger inhibitory activity than aurachin C which may be a result of the increase in solubility in the aqueous phase granted by the hydroxyl group.[24] The hydroxyl group may also increase the ability of the compound to permeate the cell walls of Gram-positive bacteria.[24] Aurachin RE was found to be a gray-brown powder which is soluble in ethanol, methanol, methyl cyanide, DMSO, ethyl acetate and moderately soluble in water.[24]

Antibiotic compounds have also been isolated from *Rhodococcus* by employing horizontal gene transfer with other bacterial species. Kurosawa *et al*. subjected a multi-drug resistant strain of *Rhodococcus fascians* that does not produce an antibiotic compound and a susceptible strain of *Streptomyces padanus* which was a stable producer of the antibiotic actinomycin to competitive co-culturing techniques.[25] A strain of *Rhodococcus,* termed 307CO, emerged from the culturing techniques and was found to produce two antibiotic compounds.[25] This strain of *Rhodococcus* was found to harbor a large segment of DNA derived from the *Streptomyces* strain indicating the occurrence of horizontal gene transfer.[25] It was found that the production of the antibiotic compounds in *Rhodococcus* 307CO correlated with the presence of *Streptomyces* DNA within its genome.[25] The two antibiotic compounds which were isolated from *Rhodococcus* 307CO were named rhodostreptomycin A and B and were found to be two isomers of a new class of aminoglycosides.[25] Rhodostreptomycins A and B were found to have a broad spectrum of activity against both Gram-negative and Gram-positive bacterial species, though B was found to be slightly more potent than A. This suggests that the stereochemistry of the compound influences biological activity.[25] The compounds were described as optically active white powders which were highly soluble in water but not chloroform or *n*-hexane.[25]

Rhodococcus sp. MTM3W5.2

A strain of *Rhodococcus,* which was isolated from soil samples collected in Morristown, Tennessee in 2011, was found to have good antimicrobial activity against *Rhodococcus erythropolis* and other *Rhodococcus* and closely related species.[19] The strain which produces this inhibitory compound was found to be similar to *Rhodococcus jostii* and most closely related to *Rhodococcus opacus* with 90% similarity; it was termed MTM3W5.2.[26] The colonies of the strain were found to grow flat on the agar surface and were initially white, but obtained a tan

pigmentation as the culture aged.[26] The initial white coloration and growth characteristics of the MTM3W5.2 colonies are depicted in Figure 3. The compound was initially produced at a temperature of 15°C but it was later found that the compound was also produced at temperatures of approximately 20°C.[19] The gene cluster responsible for production of the inhibitory molecule was found to be similar to polyketide synthase gene clusters from *Streptomyces* and other *Rhodococcus* species.[26]

Figure 3: Appearance of *Rhodococcus* sp. MTM3W5.2 colonies on an agar surface. Colonies are flat and spread over the agar. The colonies are initially white but turn tan in color with increasing age.[19]

Polyketides

Polyketides are a subgroup of natural products which have a diverse range of structural and functional diversity.[27] They have been observed to have a broad range of biological activities including antibacterial, antifungal, anticholesterol, antiparasitic, anticancer, and immunosuppressive properties.[28] This structural and functional diversity is a product of combinatorial utilization and template-directed elongation of a few simple building blocks in a process similar to the synthesis of fatty acids.[27] Synthesis of polyketides is conducted by

numerous Gram-positive bacteria which reside in soil such as species belonging to the order *Actinomycetales* including members of the genus *Streptomyces* and the genus *Rhodococcus*.[28] Some polyketide compounds have successfully been implemented as commercial antibiotic drugs including erythromycin and tetracycline which are broad spectrum drugs that inhibit bacterial protein synthesis.[27]

Polyketide Synthases

The vast structural diversity of polyketide compounds arises from the coordinated, multistep action of enzymes organized in an assembly line like manner.[28] Polyketide synthesis takes place through the addition of specific acyl originators, typically acetyl-CoA, malonyl-CoA or their derivatives, to a growing polyketide intermediate through decarboxylative Claisen condensation of thioester-activated acyl groups. After this process, the intermediate may undergo additional modification.[28,29] Enzymes responsible for the construction of polyketide compounds are termed polyketide synthases (PKSs). These PKSs are mega enzyme-complexes which operate through the action of multiple functional subunits. These subunits, called modules, are composed of several domains, each of which has a specific enzymatic function which are arranged in a manner analogous to that of fatty acid synthases.[29] There is a large assortment of domains which are involved in the synthesis of most polyketides. The domains which are obligatory for the synthesis of all polyketides are the acyltransferases (AT). AT selects the extender unit from a pool of CoA products and loads them onto an acyl-carrier protein (ACP) domain.[29] A decarboxylative Claisen condensation reaction with the upstream intermediate is conducted by ketosynthase (KS). The acyl-carrier protein (ACP) acts as a carrier for the intermediates which are tethered to the synthase complex by a thioester linkage to the phosphopantetheine cofactor of the acyl-carrier protein.[29] An example of the synthetic order of a

polyketide synthase is depicted in Figure 4. The compound may then undergo up to three reductive reactions which are carried out by the reductive domains ketoreductase (KR), dehydratase (DH), and enoyl-reductase (ER), yielding different degrees of reductions which may result in β-keto, β-hydroxy, α,β-alkene, or fully reduced intermediates.[29] Upon completion of the catalytic activity of one module, the ACP region transfers the intermediate to the next module which will then add to the growing polyketide.[29] Finally, the thioesterase (TE) domain allows the compound to dissociate from the ACP region of the final module, releasing the polyketide compound.[29] Typically the number of precursors which are incorporated into the finished polyketide compound matches the number of modules which are required for its synthesis.[27]

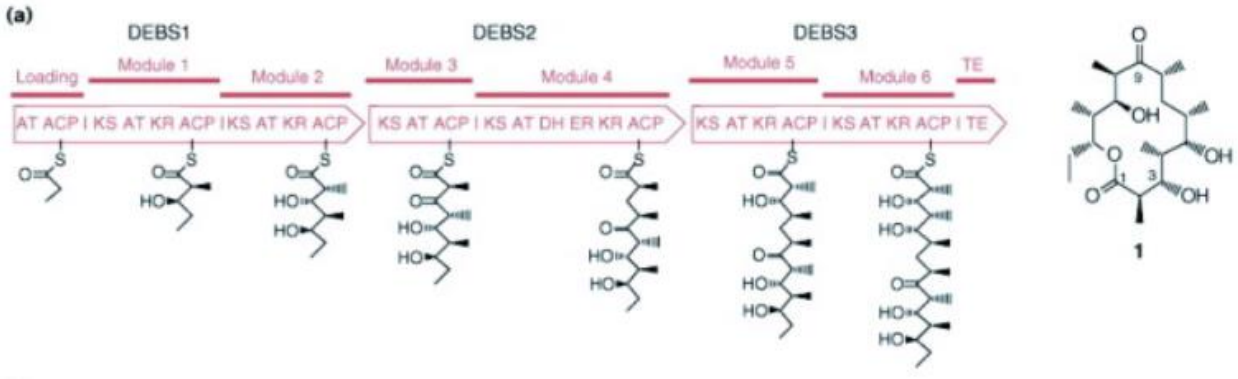


Figure 4: Arrangement of the polyketide synthase complex partially responsible for the synthesis of 6-deoxyerythronide B (DEBS) depicting the arrangement of domains within each synthetic module.[30]

Structural complexity is introduced into polyketide compounds as a result of the variation in stereochemistry and degree of reduction which takes place after each condensation reaction.[31] These factors are augmented by the ability of PKSs to conduct further downstream modification to the compound by enzymatically introducing cyclizations, oxidations, alkylations, glycosylations, methyl-transfer, halogenation, and β-branching.[29,31] Additionally, many polyketide synthase gene clusters encode for enzymes which tailor the released polyketide into

the final biologically active natural product by employing reactions such as methylation, epoxidation, and hydroxylation.[29]

Polyketide synthases resemble fatty acid synthases in form and function. In fact, the type I PKS and metazoan type I fatty acid synthase (FAS-I) have been found to have a common evolutionary ancestor.[29] They have homologous catalytic and ACP domains and both megasynthases have a common domain order.[29] The striking similarities between the KS-AT di-domains of PKS and KS-AT domain of FAS-I are perhaps the most substantial evidence for the commonality of architecture between the two megasynthases.[29] While PKS and FAS often have the same precursor molecules which include acetate derivatives, the overall addition of the precursors to the growing intermediates takes place differently.[15] In fatty acid synthesis, the carbonyl group of the acetate precursor unit is reduced during the chain assembly; whereas, the addition of precursor units in polyketide synthesis occurs through a mechanism of decarboxylative Claisen condensation followed by differing degrees of reduction of the keto groups of the resultant intermediate.[15,29] Biosynthesis of fatty acids and polyketides also differ in the overall number of precursor molecules incorporated into the growing chain and cyclization of the final products.[29]

PKSs are arranged into three broad categories which are derived from the canonical differentiations between fatty acid synthases. Type I PKSs are multifunctional enzymes which are organized into modules and minimally contain an acyltransferase, ketosynthase, and an acyl-carrier protein domain.[32] Type I PKSs are further differentiated into modular type I PKSs, which are the classical bacterial type I PKS. It has modules that act non-iteratively and are responsible for the catalysis of one cycle of chain elongation.[28] Iterative type I PKSs take advantage of the same catalytic domains, but all domains occur on a single polypeptide which is repetitively used

to generate the entire backbone of the polyketide compound.[28] Type II PKSs are multifunctional enzyme complexes comprised of multiple enzymes which carry at minimum a single set of iteratively acting enzymes.[33] Type II PKSs consist minimally of the core enzymes acyltransferase, acyl-carrier protein, and, contrary to type I PKSs, two ketosynthase regions.[33] This extra KS region denoted KS_{β} has been shown to lack an active cysteine; however, despite the lack of a cysteine active site, they have been shown to be involved. This suggests that they are not catalytically silent.[33] Type III PKSs are often referred to as chalcone synthase-like PKSs.[32] They are homodimeric enzymes that are iteratively acting condensing enzymes.[32] Unlike type I and II PKSs, they act independently of acyl-carrier protein and instead act directly upon acyl-CoA precursors.[32]

Current Work

The aim of this study is to identify the polyketide antibiotic compound produced by *Rhodococcus* sp. MTM3W5.2. This study details an efficient method for extracting the active metabolite from large batch broth cultures of the producer strain as well as an effective means for purifying the active metabolite using a combination of size exclusion chromatography and reversed-phase high-performance liquid chromatography (RP-HPLC). A description of the spectroscopic methods used to obtain data regarding the structural elucidation of the compound of interest is also included. The objectives of the project are to extract the active metabolite using the solvent extraction method set forth by Pushpavathi Manikindi, pre-fractionate the crude extract using size exclusion chromatography, isolate the inhibitory compound using semi-preparative RP-HPLC, purify the inhibitory compound by employing an analytical HPLC column and appropriate HPLC purification method, analyze the compound using high-resolution

mass spectrometry, analyze the UV-Vis spectra to yield information regarding the conjugation of the compound.[17]

Materials and Methods

Reagents

The reagents which were used throughout this study during the purification and characterization of the inhibitory compound are discussed below.

HPLC Solvents

The purification and analysis of the inhibitory compound required the use of two different solvents which were used as the mobile phase. Throughout the study solvent A was consistently chosen to be deionized water which was obtained from an Elga PURELAB UHQ water purification system. Solvent B was selected to be HPLC-Grade acetonitrile (CH_3CN) during most of the HPLC purification methods; however, HPLC-Grade methanol (CH_3OH) was also used during some HPLC separation methods. To dissolve the crude *Rhodococcus* sp. MTM3W5.2, it was extracted prior to conducting HPLC purification and diluted before it was injected. HPLC-Grade 2-propanol, dimethyl sulfoxide (DMSO), chloroform, HPLC-Grade methanol, and ethanol were also used.

HPLC Buffers

During the study two different buffers were used to improve the separation of the active compound during RP-HPLC purification. Ammonium formate ($HCOONH_4$) was used at a concentration of 20mM, and ammonium hydroxide was used in concentrations of 0.01% and 0.005%. 20mM ammonium formate was prepared by adding 0.63g of ammonium formate to 5mL of deionized water and adding 495mL of deionized water for a total volume of 500mL. 0.01% ammonium hydroxide solution was prepared by diluting 200µL of 50% ammonium hydroxide stock solution with 1L of deionized water.

Other Solvents and Reagents

During the liquid-liquid extraction of the *Rhodococcus* sp. MTM3W5.2 broth cultures, *n*-butanol was used to obtain the inhibitory compound from the culture. HPLC-Grade methanol was used as the mobile phase during size exclusion chromatography. Dimethyl sulfoxide (DMSO), 2-propanol, ethanol, and chloroform were used to dissolve the inhibitory compound prior to HPLC analysis.

Bacterial Strains and Growth Media

Bacterial Strains

The strain of *Rhodococcus* sp. MTM3W5.2, which was used throughout this study, was supplied by Dr. Bert Lampson of the East Tennessee State University Department of Health Sciences. It is a wild-type soil bacterium that produces an antimicrobial compound. It was isolated from a soil sample taken in Morristown, Tennessee in 2011.[19] The other strain of bacteria used in this study *Rhodococcus erythropolis* IGTS8 is sensitive to the antimicrobial compound of interest and was used as an indicator of antimicrobial activity during the purification of the compound.

Bacterial Growth Media

Rich media (RM) was employed during culturing of the MTM3W5.2 bacterial strain. Both RM broth and agar plates were used during the study. The RM broth was prepared by adding 5g of glucose, 4g of nutrient broth, and 0.25g of yeast extract to 500mL of water. The contents were then stirred and autoclaved at 121°C for 20 minutes. The agar plates were prepared using the same recipe and adding 7.5g of Bacto™ agar. The RM agar was autoclaved at 121°C for 20 minutes and cooled in a 55°C hot water bath before they were poured. Once the agar had

cooled, it was poured into 100mm x 15mm polystyrene Fisherbrand Petri dishes. They were allowed to cool and solidify overnight on a bench top. Afterwards, they were stored at 4°C for future use.[26]

Mueller-Hinton agar plates (MH) were used during zone inhibition assay to assess the activity of the crude MTM3W5.2 extract as well as the fractions taken during Sephadex LH-20 column chromatography and HPLC. MH agar plates were created by dissolving 19.5 g of Difco™ Mueller-Hinton agar in 500 mL of distilled water while applying heat.[26] The solution was then autoclaved at 121°C for 20 minutes. The medium was then cooled in a 55°C water bath after which time it was poured into sterile 100mm x 15mm polystyrene Fisherbrand Petri dishes. The MH agar plates were allowed to cool and solidify on a benchtop overnight. The solidified MH agar plates were then stored at 4°C for future use.[26]

Extraction Methods

A method for culturing the *Rhodococcus* sp. MTM3W5.2 bacterial strain to ensure that sufficient production of the secondary metabolite of interest was adopted from Manikindi et al.[17] Since the production of secondary metabolites varies widely based on the type of medium used and the specific microorganism that is cultured, it was highly important to employ a culturing method which would ensure that significant production of the active metabolite took place. The solvent which was chosen for the solvent extraction method was vital in obtaining a sufficient amount of the inhibitory compound to allow further purification and analysis to be conducted. The solvent used was chosen based on polarity and was adopted from an extraction method described by Pushpavathi Manikindi.[17]

RM Broth Culture Extraction of MTM3W5.2 Metabolites

The active metabolite of interest has been shown to be produced by *Rhodococcus* sp. MTM3W5.2 under stagnant growth conditions based on work by A. Ward.[26] To produce a sufficient amount of the active metabolite for further purification and analysis, 2000 mL of RM broth was prepared. 500 mL portions of the RM broth were added to four 2 L Erlenmeyer flasks, shown in Figure 5. A seed culture of the MTM3W5.2 bacteria was created by adding 10 mL of RM broth to a test tube and inoculating it with the bacteria. The seed culture was incubated at 27°C for 48 hours under constant shaking. After incubation, 2.5 mL portions of the seed culture were added to each of the flasks. The flasks were then incubated under stagnant conditions for 2 weeks at 20°C. After growth, the bacterial cultures were added to centrifuge bottles and subjected to centrifugation at 6000 rpm for 10 minutes. The supernatant was added back to the flasks and the cells were disposed. 100 mL of *n*-butanol was added to the supernatant within each of the flasks. The flasks were then shaken in a floor incubator for 30 minutes at 100 rpm. After shaking, the cultures were added to separatory funnels and allowed to settle. The bottom aqueous layer was added back to the flasks and the top organic layer which contained the *n*-butanol was retained. 50 mL portions of *n*-butanol were then added to each of the flasks containing the aqueous layer and the flasks were shaken for an additional 30 minutes. The solution was again added to the separatory funnel, and the organic layer was retained. The aqueous layer was disposed of. The *n*-butanol extract was then evaporated under a fume hood resulting in a dried crude extract recovered from the broth cultures.

Figure 5: 2 liter Erlenmeyer flasks containing 500 mL of RM broth used in the growth of *Rhodococcus* MTM3W5.2 cultures.

Zone Inhibition Antimicrobial Activity Assay

To determine whether the compound of interest had been isolated during the *n*-butanol extraction method and to determine which fractions contained active metabolites during Sephadex LH-20 column chromatography and HPLC, a zone inhibition assay was used. This test is conducted by soaking a paper disk with the antimicrobial compound and placing it on an agar plate which has been inoculated with a sensitive indicator bacterium. If the compound placed on the paper disk was inhibitory, it would prevent the growth of the indicator bacteria in the vicinity of the disk creating a zone of inhibition.

Initially, a seed culture of the sensitive indicator bacteria was created by adding 2 mL of RM broth to a 10 mL test tube. A well-formed and isolated colony of the indicator bacterium was selected from an agar plate of *Rhodococcus erythropolis* IGTS8 to ensure that no unwanted bacteria species contaminated the seed culture. The colony which was selected was used to inoculate the RM broth in the test tube by transferring the cells using a sterile loop. The seed culture was placed in a shaking water bath and incubated at 27°C for 18 hours. After suitable

turbidity was obtained, the seed culture was ready to inoculate the MH plates used in zone inhibition assay.

The MH agar plates were then inoculated with the indicator seed. This was accomplished by plunging a sterile cotton swab into the turbid *R. erythropolis* seed culture and rotating the swab several times while pressing on the inside wall of the test tube above the liquid level during removal of the swab to remove superfluous liquid. The swab was then swiped over the entire surface of the MH agar plate to create a sufficient bacterial lawn.

The compound was then added to the sterile paper disks prepared from Whatman blotting paper GB004 using a hole puncher. The newly punched paper disks were then autoclaved at 121°C for 20 minutes to ensure sterilization. The paper disks were labeled using a pencil. After labeling, the disks were soaked with 25 µL of the crude extract of chromatographic fraction. The disks were allowed to completely absorb the liquid before adding an additional 25 µL of the compound to each disk. The total volume of compound added to each disk was 50 µL for the crude *n*-butanol extract and the Sephadex LH-20 fractions as well as most HPLC fractions. When the volume of HPLC fractions were low, indicating a higher concentration of compound within the fraction, a total volume of 25 µL was added to the paper disks to ensure the resolution of the zones of inhibition was sufficient to determine which fractions contained the active compound.

The dried paper disks were then added to the MH agar plate which was inoculated with the indicator bacteria. The disks were placed a sufficient distance apart to ensure that clear zones of inhibition could develop and were pressed down to ensure complete contact with the agar surface. After adding the disks, the plate was left to grow at room temperature for approximately 48 hours before examining each plate for activity. The final plate showed a consistent bacterial

lawn of the indicator bacteria over the agar surface without the presence of individual colonies. If individual colonies were observed, the inoculum was not sufficiently concentrated, and the test was repeated. Zones of inhibition were noted by consistent circular areas surrounding a paper disk in which no indicator bacteria grew.

Chromatographic Methods

This study involved multiple chromatographic steps to ensure that a highly pure sample of the inhibitory compound was obtained upon which spectral analysis could be conducted. The chromatographic methods are outlined below from preliminary purification of the crude extract to final HPLC purification.

Size Exclusion Chromatography (SEC)

To ensure good separation of the compounds within the crude butanol extract, size exclusion chromatography was employed. This type of chromatography takes advantage of the size differences among molecules. By packing a chromatography column with the small, porous polymer beads of the stationary phase, the molecules in the crude extract are separated based on their ability to fit within the pores in the polymer beads.[34] Large molecules are unable to enter into the pores of the medium and must pass directly through the interparticle volume of the medium causing it to elute more quickly; however, small molecules are able to enter into the pores of the medium and become trapped causing them to elute slower. This provided separation between compounds found within the sample.[34] Sephadex™ LH-20 was selected as the chromatographic medium for this study based on its unique physico-chemical properties. Sephadex™ LH-20 resin is designed for use with polar organic solvents and aqueous solvent mixtures, making it useful for the isolation of the inhibitory compound of interest which has been

found to be soluble in the organic mobile phase used in SEC. The LH-20 resin is both hydrophilic and lipophilic causing it to swell in organic solvents. It is created by hydroxypropylation of a cross-linked dextran, Sephadex™ G-25.[34] This cross-linking yields a polysaccharide network with pores with an average size of 103 µm in methanol.[34] This allows for high resolution separation of the compounds in the crude extract and allows for good recovery of the compound of interest. Size exclusion chromatography relies on an isocratic elution method using a single constant mobile phase to elute the compounds within the crude extract. The size exclusion chromatography method used during this study was termed Sephadex™ LH-20 chromatography based on the medium used.

Procedure:

28.78 g of Sephadex™ LH-20 dry powder was added to a 250 mL beaker and swollen in excess methanol for 2 hours. The beaker was stirred at 30-minute intervals to release any air bubbles which may have become trapped in the medium. The swollen slurry was added to a chromatographic column through a glass rod to ensure the column was filled evenly and no air bubbles were formed. After adding the medium to the column, the solvent reservoir was attached, and the methanol solvent was allowed to flow through the column with atmospheric pressure. After ensuring that the column was tightly packed with Sephadex™ resin and devoid of air bubbles, the stopcock was closed. The dried *n*-butanol crude extract was then reconstituted by adding 10 mL of isopropanol to the beakers containing the dried crude extract and shaking them for 4 hours at 100 rpm in a floor incubator. The extract was then added to the Sephadex column and eluted using methanol as the mobile phase. Pressure was applied to the column until a flow rate of approximately 2.5 mL/min was reached to ensure uniform elution of the active compound was achieved. The eluate was collected in 15 fractions of 50 mL, each of which were tested for

antimicrobial activity using zone inhibition assay. Fractions which demonstrated antimicrobial activity were pooled and dried under a fume hood.

High-Performance Liquid Chromatography

HPLC is an effective means for purification of natural products and yields good separation between eluted compounds. HPLC employs the use of high solvent pressures to force the mobile phase and the sample through a column with small stationary phase particle sizes in a short, narrow column.[35] By decreasing the particle size of the stationary phase the surface area of stationary phase is increased allowing more association between constituents of the sample with the stationary phase media which leads to more effective separation with a higher resolution than other liquid chromatographic techniques.[35] The HPLC instrument, which was employed for the purification of the antimicrobial compound, was a Shimadzu LC-10AS instrument. The instrument was equipped with a two-solvent system allowing for the use of a gradient method of sample elution. The solvents were labeled Solvent A and Solvent B and were pumped into the column using separate solvent pumps A and B. The pumps were used to generate a maximum pressure of 5000 psi enabling the solvent and sample to move through the column. The range of solvent pressure which was used during the experiment was between 1200-4500 psi. The instrument was equipped with a SPD-10A UV-Vis detector which was set to 254 nm and allowed for the detection of organic analytes. The instrument was controlled through a SCL-10AVP system controller to manage the time programs used throughout the study. Temperature was maintained at 25°C during all HPLC purification methods.

It was necessary for the HPLC-Grade solvents used throughout this study to undergo vacuum degassing to remove excess air within them. The formation of air bubbles during HPLC purification can cause problems with solvent delivery and analyte detection. Though some more

modern HPLC instruments are equipped with internal solvent degassers, it was necessary to undertake vacuum degassing of the solvents used in this study manually. This was accomplished by placing the solvents in a HPLC solvent container, adding a stir bar, and applying a vacuum to the container. The solvents were agitated under vacuum until all excess air had been removed.

Reversed-phase high-performance liquid chromatography was used to effectively isolate the antimicrobial compound of interest. This method causes polar compounds to elute from the sample first while non-polar compounds are retained longer and elute later. To attain this type of separation the stationary phase of the HPLC column is typically a non-polar hydrophobic medium which will form associations with non-polar compounds found within the sample.[36] The solvents which are used as the mobile phase for reversed-phase high-performance liquid chromatography are usually polar. This means that water is typically the "weakest" solvent because it repels highly hydrophobic compounds into the stationary phase causing longer retention times.[36] A polar organic modifier is usually selected which is less polar than water, thereby making it a "stronger" solvent since it will no longer strongly repel the analyte into the stationary phase allowing it to move through the column more easily.[36]

To obtain greater separation of the constituents of the samples subjected to HPLC, a gradient method was used. This method utilizes the independent solvent pumps of the HPLC system to alter the ratio of each solvent in the mobile phase over time. By altering the mobile phase composition throughout the time program better separation of the constituents of the sample can be obtained, the time of separation can be significantly decreased, and column contamination can be eliminated. This method also ensures that all components of the sample are eluted decreasing sample loss.

The columns selected for this study were chosen based on their favorable properties which increased separation of the sample and aided in increasing the efficiency of the purification method. A semi-preparative column was used for initial purification of the active fractions eluted through the Sephadex™ LH-20 column. A Hamilton polymeric reversed phase-1 column was selected for the semi-preparative step. This column was packed with poly(styrene divinylbenzene) with particle sizes between 12 μm and 20 μm and a pore size of 100 Å. The inner diameter of the column was 21.2 mm and a length of 250 mm. Due to the larger particle size, the number of theoretical plates of the column decreases thus allowing for an increased mobile phase flow rate and a decreased solvent pressure. A Kinetex® phenyl-hexyl 100 Å analytical column was used for additional preparation prior to final HPLC purification. This column was packed with hexyl linked phenyl with trimethylsilyl endcapping with a particle size of 5 μm and a pore size of 100 Å. The column length was 250 mm and the internal diameter was 4.6 mm. For the final purification of the antimicrobial compound, multiple rounds of HPLC were conducted using a Kinetex® 5 μm EVO C18 100 Å analytical column with a particle size of 5 μm and a pore size of 100 Å. The internal diameter of this column was 4.6 mm and the length of it was 150 mm. Both of the Kinetex® analytical columns utilized core-shell technology in which a homogenous porous shell of stationary phase substrate envelops a non-porous compact silica core. Because the core is non-porous, less band broadening is observed leading to greater resolution between eluted compound peaks. Throughout the study a guard column obtained through Phenomenex (Torrance, CA) was utilized to remove dust and contaminants found within the solvent. This prevented damage to the HPLC instrument, eliminated the appearance of “ghost peaks”, and increased the purity of the antimicrobial compound.

The solvents used as the HPLC mobile phase throughout this study were selected based on their suitability for reversed-phase high-performance liquid chromatography. The ideal solvents were water-miscible, low viscosity, chemically unreacted, demonstrated low UV absorbance, and good solubility properties. Based on polarity and solvent strength acetonitrile was chosen to be solvent B throughout most of the study while water was used as solvent A. Solvent buffers were also used occasionally throughout the study. 20 μM Ammonium formate was added to water during initial method development. Ammonium hydroxide was also used as a buffer in both water and acetonitrile at various times during HPLC purification to improve separation of sample constituents. Since the development of the HPLC method was partially based on a trial and error approach, different combinations of solvents and buffers were used until the desired purity was obtained.

Semi-Preparative HPLC Elution Method

The extract obtained through Sephadex™ LH-20 column chromatography was subjected to initial HPLC purification through the Hamilton PRP-1 column. The solvents used were acetonitrile and water with no buffers added. Prior to injecting the sample, the column was equilibrated for 45 minutes with 80% solvent A (water) and 20% solvent B (acetonitrile) and a solvent flow rate of 4.00 mL/min. After the column had been equilibrated 1 mL of the sample was injected into the column. The flow rate was maintained at 4.00 mL/min throughout the time program. The initial solvent ratio of 80:20 was maintained for 3.20 minutes after which time the concentration of solvent B was gradually increased to 100% over the course of 60.20 minutes. The 100% concentration of solvent B was then maintained from 63.40 minutes until 93.40 minutes at which time the time program was ended, and the column was washed with 100% acetonitrile for 30 minutes. All collected peaks were tested for activity and active peaks were

combined. The time program for the gradient elution method used during the semi-preparative stage is shown in Table 1.

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	

Table 1: HPLC solvent time program for semi-preparative and preparative HPLC purification steps.

Preparative HPLC Elution Method

The combined active semi-preparative HPLC extract was then subjected to a preparative elution method which utilized the Kinetex® phenyl-hexyl 100 Å column. Prior to injection of the sample the column was equilibrated with a solvent ratio of 80% solvent A (water) and 20% solvent B (acetonitrile) for 45 minutes with a solvent flow rate of 2.00 mL/min. 500 µL of the sample was then injected into the column. The flow rate was maintained at 2.00 mL/min, and for 3.20 minutes the solvent ratio was maintained at 80:20. Then the concentration of solvent B was increased gradually from 20% to 100% between 3.20 min and 63.40 min. The mobile phase was maintained at a concentration of 100% solvent B from 63.40 min until 83.40 min at which time the controller stopped the run. All peaks collected were tested for activity and fractions containing peaks which eluted at similar times were combined. The time program for preparative HPLC purification was the same as that in Table 1.

Final HPLC Purification

During the final purification of the antimicrobial compound peaks eluted during the preparative phase of purification which displayed activity against the indicator bacteria were subjected to multiple rounds of purification using the Kinetex® 5µm EVO C18 100 Å analytical column. The column was equilibrated using 30% solvent B (acetonitrile) and 70% solvent A (water) for 45 minutes with a flow rate of 2.00 mL/min before the sample was injected. After equilibration 200 µL of the sample was injected into the column. The flow rate was maintained at 2.00 mL/min and the mobile phase concentration remained at 30% solvent B from starting until 45.20 minutes after which time it was gradually increased to 100% solvent B from 45.20 min until 75.20 min. The solvent ratio was maintained at 100% solvent B until the controller stopped the run at 95.20 min. Fractions containing peaks which eluted at similar times during the final purification were combined and subjected to the same final purification method until desired purity was attained. The time program used during analytical HPLC purification is depicted in Table 2.

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

Table 2: Solvent time program utilized during analytical HPLC purification of the compound.

Structural Elucidation Methods

The methods used during the determination of the structure of the antimicrobial compound produced by *Rhodococcus* sp. MTM3W5.2 are outlined below.

Spectroscopic Methods

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

High-resolution mass analysis was conducted using a Bruker maXis mass spectrometer. The purified compound was dissolved in a mixture of acetonitrile and water with a ratio of 50:50 and 0.1% formic acid. The sample was ionized by electrospray ionization in positive mode and subjected to time of flight mass spectrometry to determine the exact mass of the compound.

Results

Crude Extraction

The large batch RM broth cultures were successful in growing the *Rhodococcus* MTM3W5.2 bacteria, and under stagnant conditions where the bacteria were allowed to form a biofilm there was sufficient production of the active compound for purification and characterization. Using the method of *n*-butanol crude extraction was effective at extracting the antimicrobial compound. Each of the *n*-butanol extracts demonstrated antimicrobial activity against the sensitive indicator bacteria *Rhodococcus erythropolis* IGTS8. The crude extract was dried and reconstituted in isopropanol before subjecting it to Sephadex LH-20 column chromatography. The media phase of the RM broth culture was tested for activity after undergoing the second *n*-butanol extraction and did not demonstrate any antimicrobial activity. The zone inhibition plate obtained for the crude *n*-butanol extract is depicted in Figure 6.

Figure 6: Zone inhibition plate of the crude *n*-butanol extract demonstrating the antimicrobial activity of the crude extract against the indicator bacteria.

Sephadex LH-20 Chromatography

Sephadex™ LH-20 column chromatography was effective as a preliminary method of purification of the crude extract. By subjecting the crude extract to fractionation through the

Sephadex column, impurities in the sample which were not a similar size to the antimicrobial compound were removed from the sample. Sephadex column chromatography was conducted on all crude extracts obtained during this study. Though there was some variation in the retention time of the active compound throughout the study, it was consistently eluted early during the preliminary Sephadex purification. The active Sephadex fractions were dried under a fume hood and combined using a minimum amount of methanol and isopropanol. The zone inhibition plates for the Sephadex LH-20 fractions obtained from the fractionation of the crude extract are shown in Figure 7.

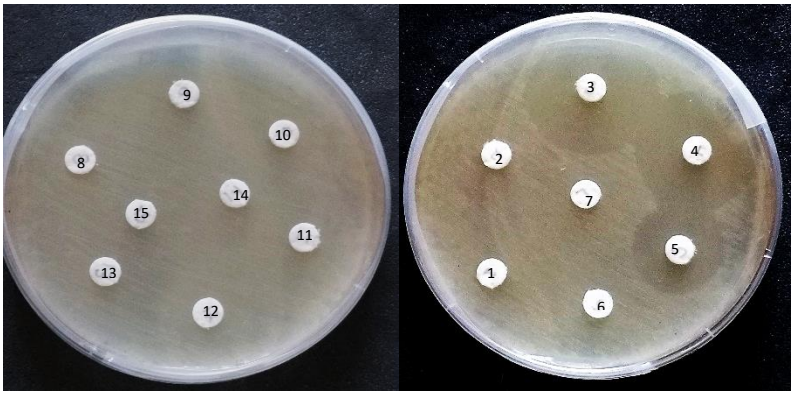


Figure 7: Zone inhibition plates for the Sephadex LH-20 extract demonstrating the activity of fractions 3, 4, and 5 while all other fractions are inactive against the indicator bacteria.

Semi-Preparative HPLC

The active Sephadex LH-20 extract was then subjected to semi-preparative high-performance liquid chromatography. The solvents used were water (solvent A) and acetonitrile (solvent B). The column which was used was a Hamilton polymeric reversed phase-1 column. A gradient elution method was used to ensure good separation of the active compound was achieved. After injection of 1 mL of the Sephadex extract, the solvent ratio was maintained at 20% solvent B until the 3.20-minute mark. The concentration of solvent B was then steadily increased to 100% solvent B by the 63.40-minute mark. The solvent concentration was

maintained at 100% solvent B until the end of the collection period at 93.40 minutes. Since multiple rounds of semi-preparative HPLC were conducted the fractions which eluted were tested for activity using zone inhibition assay and combined to undergo the preparative HPLC method. The active peaks which were eluted demonstrated a need for further purification of the compound due to the band broadness of the active peaks and the peak overlap that was observed. A representative chromatogram obtained during the semi-preparative HPLC purification step is shown in Figure 8 and demonstrates the band broadness and peak overlap which characterized much of the preliminary HPLC purification. The zone inhibition plates obtained from the representative chromatogram are shown in Figure 9.

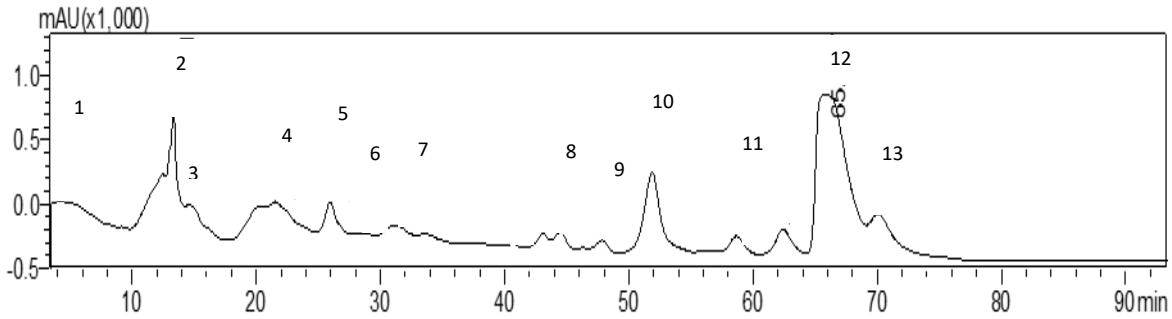


Figure 8: A chromatogram of the semi-preparative HPLC purification step depicting the peaks obtained from the active Sephadex extract. Numbers represent the peaks collected.

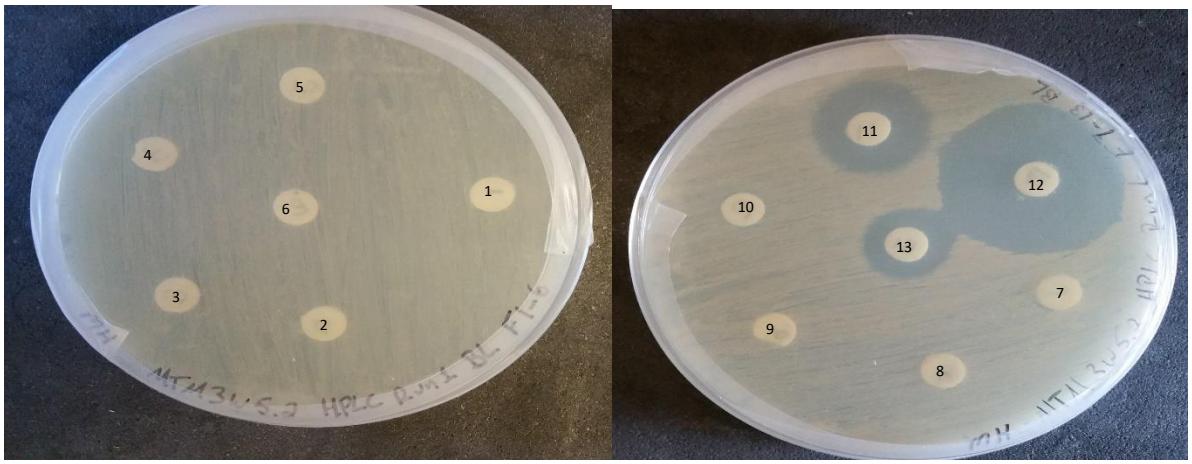


Figure 9: The zone inhibition plates of the peaks obtained from the semi-preparative HPLC step depicting the activity of peaks 11, 12, and 13.

Preparative HPLC

The combined active semi-preparative fractions were subjected to preparative HPLC on a Kinetex® phenyl hexyl 100 Å analytical column using water and acetonitrile as the solvents. The preparative purification followed the time program outlined above using the gradient elution method described. Multiple rounds of preparative HPLC were conducted and activity was determined using zone inhibition assay. A representative chromatogram obtained during the preparative HPLC purification step is depicted in Figure 10. Due to the lack of peak resolution obtained during the preparative phase of HPLC purification, it was determined that further purification was necessary for structural elucidation methods to be effective. Using the program outlined above, peaks with antimicrobial activity were eluted between 38.15 minutes and 51.10 minutes.

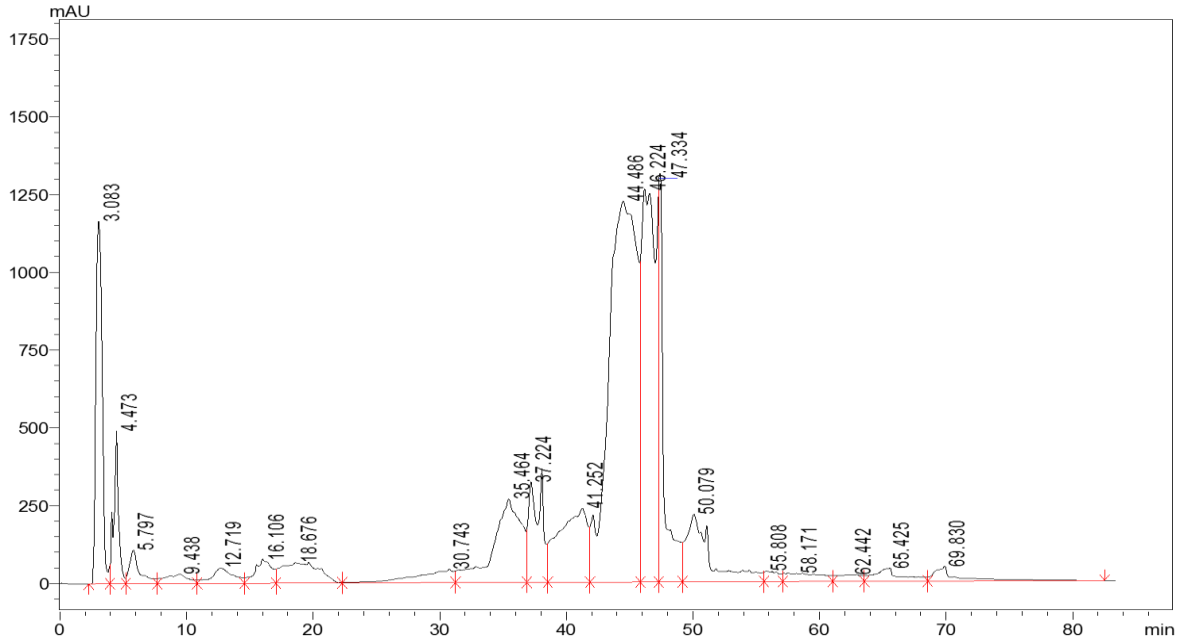


Figure 10: Chromatogram obtained during preparative HPLC purification of the antimicrobial compound demonstrating the need for better peak resolution.

Analytical HPLC Purification

The combined active fractions obtained from the preparative HPLC method were then subjected to multiple rounds of analytical HPLC purification. The time program and solvents used during this phase of purification is depicted above. Analytical HPLC purification yielded two compounds of interest which eluted at differing times. The active antimicrobial compound of interest was eluted at approximately 58 minutes using the time method described above, while a second peak of interest was eluted at approximately 72 minutes. The peak eluted at 72 minutes did not demonstrate activity against the sensitive indicator bacteria. Both compounds were subjected to several rounds of analytical HPLC until a desired purity was obtained. A representative chromatogram of the peak eluted at 58 minutes is shown in Figure 11 while Figure 12 depicts a chromatogram of the peak which was eluted at 72 minutes.

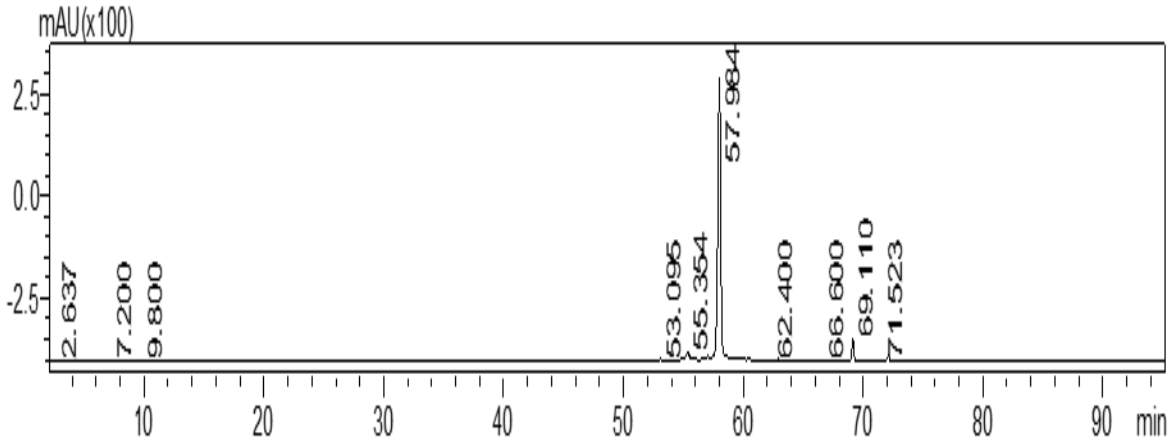


Figure 11: A representative chromatogram of the final purification step of the active peak which eluted at 57.98 minutes.

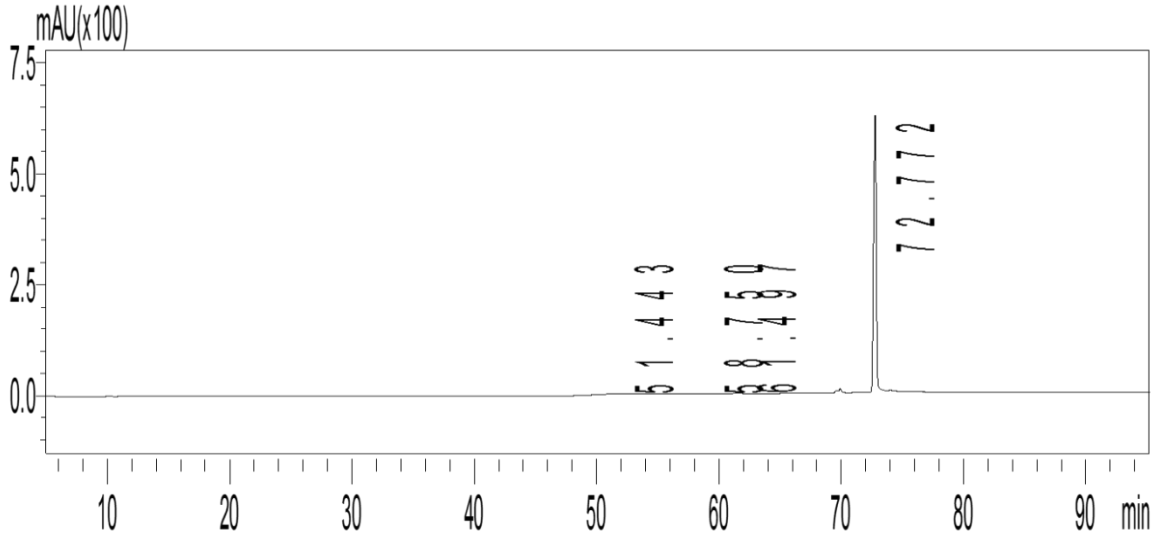


Figure 12: A representative chromatogram of the compound eluted at 72.77 minutes which depicts good resolution of the peak of interest.

UV-Visible Spectrum

The purified compounds which were eluted at 58 minutes and 72 minutes during the analytical HPLC purification method were subjected to UV-Visible spectroscopy. The absorption of the compounds over a range of wavelength was measured using a UV-Visible spectrophotometer. The UV-Vis spectrum of the 58 minute compound depicts broad absorbance in the UV range with absorbance maxima at 277 nm and 327 nm. The UV-Vis spectrum of the 58 compound is depicted in Figure 13. The UV-Vis spectrum of the compound which eluted at 72 minutes likewise showed strong absorbance in the UV region. It had absorbance an maximum at 242 nm. The UV-Vis spectrum of the 72-minute compound is shown in Figure 14.

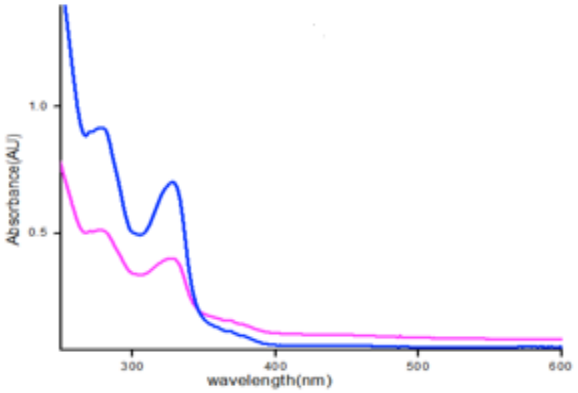


Figure 13: UV-Visible spectrum obtained for two different concentrations of the compound eluted at approximately 58 minutes with absorbance maxima at 277 nm and 327 nm. The blue line indicates a concentration of 160 µg/mL while the pink line indicates a concentration of 80 µg/mL.

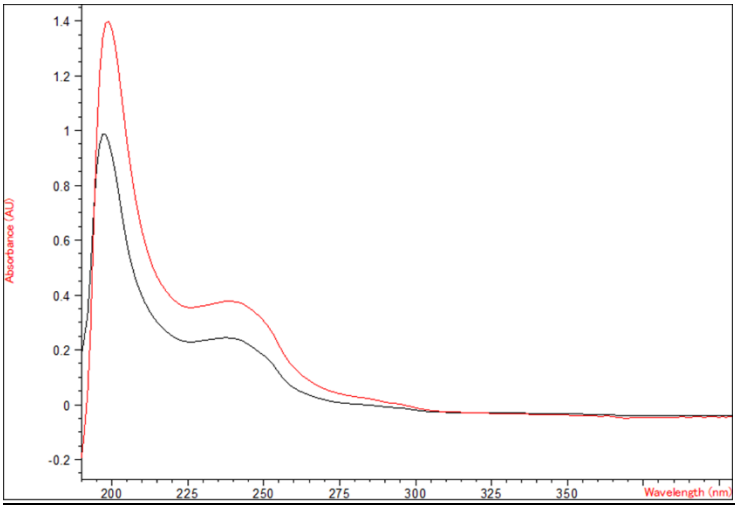


Figure 14: UV-Visible spectrum of the compound eluted at approximately 72 minutes at two different concentrations depicting an absorbance maximum at 242 nm. The red line indicates a concentration of 160 µg/mL while the black line indicates a concentration of 80 µg/mL.

High-Resolution Mass Spectrum

The antimicrobial compound of interest which were obtained from HPLC purification were subjected to high-resolution mass spectroscopy. The exact mass of the compound eluted at 58 was found at *m/z* 911.5490 $[M+H]^+$. The high-resolution mass spectrum which was obtained for this compound is depicted in Figure 15 with a full spectrum obtained from the sample along

with a zoomed isotope cluster for the molecular ion peak. The molecular formula for the compound was found to be $C_{52}H_{78}O_{13}$.

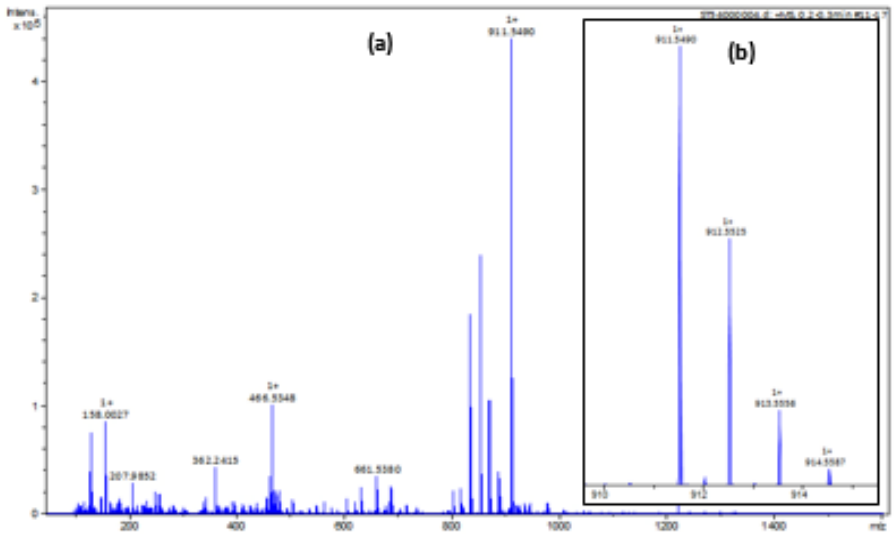


Figure 15: The high-resolution mass spectrum of the compound eluted at 58 minutes containing the full scan spectrum of the sample (a) and a zoomed isotope cluster for the molecular ion peak of the compound (b).

Discussion

Extraction and Purification

Crude Extraction

Since *n*-butanol is immiscible in water it forms an organic layer above the aqueous media phase making extraction relatively simple via a separatory funnel. Because of the moderate polarity of the active antimicrobial compound, *n*-butanol was successful at extracting it while minimizing the extraction of other unwanted compounds of greater or lesser polarity. The aqueous media phase of the RM broth cultures was tested for activity against the sensitive indicator bacteria, and did not demonstrate any activity. This demonstrated the effectiveness of the crude *n*-butanol extraction method at obtaining the maximum amount of antimicrobial compound possible. Given the success of the RM broth culture method, several large batch cultures were created to obtain a more significant amount of the compound of interest. Throughout the study the large batch cultures were grown for at least 2 weeks; however, under conditions which the cultures could not be extracted due to time constraints, they were left for up to 3 weeks. Due to the volume of media contained within each Erlenmeyer flask the *Rhodococcus* MTM3W5.2 cultures grown for this extended period did not show evidence of entering into the death phase, and the antimicrobial compound was still present upon the *n*-butanol extraction. The *n*-butanol extract demonstrated antimicrobial activity from each of the large RM broth cultures allowing the cultures to be further purified.

Sephadex Chromatography

Since the antimicrobial compound consistently eluted early during the Sephadex purification step, the relative size of the molecule can be determined. Based on the retention time

of the compound of interest, it can be determined that the compound is relatively large, too large to associate with most of the pores of the Sephadex media, causing it to pass through the column more quickly than the smaller molecules found in the crude extract. Sephadex LH-20 column chromatography was shown to be an efficient method of purification as there was minimal loss of the antimicrobial compound of interest.

HPLC Purification

Based on the chromatograms obtained throughout the HPLC purification methods outline, it was determined that the final pure 58-minute compound and the 72-minute compound were sufficiently pure for spectral analysis. By obtaining a sharp peak with no detected overlap indicated by the presence of shoulders within the peak of interest it was determined that the reversed-phase high-performance liquid chromatography method which was employed was successful in purifying the compounds of interest. The compound eluted at 58 minutes was found to be moderately polar based on the retention time during RP-HPLC. This determination was based on the ability of the compound to associate with the stationary phase of the Kinetex® 5 µm EVO C18 100 Å column early in the analytical HPLC time program, but was eluted before the mobile phase concentration had reached 100% acetonitrile during which more non-polar compounds were eluted. The compound eluted at 72 minutes was found to be less polar than the active antimicrobial compound as it associated with the stationary phase until the mobile phase was a much higher concentration of acetonitrile, the less polar solvent used.

Spectroscopic Characterization

UV-Visible Spectra

Based on the UV-Visible spectrum of the compound eluted at 58 minutes it was determined that conjugated systems were present within the compound. The strong absorbance in the UV range along with the absorbance maxima at 277 nm and 327 nm were indicative of the polyketide nature of the compound. Most polyketides produced by type I PKSs have small conjugated systems which exhibit absorbance in the UV region with bands of approximately 230-300 nm.[37] Since the absorbance of the compound and the maxima of the 58- minute compound is similar to those which are typical of polyketides, it is likely that the compound is a polyketide. The UV-Visible spectrum of the compound eluted at 72 minutes indicated the presence of some chromophores, and it may indicate some conjugation within the compound as well. The strong absorbance in the UV region indicates the compound has fewer conjugated systems than the compound eluted at 58 minutes. The absorbance maximum 242 nm indicates that it is less likely that the compound is a polyketide.

High-Resolution Mass Spectrum

The high-resolution mass spectrum obtained for the 58 minute compound indicated that the molecular ion peak of the compound at *m/z* 911.5490 $[M+H]^+$ corresponding to a molecular formula of $C_{52}H_{78}O_{13}$. Based on the molecular formula obtained through ESI Time-of-Flight Mass Spectroscopy, it was determined that the compound exhibited a high degree of unsaturation. It was estimated that the degree of unsaturation of the compound was 14 based on Equation 1. This high degree of unsaturation is in agreement with the UV-Visible spectrum obtained for the compound as it indicates the presence of conjugated systems within the molecule. The exact mass which was determined for the compound was in agreement with the retention time of the compound during Sephadex LH-20 chromatography.

$$\text{Degree of Unsaturation}=C+1-[1/2(H+X-N)]$$

(Equation 1)

Structural Fragments of the Compound

Based on work by the research group using 1-D nuclear magnetic resonance spectroscopy method such as, ^{1}H NMR, ^{13}C NMR, and 2-D nuclear magnetic resonance spectroscopy such as Heteronuclear Multiple-Bond Correlation, Correlation Spectroscopy, and Total Correlated Spectroscopy, several major fragments of the compound were constructed.[17] The determination of several key spin systems using ^{1}H-^{1}H COSY which were used to construct the structural fragments. Correlations between hydrogen and carbons within the molecule was also determined using a ^{1}H-^{13}C NMBC spectrum. The major fragments which were determined by the PI and the research team are depicted in Figure 16. Each spin system is shown in a different color and arrows within the figure indicate the hydrogen-carbon correlations found within the ^{1}H-^{13}C HMBC spectrum. The fragments which were determined by the NMR data indicate that the molecule has a polyketide structure.

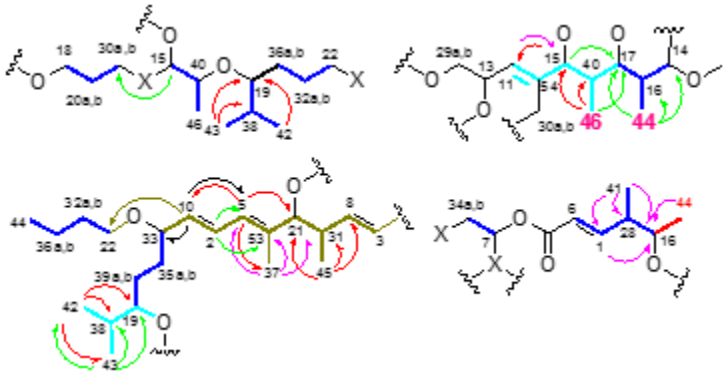


Figure 16: Major fragments which were assigned based on the various spin systems obtained from the ^{1}H-^{1}H COSY spectrum with each spin system depicted in a different color. Arrows indicate hydrogen-carbon correlations.

Conclusion and Future Work

Based on the results of this study it was found that the antimicrobial compound of interest may in fact have a novel polyketide structure; however, full structural identification will be necessary to determine whether the chemical scaffold of the compound is a novel scaffold. It was determined during this study that the method of RM broth extraction using *n*-butanol was effective at removing the antimicrobial compound from the RM broth, and that the purification method was effective in yielding a purified compound. By utilizing different HPLC time programs and adding modifiers based on the chromatogram obtained from the purification of the sample, an effective method of purification was established which caused minimal degradation to the compound. Due to low rates of the production of the antimicrobial compound spectral analysis by NMR was difficult, leading to NMR spectra which contained too much noise to elucidate the entirety of the compound of interest. However, based on the major fragments which were constructed from the spin systems obtained through ^{1}H-^{1}H COSY it was determined that the compound is a polyketide. It was also determined that the compound has a high degree of unsaturation—14—indicating the presence of the conjugated systems depicted in the fragment which were constructed. The molecular formula of the antimicrobial compound was found to be $C_{52}H_{78}O_{13}$ with an exact mass of 911.5490.

The compound which was isolated at 72 minutes during the HPLC purification method was not found to be active against the indicator bacteria; however, due to the presence of the peak in preliminary analytical HPLC trials it was determined that the peak could be of some interest in determining the structure of the antimicrobial compound. Preliminary spectral results indicate that the compound contains less conjugated systems than the active compound and

further structural identification should be done to elucidate the structure of the 72-minute compound.

Future Work

Because of the time constraints on this study the entire structure of the antimicrobial compound was not able to be elucidated. Further, only preliminary structural data was obtained for the compound which eluted at 72 minutes. To elucidate the final structure of the antimicrobial compound of interest it will be necessary for further production of the compound to be conducted using the method outlined above. Further purification of the compound will be necessary to obtain the spectral data required to determine the complete structure of the compound. Scaling up the production of the antimicrobial compound may also be necessary to obtain a compound concentration high enough to avoid the introduction of noise into the NMR spectra. Since the compound that eluted at 72 minutes may be helpful in determining the structure of the antimicrobial compound, structural elucidation should be undertaken for it as well. It will be necessary to obtain a high-resolution mass spectrum for the compound, and subject it to 1D and 2D NMR spectroscopy so that the molecular structure can be obtained.

Upon structural elucidation of the antimicrobial compound a bioactivity profile against a large library of bacteria should be constructed. This could give insight into the specificity of the compound and may help indicate its mechanism of action. By understanding the mechanism of action, it may be possible to determine the practicality of using the antimicrobial compound produced by *Rhodococcus* MTM3W5.2 as a potential antibiotic drug.

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