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Extraction, Purification and Characterization of an Antibiotic-like Compound Produced by Rhodococcus sp. MTM3W5.2

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Extraction, Purification and Characterization of an Antibiotic-like Compound Produced by *Rhodococcus* sp. MTM3W5.2

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by

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ABSTRACT

Extraction, Purification, and Characterization of an Antibiotic- like Compound Produced by *Rhodococcus* sp. MTM3W5.2

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The bacterium *Rhodococcus* is a potential source for novel antimicrobial metabolites. Recently, the *Rhodococcus* strain MTM3W5.2 was isolated from a soil sample collected from Morristown, East Tennessee and was found to produce an inhibitor molecule that is active against similar *Rhodococcus* species. The aim of this research is to extract, purify, and characterize the active compound. The compound was obtained from both agar and broth cultures of strain MTM3W5.2 and purified by primary fractionation of crude extract on a Sephadex LH-20 column, followed by semi-preparative reversed phase column chromatography. Final purification was achieved using multiple rounds of an analytical C_{18} HPLC column. Based on the results obtained from UV-Vis, FT-IR, and HR-MS, the molecule is a polyketide with a molecular formula of $C_{52}H_{78}O_{13}$ and an exact mass of 911.5490 amu. The partial structure of this compound has been determined using 1D and 2D NMR spectroscopy.

DEDICATION

To my husband and my parents

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
CDC	Centers for Disease Control and Prevention
OND	Office of New Drugs
MRSA	Methicillin-Resistant *Staphylococcus Aureus*
CRE	Carbapenem Resistant *Enterobacteriaceae*
IDSA	Infectious Diseases Society of America
FDA	Food and Drug Administration
NMEs	New Molecular Entities
GNB	Gram-Negative Bacilli
NCEs	New Chemical Entities
IPP	Isopentenyl Pyrophosphate
PKS	Polyketide Synthases
NRPS	Nonribosomal peptide Synthases
KS	Ketosynthase
AT	Acyltransferase
ACP	Acyl Carrier Protein
DEBS	6-Deoxyerythronolide B Synthase
DH	Dehydratase
ER	Enoyl Reductase
CoA	Coenzyme A
TE	Thioesterase
DMSO	Dimethyl Sulfoxide

DMSO-d6	Deuterated Dimethyl Sulfoxide
EtOAc	Ethyl Acetate
PCR	Polymerase Chain Reaction
HPLC	High-Performance Liquid Chromatography
HR-MS	High-Resolution Mass Spectrometry
NMR	Nuclear Magnetic Resonance
1D	One-dimensional
2D	Two-dimensional
CH_3OH	Methanol
CH_3CN	Acetonitrile
CH_3OD	Deuterated Methanol
CH_2Cl_2	Dichloromethane
FT-IR	Fourier Transform Infra-Red
NH_4OH	Ammonium Hydroxide
KH_2PO_4	Potassium dihydrogen phosphate
$HCOONH_4$	Ammonium Formate
HCOOH	Formic Acid
MH	Mueller-Hinton Medium
RM	Rich Medium
RPM	Revolutions Per Minute
FCC	Flash Column Chromatography
SEC	Size Exclusion Chromatography
Prep-HPLC	Preparative High-Performance Liquid Chromatography

RP	Reversed-Phase
LC	Liquid Chromatography
UV	Ultraviolet
C18	Octadecyl
Mwt	Molecular weight
µL	Microliter
mL	Milliliter
cm^{-1}	Per Centimeter
Da	Dalton
g	Grams
hr	Hour
min	Minute
mM	Millimolar
NCE	Normalized Collision Energy
ESI	Electrospray Ionization
COSY	Correlation Spectroscopy
HMBC	Heteronuclear Multiple-Bond Correlation
HSQC	Heteronuclear Single-quantum Correlation Spectroscopy
NOESY	Nuclear Overhauser Enhancement Spectroscopy
ROESY	Rotating Frame Nuclear Overhauser Effect Spectroscopy
TOCSY	Total Correlated Spectroscopy

CHAPTER 1

INTRODUCTION

Antibiotic Use and Resistance

Drug-resistant infections have been increasing for many years. Resistance develops when a microorganism evolves and prevents the introduced antibiotics from being effective.[1] The discovery of penicillin in 1928 led to the treatment of millions of bacterial infections and resulted in it being called a "miracle drug".[1] Now, more than half a century later, humans have reached a crisis level in treating antibiotic resistant infectious diseases. No drugs have been developed to keep apace with the natural capability of bacteria to advance and defend themselves against antibacterial drugs.[2] Overuse of antibiotics and the natural evolution of bacteria to change themselves to resist the effect of the drugs have led to this growing threat.[2]

Alexander Fleming warned of the development of resistance to penicillin as early as 1945 by exposing microbes to lower concentrations of penicillin.[3,4] Since then simultaneously with the development of new drugs, resistance in both pathogenic and nonpathogenic bacteria has been observed (Figure 1).[5]

Microbes develop resistance through various mechanisms such as altering the target, hydrolysis, efflux, glycosylation, phosphorylation, reprogramming peptidoglycan biosynthesis, ADP-ribosylation, nucleotidylation, monooxygenation and acetylation.[6] Resistant infections are turning deadly. The CDC in 2013 reported that antibiotic resistant infections result in 25,000 deaths per year and an additional 2.5 million hospitalizations in the European Union.[1] As a result of resistant bacterial infections passed from their mothers, more than 58,000 babies die in India per year.[7,8] These infections cause 3.2 million illnesses and over 38,000 deaths per year in

Thailand[9] and over 23,000 deaths, and more than 2.0 million illnesses in the United States yearly.[1]

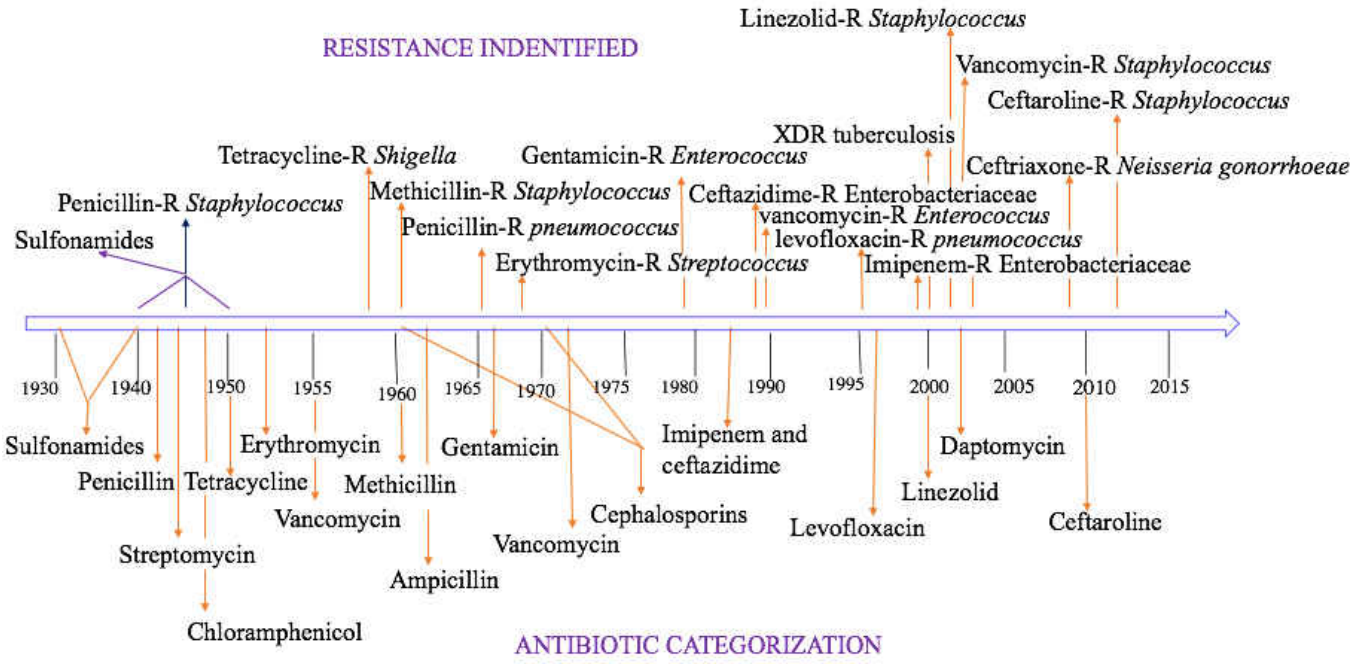


Figure 1. Timeline of antibiotic categorization and the identification of antibiotic resistance. (Adopted from references.[1,5])

In the process of discovering novel antibiotics, concomitant occurrence of antibiotic resistance has been developed.[6] Figure 2 shows the evolution of antibiotic resistance for the leading antibiotics and the events in eras. Primeval era was the beginning of chemotherapy through sulfonamides. The broadest discovery of antibiotics occurred in the golden years of research between 1945 and 1955.[6] Pharmacologic effects were made to understand the use of antibiotics by research and management.[6] The biochemical activities of antibiotics and resistant mechanisms were used to modify the structure of drug compounds to chemically combat resistance.[6] Genomic studies led researchers to propose novel molecules and predicted the

fundamental targets.[6] Disappointment with massive investment in genome-based methods, many pharmaceutical companies dropped their antibiotic discovery programs.[6] The important highlights in this history include the creation of the Office of New Drugs (OND) which introduced harsher requirements for the drug safety. These requirements reduced the introduction of new antimicrobial compounds by pharmaceutical companies.

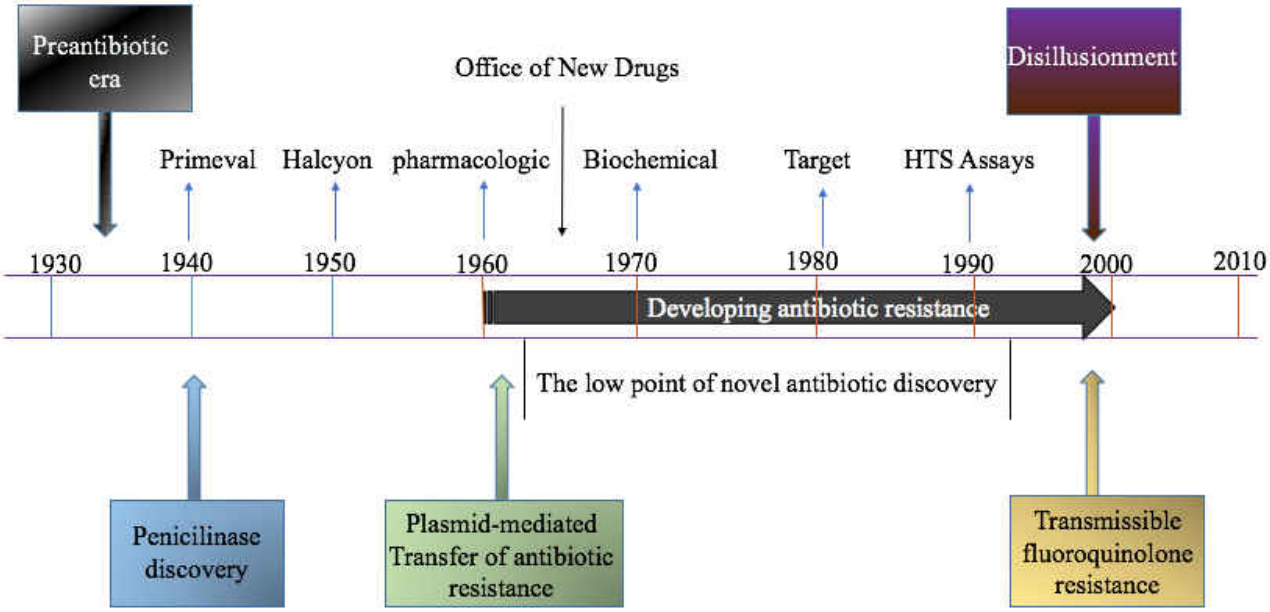


Figure 2. Events in the history of antibiotics and the development of antibiotic resistance. (Adapted from the source[6] with permission from the publisher)

The Need for New Antibiotic Classes

In the society, antibiotic-resistant strains of infectious bacteria are increasingly common resulting in the appearance of multidrug resistance among the newest generation of pathogens.[10] The CDC reports dangerous levels for antibiotic resistance amongst pathogens. For example, Methicillin-resistant *Staphylococcus aureus* (MRSA), a gram-positive microbe is associated with severe hospice illnesses, and is considered a serious threat.[11] Others such as Carbapenem-

Resistant *Enterobacteriaceae* (CRE), like *Klebsiella* and *Eschericha coli* have quickly developed as major hazard being their resistant to nearly all modern antibiotics.[11] There is an urgent need to find novel antibitics to fight against these microbial pathogens. However, the progress in developing them has been slow.[10] The Infectious Diseases Society of America's (IDSA) 2009 antibiotic pipeline status report tracked a continual drop in the development of new antibiotics.[12] The fall from a high sixteen new antimicrobial agents approved during the period of 1983 to 1987 to only two new antibiotics (ceftaroline-fosamil, and telavancin) since 2008 is problematic.[12] Between 1998 and 2002, the approval rate of new antibacterial agents by FDA declined by 56% compared to the period from 1983 to 1987.[12] Of the total fourteen drugs approved since 1998, only four exhibited a unique mechanism of action.[12,13] Diseases caused by exclusively the "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species), cause substantial morbidity and mortality.[12] Worldwide and especially in the United States, "ESKAPE" and other drug-resistant gram-negative bacilli (GNB) infections negatively impact the health of hospitalized patients undergoing clinical treatments and additional procedures and also on healthy people outside the hospital.[12] The requirement for new antibacterial agents to treat infections instigated by GNB resistance to available agents today is much more important than in the past.[12]

Most clinically used antibiotics have come from a relatively small set of chemical structures. However, chemical groups of antibiotic scaffolds have been extended by the modification of their structures through synthetic tailoring.[10] More than 73% of antibiotic-new chemical entities (NCEs) filed between 1981 and 2005 are derived from just four scaffolds - penicillins, cephalosporins, quinolones, and macrolides discovered between the mid-1930s and

the early 1960s.[10] Although all antibiotics clinically approved between the 1960s and 2000 were derived synthetically from naturally existing scaffolds, synthetic tailoring is the primary method used for restocking of the antibiotic pipeline. The logical fashion to battle against resistance is to discover new scaffolds.[10]

Natural Products as a Possible Source of Novel Antibiotics

Over two-thirds of clinically-approved antibiotics are natural compounds or their semisynthetic derivatives.[10] Living organisms generate three specifically distinctive types of organic products: primary metabolites, high molecular weight polymeric compounds, and secondary metabolites.

Primary metabolites are produced in every part of cells and play a fundamental role in the metabolism and reproduction of those cells. These compounds include common amino acids, sugars, and nucleic acids.[14] The high molecular weight polymeric compounds are involved in the formation of cellular structures. These include cellulose, lignins, and proteins.[14]

Interestingly, secondary metabolites can show biological effects on other cells or even other organisms.[14] This feature of these natural products makes them function as regulators, resistance and defensive substances.[14] The organism which produces these organic secondary metabolites will get an advantage from their biological activity. However, it is often dangerous to other species, including humans.[15] More than 40% of the bioactive secondary metabolites of plants and microbes are widely used as drugs.[14]

The Classes of Secondary Metabolites

Secondary metabolites belong to one or more families. The structures of these compounds are diverse.[14] It is not possible to distinguish primary and secondary metabolites based on their structure and biochemical processes; the differentiation principally depends on functionalities of the compounds.[14,16] Based on the way they are produced by biosynthesis pathways in different organisms, they can be categorized into few major classes (Figure 3).[14]

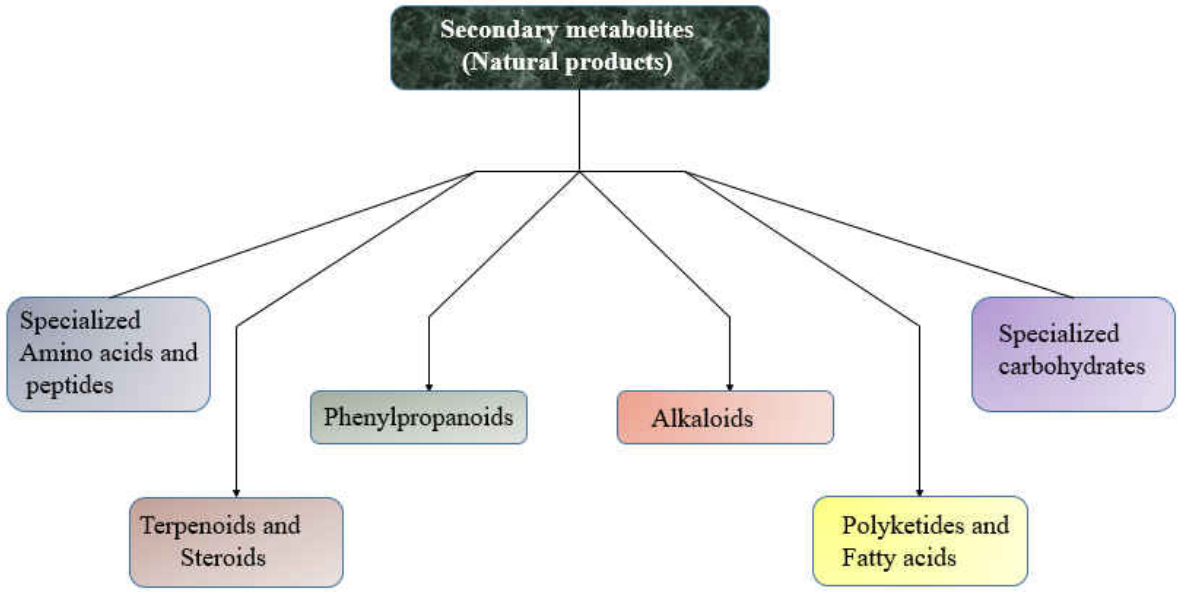


Figure 3. Flow chart of the categorization of secondary metabolites.[14]

Terpenoids and Steroids

Terpenoids are the most structurally diverse class of natural plant products derived from a repetitive combination of isopentane units indicated as isoprene monomers.[17] Isopentane units usually originated from isopentenyl pyrophosphate (IPP) (Figure 4).[14] As per Ruziicka's isoprene rule, they are monomers are bonded in a head-to-tail fashion.[14] Based on the number of isoprene units (C_5), the terpenes are classified as monoterpenoids, (C_{10}), sesquiterpenoids, (C_{15}),

diterpenoids, (C_{20}), sesterterpenoids, (C_{25}), triterpenoids, (C_{30}), and carotenoids (C_{40}). The steroids naturally come from the tetracyclic triterpenoid units.[14]

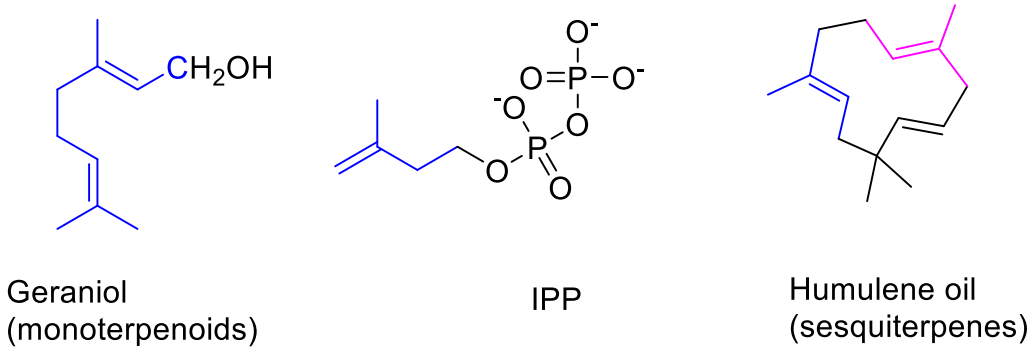
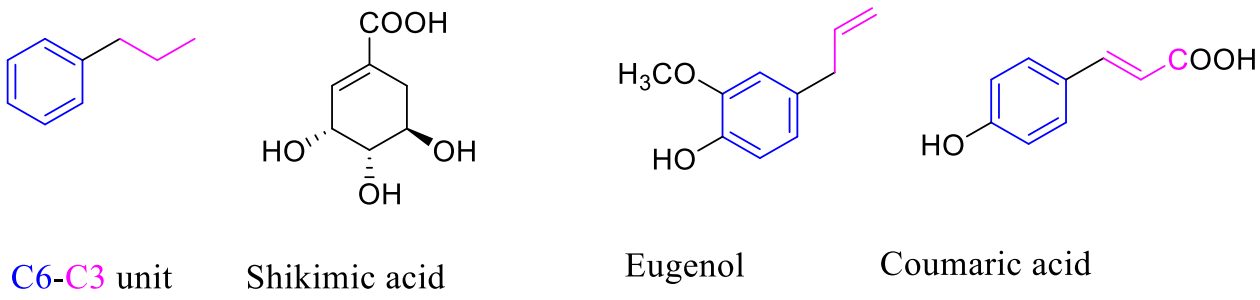


Figure 4. The examples of terpenoid metabolites.[14]

Phenylpropanoids

The phenylpropanoids are well-known plant natural products with a six-membered aromatic ring structure plus a three carbon chain attached to it (C_6-C_3 unit).[14] The Shikimic pathway,[17] is an important biosynthetic pathway found in plants and microorganisms but not in animals.[14] This pathway provides an unconventional way to synthesize aromatic compounds such as phenylpropanoids, and the aromatic amino acids like L-phenylalanine, L-tyrosine, and L-tryptophan.[17] The intermediate in the biosynthesis of these amino acids is shikimic acid (Figure 5) which is extracted from *Illicium* plant species, also called shikimi plant in Japan.[17] The phenylpropanoids biosynthesis pathway at oxygenation step differed from polyketide biosynthesis by this Shikimic route.[14] The chemical structures of different phenylpropanoid metabolites appear in Figure 5.



C6-C3 unit Shikimic acid Eugenol Coumaric acid

Figure 5. The examples of phenylpropanoids metabolites.[14]

Alkaloids

Alkaloids were the leading natural products to be isolated from medicinal plants in the 19th century.[14] They were initially described as pharmacologically active, nitrogen-containing compounds of plant origin.[17] Due to the basic nature of nitrogen which is present in all alkaloids, they form salts from their reaction with acids, thus, they are also known as vegetable alkali.[14] Alkaloids are classified in three ways;

(i) Based on biosynthesis from amino acid precursors: For example, relatively limited amino acids are involved in the biosynthesis of alkaloids such as lysine, nicotinic acid, ornithine, anthranilic acid, phenylalanine, tyrosine, histidine, and tryptophan.[17]

(ii) According to their sources: According to the plant sources from which alkaloids are extracted, they grouped are as Aconitum, Cinchona, Curare, Ergot, Opium, Amaryllidaceae, Senecio and Vinca.[14]

(iii) Based on the nature of nitrogen present in them: Another important classification is made based on the nature of nitrogen positioned in the alkaloid structure. They include pyrrolidine, indole, benzylisoquinoline, piperidine, quinoline, and isoquinoline.[17]

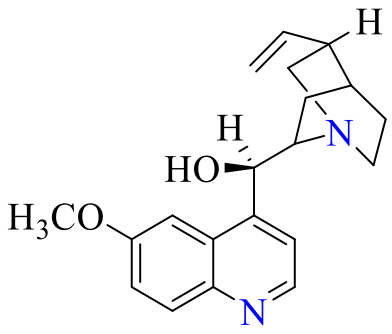


Figure 6. Quinine alkaloid (antimalarial drug).[14]

Polyketides

Polyketides are the most distinct subgroup of natural products, produced by fungi, bacteria, and plants.[18] They engage in a broad range of biological activities such as antibacterial, antifungal, anticholesterol, antiparasitic, anticancer, and immunosuppressive properties.[19] The activity of polyketides is represented in Figure 7.

The diversity in the chemical structure of polyketides is a key factor causing their diverse bioactivities.[18] Numerous polyketides are biosynthesized by gram positive, soil residing microorganisms of the genus *Streptomyces* which belongs to the *Actinomycete* family.[20]

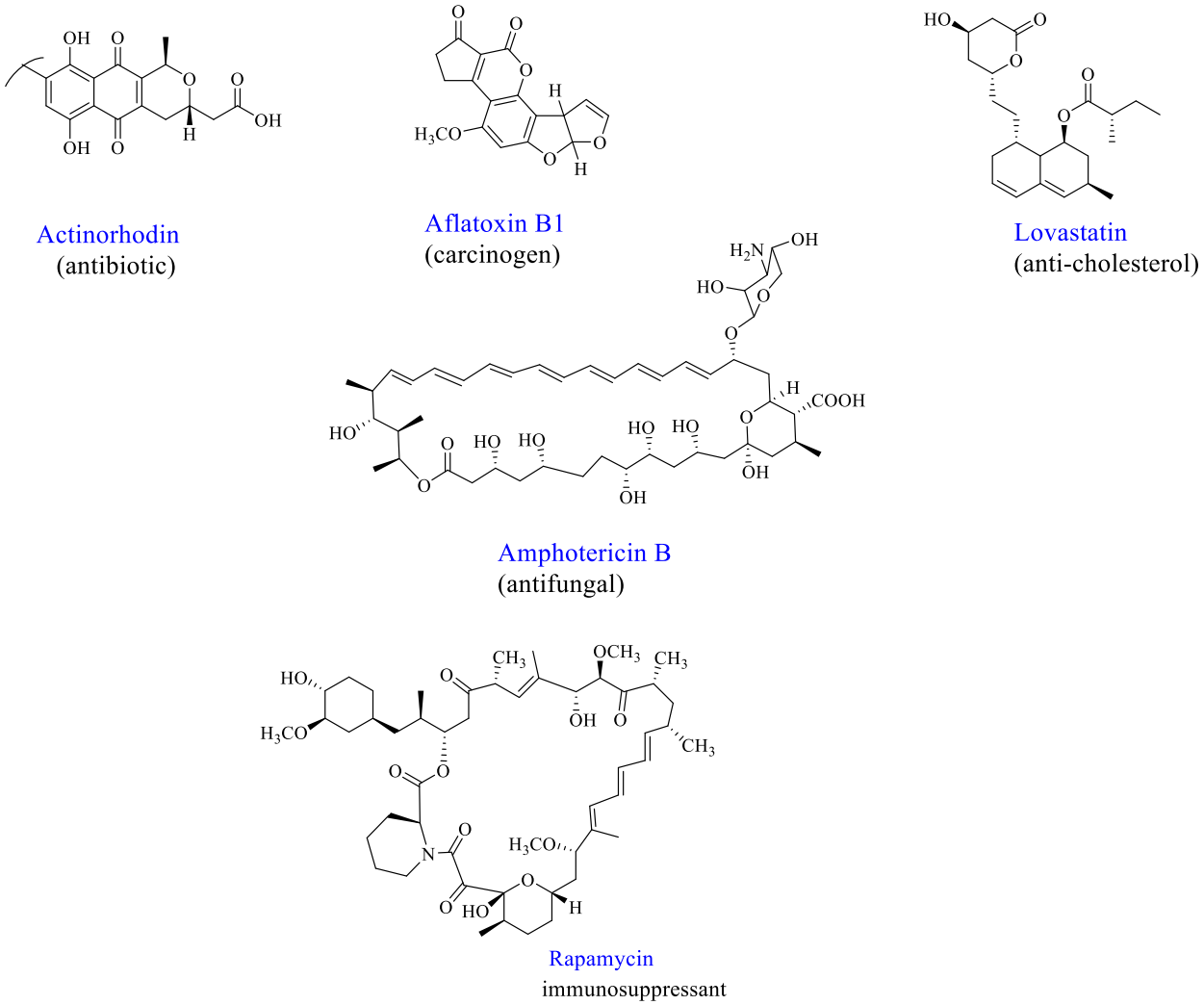


Figure 7. Different kind of polyketides and their pharmaceutical activity.[21]

Polyketide Synthases (PKSs)

The assemblies of polyketides vary widely; but they are all biosynthesized by a mechanism in their initial stages that is very similar to fatty acid biosynthesis.[22] Polyketides are formed by sequential condensation of acyl originators such as acetyl and malonyl units.[22] These condensation reactions are catalyzed by large mega enzyme-complexes known as polyketide synthase (PKS). Polyketide synthases can contain several protein domains (catalytic regions),[14] which are organized into sections called modules.[23] The domains function in an assembly-line

manner to form a polyketide chain (Figure 8).[23] Generally, the number of modules essential for the synthesis of the compound matches the number of precursors that are incorporated into the product polyketide chain.[24] A typical intermediate in a polyketide biosynthesis is a polyketone.[25] Assembly of a basic polyketide involves three discrete steps:

(i) loading of a precursor molecule,

(ii) the addition of multiple chemical building blocks to elongate the polyketide chain, and

(iii) the release of the final condensed chain of the polyketide occurs.[25]

Biosynthesis of a polyketides is very similar to that of fatty acid biosynthesis, often having the same type of building blocks. In the fatty acid chain assembly process, the carbonyl group of acetate undergoes reduction.[14] Subsequent degradations and oxidation produces unsaturated fatty acids. Polyketide and fatty acid biosynthesis primarily differs in the number and type of acyl building blocks used, the extent and position of keto group reductions, and the cyclization arrangement of the finalized products.[25]

PKSs are cataloged into three types, PKS I, PKS II, and PKS III. Type I PKSs contain a set of catalytic core domains, such as keto synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) assembled as a module (Figure 8) that regulates the assimilation of starting molecules into the polyketide chain.[19] Unlike the type I PKSs, type II PKS contain an additional KS domain along with the core domains. The extra KS controls the elongation and enzymatic activities.[19] Type III PKSs, on the other hand, are typically deficient in multiple catalytic domains and employ an ACP-independent mechanism.[19]

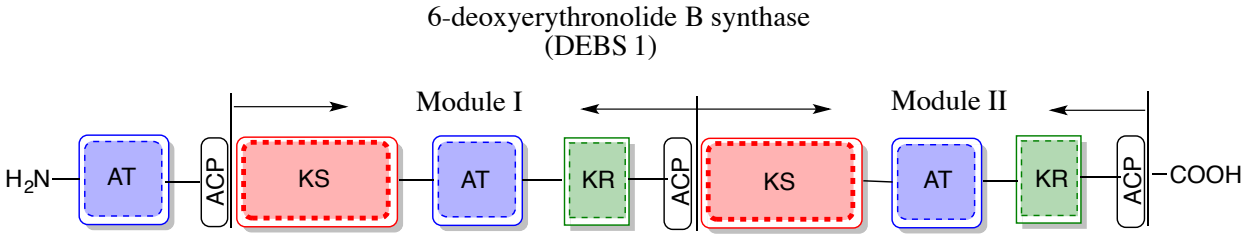


Figure 8. The arrangement of domains in the DEBS 1.
Different colored boxes represent the keto synthase (KS), acyltransferase (AT), ketoreductase (KR), and acyl carrier protein (ACP) domains. (Adopted from the reference.[21])

Type I Modular Polyketide Synthase

The classic bacterial type I PKS comprises multi-functional modules. Thus, they are named a 'modular' PKS.[21] The 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea*[26] is the archetype of modular[27] PKS responsible for making the 6-deoxyerythronolide B (6-DEB) structure of erythromycin A.[19] This PKS has three large proteins, DEBS 1, DEBS 2, DEBS 3, that accommodate 28 domains organized into seven functional modules.[21] Each module contains the three domains KS, AT, and ACP, which co-operate to catalyze C–C bond formation by Claisen condensation.[21] Catalysis of one cycle of chain addition is done by core domains as well as an adaptable set of domains (ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER)) along with the modification of the keto functional group.[21,26,28] The polyketide biosynthesis starts with DEBS 1 by a loading N-didomain (AT and ACP), with the precursor propionate from propionyl-CoA.[21] The AT domain recongnizes the particular extender unit to be integrated into the growing polyketide chain,[19] while DEBS 3 terminates with a thioesterase (TE) by off-loading followed by cyclization of the fully-formed heptaketide intermediate to provide 6-DEB 16.[21]

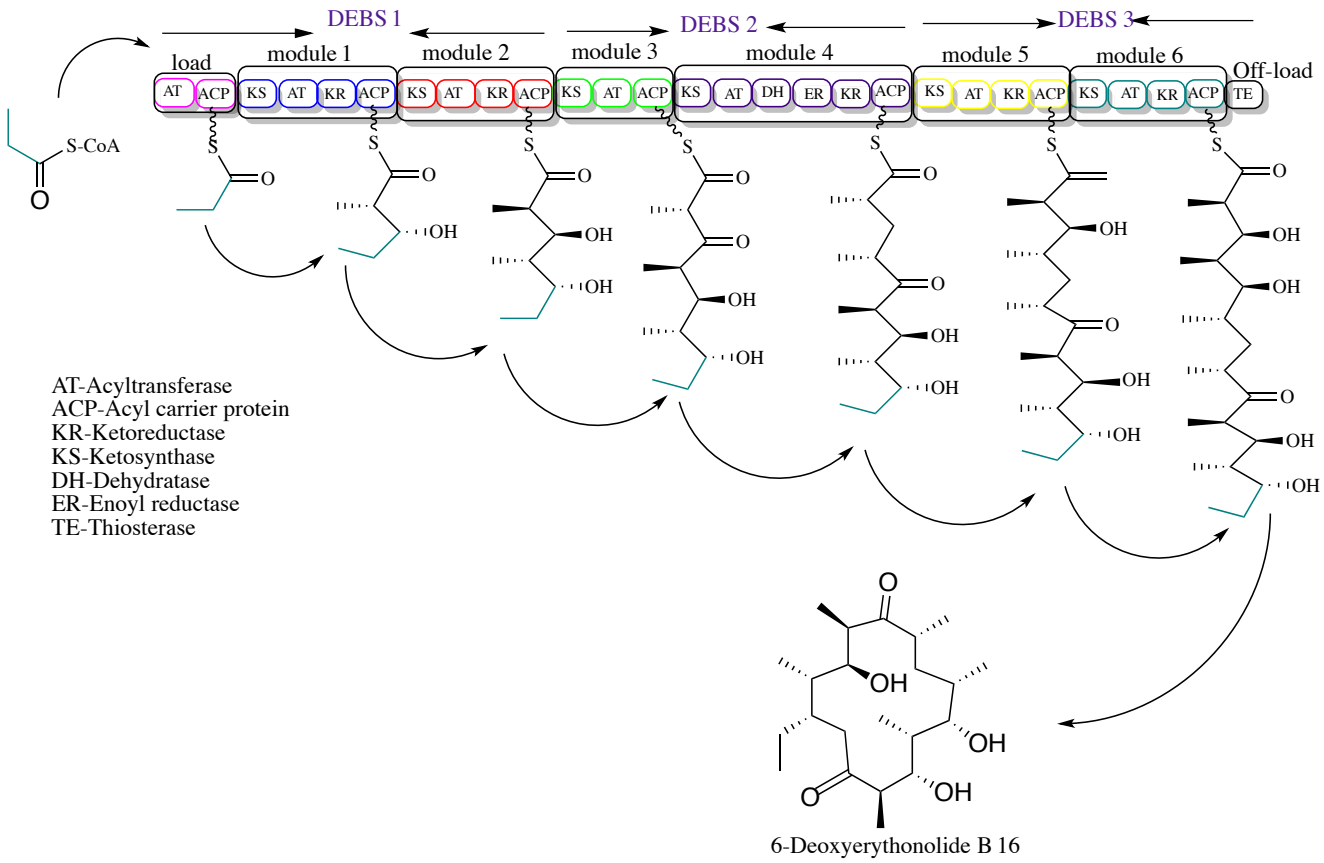


Figure 9. Organization of modules in 6-deoxyerythronolide B synthase (DEBS). Three mega proteins (DEBS 1, DEBS 2, and DEBS 3) are organised into a total of six modules depicted by large rectangular boxes in the figure. Each module has core enzyme domains (KS, AT, and ACP) shown in small rectangular boxes including variable domains (KR, DH, and ER). The starter unit is loaded at the N-terminal of DEBS 1, followed by elongation with extender methyl malonyl-CoA units, then the polypeptide chain is terminated at the C-terminal of DEBS 3 (Adopted from the reference.[21,19]).

The *Actinomycete* Genus *Rhodococcus*

At the moment, there are about 56 species classified in the genus *Rhodococcus*.[29] The *Actinomycete* genus *Rhodococcus* is described as an aerobic, GC-rich nonsporulating, and nonmotile Gram-positive bacteria that also contain mycolic acids in their cell envelope.[30] The cell walls of the rhodococci are chemotype IV, which has *meso*-diaminopimelic acid containing

peptidoglycan, and arabinose, galactose as major sugars.[31] Their cell morphology changes during the different stages of their growth cycle; the *cocci* of some strains turn into short rods, while others continue transforming into filamentous rods.[32] Some rods start branching out in a simple or extensive manner.[32] Rhodococci are found in soil, rocks, boreholes, groundwater, animal dung, marine sediments, the guts of insects and from healthy and diseased plants and animals.[33, 34]

Environmental and Biotechnological Importance

The mycolic acid containing cell wall of *Rhodococcus* may contribute to their exceptional ability to degrade a broad range of compounds, including pollutants.[35] The catabolic versatility of *Rhodococcus* and their exceptional stress tolerance[36] and rapid growth rates have led to their use in numerous applications in bioremediation and biocatalysis. For instance, *Rhodococcus rhodochrous* J1 is used in the production of acrylamide from acrylonitrile.[30]

Antibiotics Produced by *Rhodococcus*

In the past few decades, scientists have explored the genus *Rhodococcus* for its ability to produce antibiotic like compounds.[37] Since 1999 other antimicrobial compounds have been produced from the *Rhodococcus* genus. Different types of antibiotic like compounds produced by this genus published to date are described below.[38-44]

Rhodopeptins. Chiba *et al.* found a strain of *Rhodococcus* that produced a series of cyclic tetrapeptides that inhibited the growth of *Candida albicans* but did not show antibacterial activity.[38] Five of these rhodopeptins C1, C2, C3, C4, and B5 were purified from the metabolites

produced by *Rhodococcus sp.* Mer-N1033.[38] This strain was isolated from the soil sample collected at Mt. Hayachine, Prefectur, Japan.[38] These novel type of cyclic tetrapeptides are made up of lithophilic β-amino acids and α-amino acids. The rhodopeptins are white residues or colorless solids and are soluble in methanol, dimethyl sulfoxide (DMSO), acetic acid and water.[38]

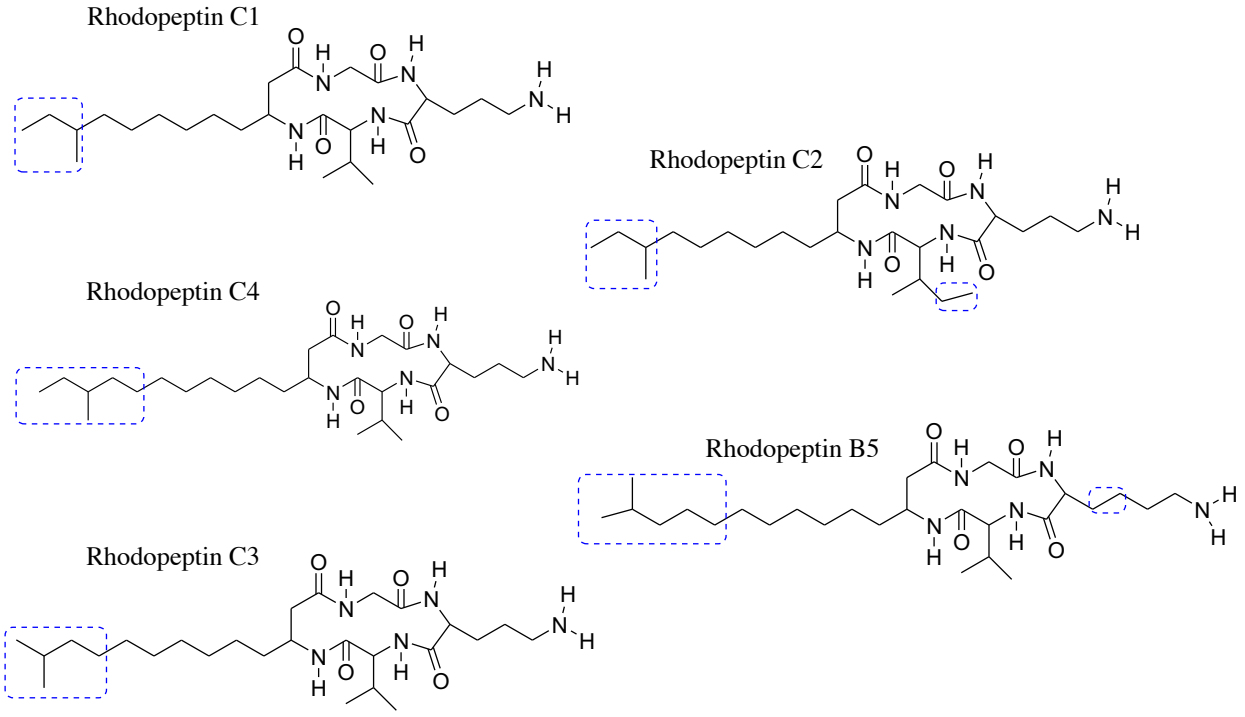


Figure 10. The structural differences of rhodopeptins C1, C2, C3, C4, and B5.[38]

Lariatins. In 2006, Iwatsuki *et al*. discovered the antimicrobial agents named lariatins A and B (Figure 11) while screening microbial metabolites that showed inhibitory activity towards Mycobacteria. These agents are specific cyclic peptides produced by *Rhodococcus sp*. K01-B0171. They inhibit the growth of *Mycobacterium smegmatis* and also inhibited the growth of *M. tuberculosis*.[39] The scientists also studied the structure of lariatins A and B and discovered a

'Lasso' peptide structure, which consists of 18 and 20 L-amino acid residues with a linkage between the γ-COOH group of Glu8 and the α-NH_2 group of Gly1 (Figure 11).[39] They correspondingly isolated other similar compounds from microbes having similar internal bonding seen in the lariatins. These were classified into three groups; siaycin, anantin, and lariantin.[39]

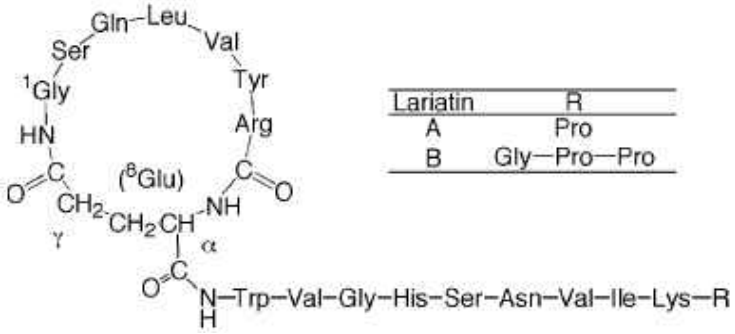


Figure 11. Structure of lariatins A and B.[39]

In 2008, a research team from Japan (Kitagawa and Tamura)[40] investigated three groups of antibiotic producing *R. erythropolis.* They screened 80 strains of the genus *Rhodococcus* for antibiotic-producing ability. *Eschericcoli*, *Pseudomonas*, *Streptomyces*, *Corynebacterium*, *Sinohizonium*, *Arthrobacter,* and *Rhodococcus* were used as test strains for the first screening. 14 *R. erythropolis* strains and one *R. globerulus* strain exhibited inhibition against the test strains. These 15 strains were then extensively studied using 52 test strains. As a result the *Rhodococcus* strains showed antibiotic activity against gram-positive test strains but did not exhibit same activity against gram-negative bacterial test strains.[40] They classified these 15 strains into three groups;

(i) Group I (R01-R05) strains exhibited the antibiotic activity against gram-positive bacteria,

(ii) Group II (R06-R08) showed antibiotic activity against *Rhodococcus* and gram-positive bacteria, and

(iii) Group III consists of R09-R15 and exhibited antibiotic activity against *R. erythropolis*.[40]

Based on their results they concluded that the antibiotic spectrum of the 3 groups was different from the antibiotics which were previously reported from *Rhodococcus* strains.[40]

Aurachins. Further research on three groups of *R. erythropolis* strains which exhibited the antibiotic activity. The researchers isolated an antibiotic from *R. erythropolis* JCM 6824 strain and determined the structure based on NMR and mass spectrometric analysis.[41] The antibiotic was a quinoline called aurachin RE and exhibited strong antibiotic activity against gram-positive bacteria. It appeared as gray-brown and was soluble in ethanol, methanol, methyl cyanide, DMSO, and EtOAc and was barely soluble in water.[41] Excitingly, they found that the structure of aurachin RE was similar to the structure of the antibiotic aurachin C shown in Figure 12. aurachin C was isolated from a gram-negative myxobacterium, *Stigmotella aurantiaca*.[42] They both exhibited the antibiotic activity against gram-positive bacteria but aurachin RE showed considerably stronger activity.[41]

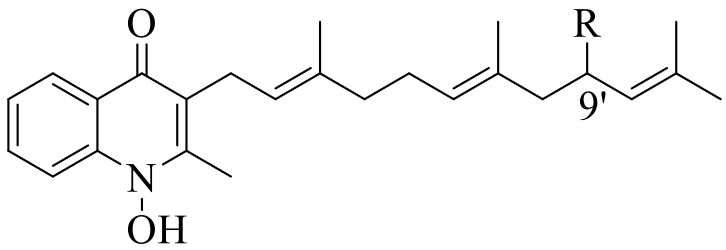


Figure 12. The structures of aurachin RE (9'(R)-OH) and aurachin C (9'(R)-H)[41,42]

Horizontal Gene Transfer in Antibiotic Production. In January 2008, Kurosawa *et al.* isolated the antibiotic producing strain *Rhodococcus* 307C0 by co-culturing a strain of *Rhodococcus fascians* and a strain of *Streptomyces padanus*.[43] *R. fascians* was not an antibiotic producer while *S. padanus* was a known producer of antibiotics. After co-culturing they recovered *Rhodococcus* 307C0 and found that it has a large segment of DNA derived from the *Streptomyces* strain.[43] Two antibiotics named rhodostreptomycin A and B (Figure 13) were isolated from culture broths of *Rhodococcus* 307C0.[43]

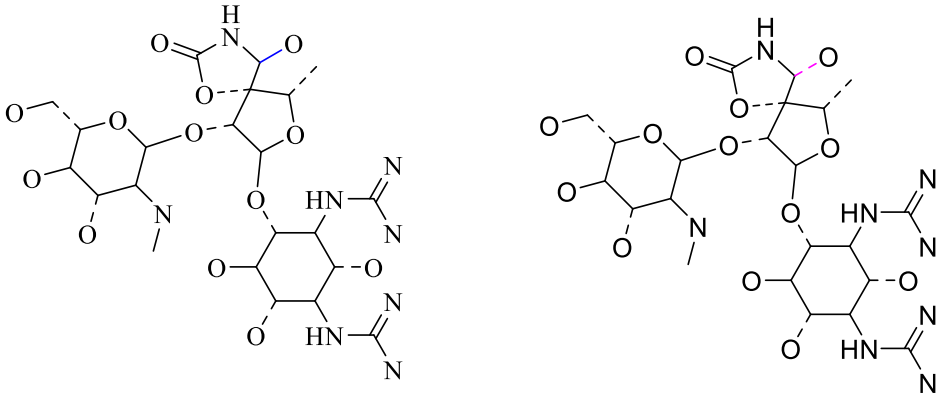


Figure 13. The isomers of rhodostreptomycins. The configuration of carbon attaching hydroxyl group in the oxazine ring is 'R' in A and 'S' in B.[43]

They described these two antibiotics as two isomers of a class of aminoglycosides, differing in structure from actinomycins (polypeptide antibiotics produced by *Streptomyces*), and showed that they had better antibiotic activities against gram-negative and gram-positive bacteria.[43]

With the aim of detecting novel drugs for pharmaceutical applications Nachtigall *et al*. examined *Actinomycete* strains from terrestrial and limnetic habitats.[44] Interestingly, they found a new strain Acta 2259 which exhibits an unusual UV-Vis spectrum and showed a prominent peak

from a mycelium extract at a retention time of 12.9 min in their optimized gradient elution method.[44] During isolation and purification, four aurachins eluted in the same peak at retention time of 12.9 min. They determined the structures as aurachin Q, aurachin C, aurachin D, and aurachin R (Figure.14).

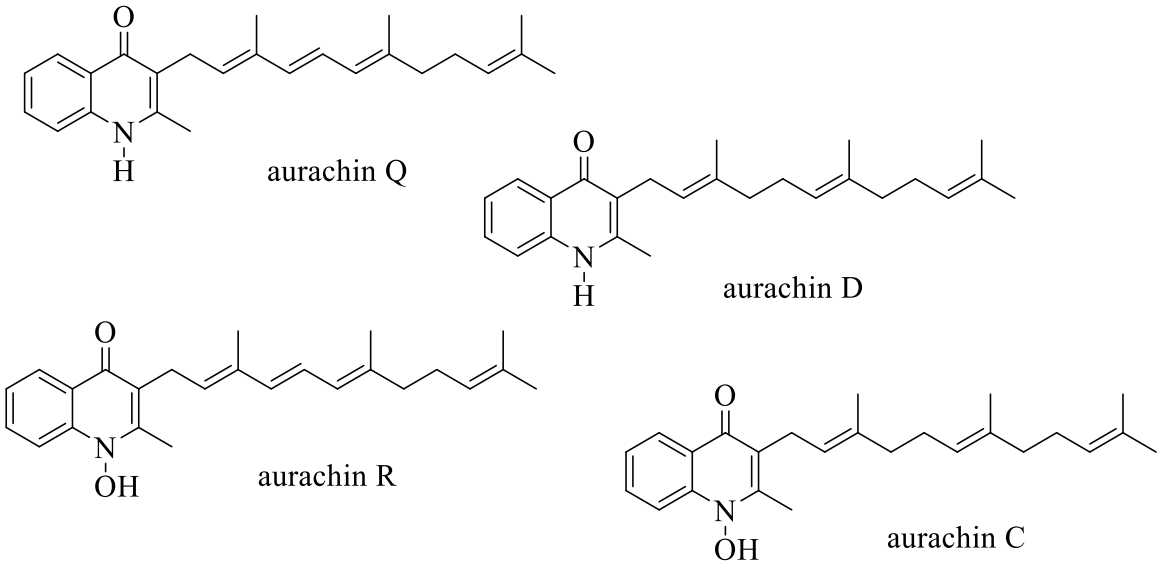


Figure 14. The structures of aurachin Q, D, R, and C.[44]

These compounds showed for antibiotic activity against gram-positive and gram-negative bacteria. Aurachin R and C exhibited moderate antimicrobial activity against *Staphylococcus epidermidis* DSM 20044, *Bacillus subtillis DSM* 347 and *Propionilbacterium acnes* DSM 1987, whereas aurachin Q and D were inactive up to a concentration of 100 μM.[44]

The first example of the isolation of an antibiotic-producing gene from *Rhodococcus* was done using a transposon mutagenesis method by Kitagawa *et al*.[45] They identified a new functional P450 monooxygenase, which catalyzes N-hydroxylation in the quinoline ring skeleton of the aurachin originator. Based on their results, they concluded that the unique function of

P450 monooxygenase might be useful for the development of new antibiotic products with quinoline compounds as a precursor.[45]

Rhodococcus sp. MTM3W5.2

In 2011, soil samples from across East Tennessee were tested for antimicrobial compounds. One of the samples from Morristown, Tennessee, yielded a microbe that showed good inhibition against *Rhodococcus erythropolis* and other *Rhodococcus* species.[32] The soil bacterium that produces this inhibitory compound is similar to *Rhodococcus jostii*.[32] The strain was given the name MTM3W5.2.[32] The inhibitory compound was tested against many different bacteria species, but the best results were against members of the related species within the genus. *Rhodococcus erythropolis* showed a well defined inhibition zone with a size of 36-50 mm.[32] The compound of interest was initially produced at about 15 ^{o}C,[32] but later was produced at a slightly higher temperature than previously reported (approximately 20 ^{o}C).[46] Part of a gene required to produce this inhibitor molecule was previously discovered and found to be similar to a polyketide synthase gene from *Streptomyces*.[46] At that time, research publications clearly indicated that few antibiotics had been identified in the genus *Rhodococcus*.

Figure 15. The colonies of *Rhodococcus* sp. MTM3W5.2.[32]

Research Objectives

Natural products obtained from microbial sources have been a major source of antibiotics which are in the market today. Advances in natural product based screening and developments in NMR techniques for the structural elucidation process have contributed to a revival of interest in natural products for antibiotic discovery.[47] The bacterial genus *Rhodococcus* has recently shown potential to produce new active metabolites.[38-44] The aim of this research work is to exploit genus *Rhodococcus* for the discovery of novel bioactive molecules. This project describes an efficient extraction method and purification of an inhibitor compound from *Rhodococcus* using reversed-phase high-performance liquid chromatography (RP-HPLC) as well as possible structural elucidation of the purified compound of interest. The proposed objectives of this project are outlined and described below

1. Identify inhibitory secondary metabolites from culture extracts produced by *Rhodococcus* sp. MTM3W5.2.
2. Develop an efficient solvent extraction method to give sufficient amount for the purification and characterization of an inhibitory compound.
3. Pre-fractionate crude extract using flash column chromatography or size exclusion chromatography.
4. Compare wild-type and mutant strain of MTM3W5.2 using HPLC profile
5. Isolate the inhibitory compound using semi-preparative RP-HPLC.
6. Purify the active compound by using analytical HPLC column.
7. Analyze the pure compound using high-resolution mass spectrometry to determine the elemental composition of the inhibitory compound.
8. Validate the inhibitory activity of the purified compound.
9. Characterize the compound using IR spectroscopy to detect the functional groups present in the structure of the compound.
10. Analyze the UV-Vis spectra which provides information about any conjugated unsaturated chromophores present in the compound.
11. Analyze the 1D NMR and 2D NMR spectra which can give complete information about the structure of the compound.
12. In the end, crystallize the compound to determine the structure using X-ray crystallography.

CHAPTER 2

EXPERIMENTAL METHODS AND MATERIALS

Reagents

The reagents, stock solutions, and solvents used for the purification, and characterization procedures are discussed below.

HPLC Solvents

Two different mobile phases were used during HPLC separation and analysis; solvent A and solvent B. Water (HPLC-Grade) obtained from Fisher Scientific and deionized water from the Elga PURELAB UHQ water purification system were used as solvent A in some HPLC purification Methods. HPLC-grade methanol (CH_3OH), HPLC-grade acetonitrile (CH_3CN), and HPLC-grade 2-propanol from Fisher Scientific, were used as solvent B in different HPLC separation methods. 2-propanol (HPLC-grade) and DMSO were also used to dissolve crude extract of *Rhodococcus* sp. MTM3W5.2, and to make dilutions of the sample before it was injected into the HPLC system.

Buffers Used in RP-HPLC

Different types of buffers such as 10 mM ammonium formate ($HCOONH_4$), 0.1% potassium dihydrogen phosphate (KH_2PO_4), and 0.1% ammonium hydroxide (NH_4OH) were used in HPLC purification. 10 mM ammonium formate was prepared by dissolving 0.3153 g of ammonium formate in 5 mL of dH_2O to a volume of 500 mL by using 495 mL of dH_2O. The pH of the solution was adjusted to 3.0 using formic acid (HCOOH). 0.1% NH_4OH was prepared by diluting 2 mL of 50% stock NH_4OH with 1 L of HPLC-grade water. 0.1% KH_2PO_4 was

prepared by dissolving 0.5 g of KH_2PO_4 salt in 500 mL of deionized water, and was adjusted to pH of 3.5.

Other Solvents and Reagents

1-pentane and 1-butanol solvents were used in liquid-liquid extraction method to obtain antimicrobial compound from the bacterial strain. Dichloromethane (DCM) was used in column chromatography for the fractionation of the crude extract. Dimethyl sulfoxide (DMSO), ethanol, and acetonitrile from Fisher Scientific were used to dissolve the inhibitor molecule before analyzing by HPLC. Methanol (semiconductor grade) was also used for the HPLC purification. Every other chemical used was acquired from Sigma-Aldrich or Fisher Scientific.

Bacterial Strains

The bacteria used in this study was obtained from Dr. Bert C. Lampson's research lab, Department of Health Sciences, East Tennessee State University. *Rhodococcus* sp. MTM3W5.2 is a wild-type bacterium, that produces the inhibitory compound, was isolated from surface soil.[32] *Rhodococcus* sp. RMP2.31 is a mutant strain that is no longer producing the inhibitory compound of interest.[46] *Rhodococcus erythropolis* IGTS8 was used as sensitive indicator strain for the determination of antimicrobial activity of crude extracts from wild-type and mutant-type bacteria described above.

Types of Culture Media

Rich Medium (RM)

Rich medium[32,46] used for the growth of MTM3W5.2, and RMP2.31 bacteria. The composition and the preparation procedure of the medium adopted from reference.[46]

Mueller-Hinton Medium (MH)

Mueller- Hinton media was used to test the sensitivity of the organism to the inhibitory compound. MH agar plates were prepared by dissolving 19.5 g of Difco™ Mueller-Hinton agar in 500 mL of dH_2O with heating.[46] The dissolved medium was autoclaved for 20 min then transferred the MH medium in the sterile Petri dishes at 55 °C. Solidified MH agar plates were stored at 4 °C for further use.[32] MH broth was also prepared using 11 g of BBL™ Mueller-Hinton broth and 500 mL of dH_2O with the same process.[32, 46]

Extraction Methods

The production of secondary metabolites varies depending on the culture medium and the microorganism cultivated.[14] The solvents used for the extraction were chosen based on increasing polarity. In order to isolate the pure compound in sufficient concentration, the compound has to be extracted several times using different methods. The type of extraction methods used in this research are described below.

Extraction from RM Agar Plates

Mini Scale Extraction. Primarily, the antibacterial secondary metabolite was extracted in small quantities using the agar extraction method, which was adopted from Carr *et al*.[46, 48] *R*. sp. MTM3W5.2 bacterial seed culture was prepared by inoculating a single colony into a 2 mL RM broth, then incubated in a shaking water bath for 18 hours. The seed culture was grown at 27 °C. To produce the compound on a small scale, five (100 mm ×15 mm) RM agar Petri dishes were used. The seed inoculum of the MTM3W5.2 was streaked on RM agar plates by using a sterile cotton swab. Then the plates were kept in an incubator at 19 °C for two weeks. After growth, the agar plate was sliced into small square (1 cm × 1 cm) pieces (Figure 16). The agar square pieces were collected in a 250 mL beaker and ethyl acetate was added to soak the agar pieces. The beaker was wrapped with parafilm and let stand for one day. The ethyl acetate extract was transferred into a 100 mL beaker and was left under fume hood to evaporate the organic solvent. The left over agar slices were again soaked in a small amount of ethyl acetate solution to remove all the active compound from the agar. Once the collective ethyl acetate extract was evaporated to dryness, the dried extract was dissolved in 1 mL of methanol (MeOH) and transferred to an Eppendorf tube and stored at 4 °C for later use.

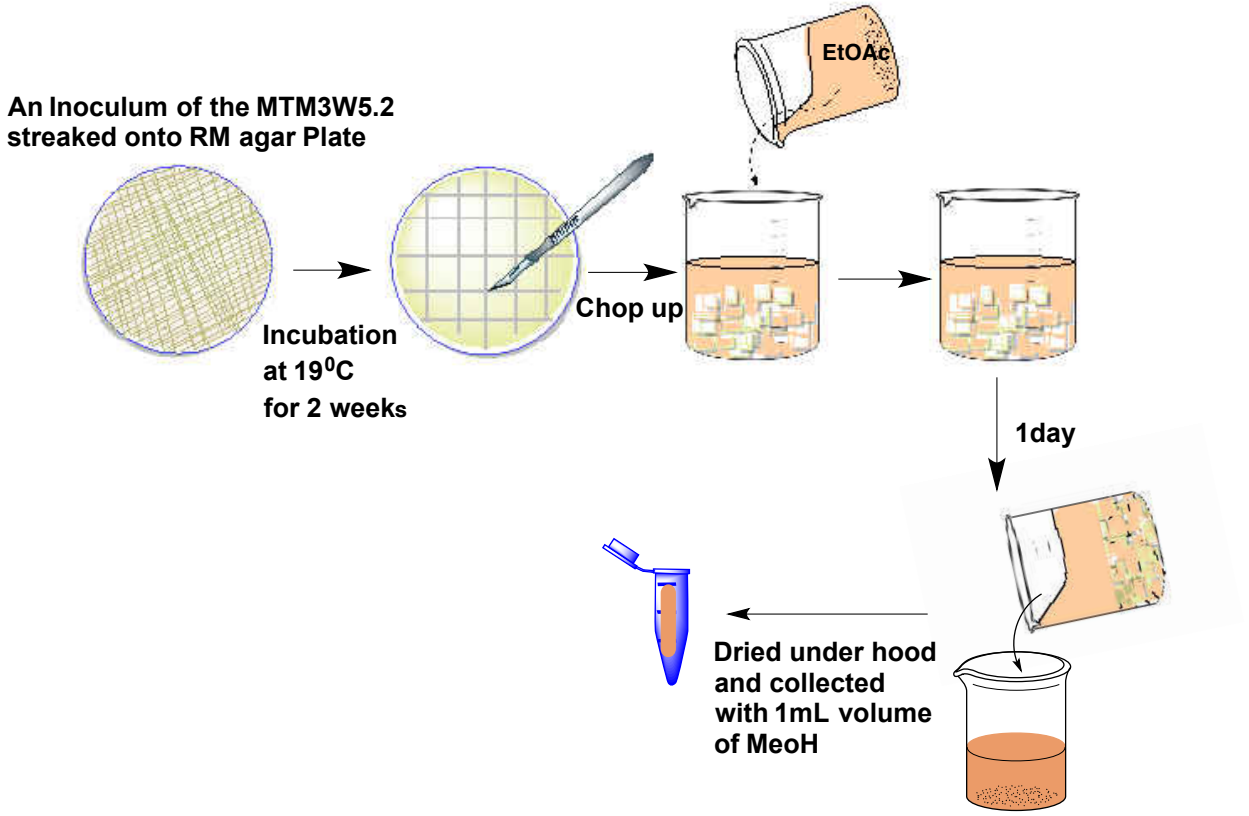


Figure 16. Agar extraction method.[46] An inoculum of *Rhodococcus* sp. MTM3W5.2 was streaked on RM agar plate and incubated at 19 ^{o}C for two weeks. The agar plate was then chopped into small pieces, and metabolites were extracted using ethyl acetate.

Large Scale Extraction. For the purification analysis, a larger amount of the inhibitory compound was needed. A large scale up of agar extraction method[48] was done. Fifteen large (150 mm × 15 mm) RM agar Petri dishes were used. After appropriate growth at 19 ^{o}C for two weeks, the agar plates were chopped into small square (1 cm × 1 cm) pieces. The agar squares were collected in a 2 L beaker, and ethyl acetate (1 L approximately) was added to soak up the agar chips. The beaker was wrapped with the parafilm and left for one day. The ethyl acetate solution was transferred into another 1 L sterile beaker and left under a fume hood to evaporate the organic solvent.

The left over agar chips were again soaked in a minimum amount of ethyl acetate solution to remove all the active compound from the agar. All the ethyl acetate extract was combined and kept under the hood for dryness. The dried extract was dissolved in a small volume of MeOH. It was transferred to polystyrene tubes and centrifuged for 30 min at 8000 RPM speed to remove any solid particles. The Methanol extract was then washed with a 6 mL volume of deionized water for three rounds to remove polar impurities. The leftover organic layer (MeOH) was then again washed with 10 mL of pentane for three times to remove any nonpolar impurities. The separated organic extract was dried under a fume hood. The dried natural product was finally dissolved in a minimum volume of MeOH (1 mL approximately) and stored at 4 °C.

Extraction of MTM3W5.2 Metabolites from RM Broth Culture

Based on work by A. Ward,[46] production of inhibitory compound occurs in stagnant broth cultures of MTM3W5.2. 1500 mL of RM broth was divided into three different 1 L flasks, each containing 500 mL of RM broth were inoculated with 5 mL of the MTM3W5.2 seed culture. The stagnant broth cultures was grown at 19 ^{o}C for two weeks. After two weeks, a 300 mL of 1-Butanol was added to a 1500 mL of entire culture. 1-Butanol is immiscible with the aqueous solution. The flasks were then allowed to shake in an incubator for 1 hr. After shaking, the culture was transferred to centrifuge bottles then subjected to centrifugation at 6000 RPM for 10 min. The centrifuged culture was poured into a separating funnel, the top organic layer (1-butanol) was collected into a beaker, and both layers (organic and the aqueous) tested for activity to ensure the all of the compound had been extracted from the broth culture. The butanol extract was evaporated using a rotor evaporator at 25 °C. The result was 1.51 g of a dried compound had

been recovered from 1500 mL of broth culture.

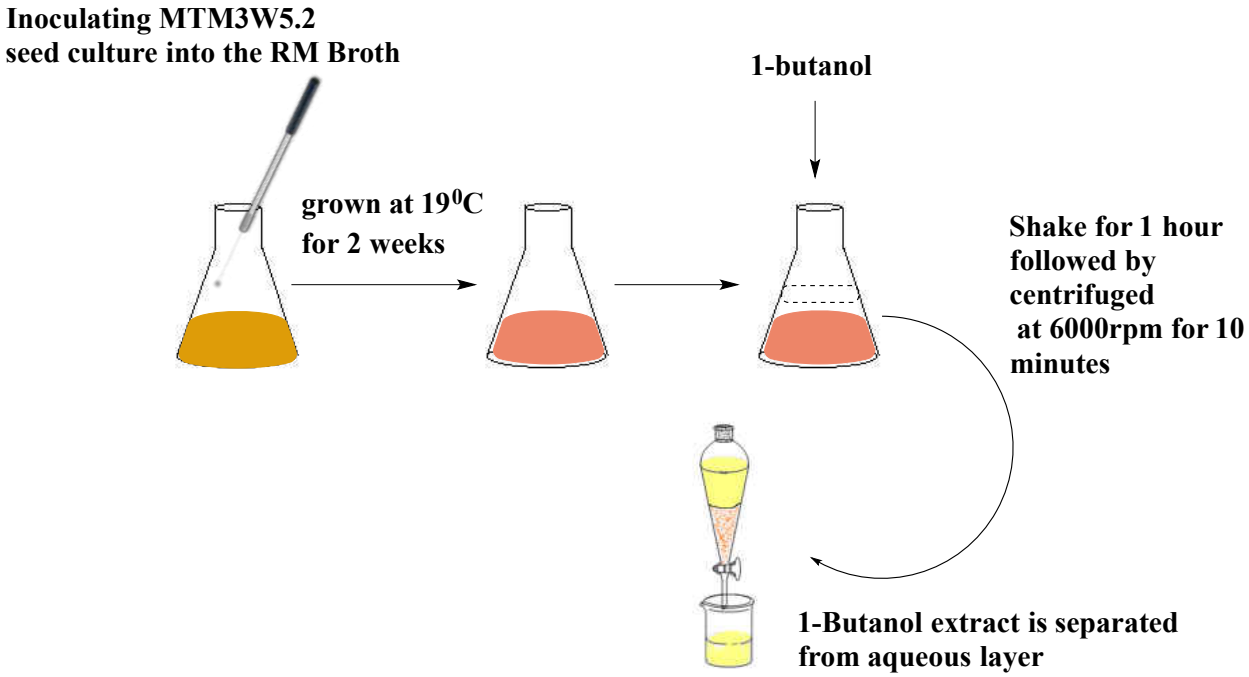


Figure 17. The extraction of the crude sample from a liquid RM broth culture.

Antimicrobial Activity Test

An antimicrobial activity was detected using the disc diffusion method.[49] In this test, a paper disk soaked with an antimicrobial compound was placed on an agar plate where a sensitive indicator bacterium had been inoculated, and the plate was incubated. If the compound stopped the indicator bacteria from growing, then there would be areas of no progress of growth forming zone of inhibition.[49]

Preparation of Seed Culture for *R. erythropolis* IGTS8

R. erythropolis strain IGTS8 was used as a sensitive indicator bacterium to detect the inhibitory compound from culture extracts. First, well-formed isolated colonies of the same type

were selected from an agar plate culture of *R. erythropolis* IGTS8. The single colony of indicator strain was inoculated using a sterile loop into a 10 mL test tube which contains 2 mL of RM broth. The test tube was placed in a shaking water bath at 27 °C for 18 hrs. The suitable turbid seed culture was then used to inoculate the MH plates for the disk diffusion assay.[49]

Inoculation of MH Agar Plate

A sterilized cotton swab was plunged into the adjusted turbid *R. erythropolis* IGTS8 seed culture. The swab was rotated several times and pressed decisively on the inside wall of the tube above the liquid level to remove superfluous liquid.[49] The dried surface of a Mueller-Hinton agar plate was inoculated by swipe the swab over the entire sterile agar surface.[49]

Preparation of Antimicrobial Disks

Sterilized autoclaved paper disks were prepared from whatman blotting paper GB004 with a hole puncher, and were labeled using a pencil. Each disk was soaked with 25 microLiter (μL) of an antimicrobial extract or HPLC column fractions. After complete absorption of the compound, another 25 μL of the antimicrobial compound was soaked onto each disk.

Application of Disks onto Inoculated Agar Plates

Prepared and dried disks were placed onto the surface of the agar plate, previously inoculated with the indicator strain. Each disk was pushed down to ensure complete contact with the agar surface. After 16 to 18 hrs of incubation, each plate was examined. If the plate was adequately streaked, the developing zones of inhibition would be consistently spherical, and there was a confluent lawn of growth.[49] If individual colonies appeared, the inoculum was too

low in concentration and the test was repeated.[49]

Chromatographic Methods

In the present work, several chromatographic techniques were used to separate the antimicrobial compound from the crude extract.

Flash Column Chromatography (FCC)

Silica gel flash chromatography is a popular method of preliminary separation in drug discovery.[50] The prominence of flash chromatography is predominantly due to its simple packing procedure,[51] low operating pressure,[50] low cost for instrumentation.[51] The main purpose of FCC is for pre-workup because its high loading capacity, and its operability at low to medium pressures.[52] The principle involved in FCC is that the mobile phase was rapidly pushed through a glass column with a large inner diameter under low pressure.[51] The basic theory is the importance of partitioning between a mobile phase and a stationary phase to separate the compounds in a mixture. The molecules in the crude mixture had a different affinity with the solid phase,[50] which can be caused by a charge or adsorption.[50] The glass column was packed with the stationary phase of defined-particle size silica gel (40-63 μm).[51] The common stationary phases are silica gel (SiO_2) (silica gel 60 or silica gel 230-400) and alumina (Al_2O_3).[53] In the current work, silica gel was preferred because of the slight acidity in nature, so good separation would be expected.

Procedure. A chromatography column was sealed with a piece of cotton wool on the bottom. A small layer of sand was added across the diameter of the column (approximately 1-2

cm). Silica gel 60 was loaded onto the column. Vaccum was applied through the stopcock at the bottom of the column. The vacuum condensed the silica gel and packed the column tightly. Sodium sulfate (Na_2SO_4) was added on top of the column as a protecting agent. The mobile phase, dichloromethane (DCM) was passed onto the column with a vacuum till all the silica adsorbent packed perfectly, and the all the solvent eluted. The column was filled with sufficient eluent to prevent the column drying. The flash column should not have any air bubbles[54] before applying the crude extract on it. The dried butanol extract was mixed with silica and the solution was prepared with a minimum amount of DCM. This crude mixture was loaded atop of the column using a pipette then a protecting agent was added. Purification was carried out with a combination of two solvents, polar (methanol), and nonpolar (dichloromethane). Different ratios of volumes of DCM to methanol was used to elute the various compounds from the column (Table 1).

Table: 1 Ratios of DCM and Methanol Used in Flash Column Chromatography.

DCM (volume in mL)	Methanol (volume in mL)	DCM/MeOH
300	0	1:0
300	10	30:1
300	20	15:1
300	30	10:1
250	50	5:1
150	150	1:1
0	300	0:1

A total of 21 column fractions, each containing 100 mL of eluent, were collected. UV-Vis absorption values were recorded for 21 fractions using Carey 8454 UV-Vis

spectrophotometer. The fractions were further tested for antimicrobial activity by the disk diffusion method. The active fractions were pooled, and the solvent was evaporated to dryness using N_2 Gas. The dried mixture was dissolved in 2-propanol and stored at 4 °C for further purification.

Size Exclusion Chromatography (SEC)

Size exclusion or gel filtration chromatography was also performed for better separation of the compounds in the crude butanol extract. In this chromatography, molecules were separated based on their size as they passed through the stationary phase packed in a column.[55] The larger size molecules cannot fit in the pores of the media and run faster through the stationary phase with the mobile phase, whereas the smaller molecules can easily settle in the pores of medium and take a longer time to elute out of the column. Sephadex™ LH-20 was used for the purification of secondary metabolites using organic solvents as mobile phases. The Sephadex is prepared by hydroxypropylation of Sephadex G-25 (cross-linked dextran), a bead with pores of different sizes.[55] It has both hydrophilic and lipophilic characteristics, so it swells in aqueous solutions and organic solvents.[56] Molecular exclusion chromatography is a simple isocratic elution of liquid chromatography.[56] Sephadex™ provides high-resolution separation with short elution times, and substantial recovery.[55] Since the medium is Sephadex LH-20, the method is termed as Sephadex LH-20 column chromatography.

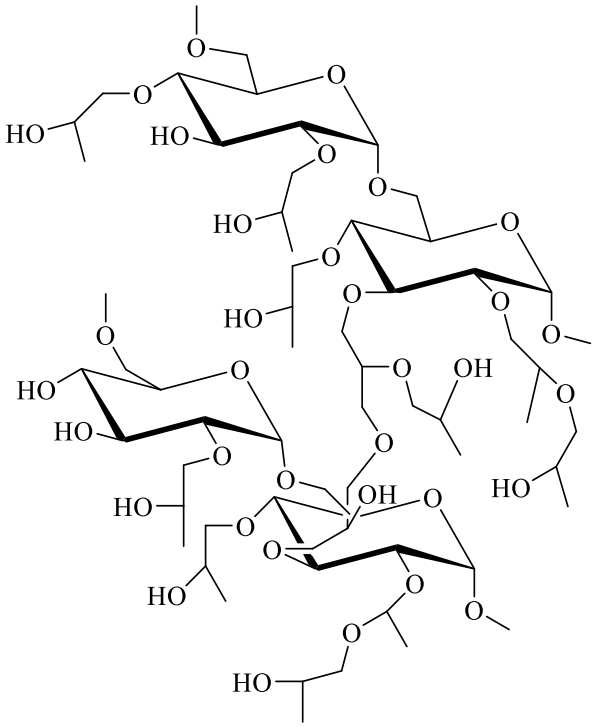


Figure 18. Structure of Sephadex LH-20 (cross-linked hydroxypropylated dextran).[56]

Procedure. 28.78 g of Sephadex™ LH-20 dry powder (particle size range 18 µm-111 µm)[55] was taken into a 250 mL beaker and swollen in excess methanol for 2 hrs. The beaker was shaken every 30 minutes to remove any air bubbles trapped in the medium. The Sephadex slurry was poured onto the chromatographic column through a glass rod to fill the column evenly and without air bubbles forming. After filling the column with the medium, the top of the column was connected to the solvent reservoir, which allowed it to flow through the medium with atmospheric pressure. Once the column was tightly packed with Sephadex resin, the outlet was closed. The dried 1-butanol extract was dissolved in a minimum amount of isopropanol and applied to a Sephadex column eluted with methanol as a mobile phase. The fractions collected were tested for antimicrobial activity, and UV-Vis absorption values were recorded using Carey 8454 UV-Vis spectrophotometer. The active antimicrobial fractions were pooled and dried using

rotor evaporator. The dried compound was then subjected to semi-preparative HPLC followed by analytical HPLC purification.

High-Performance Liquid Chromatography

HPLC works at high pressure to push the mobile phases through a column containing stationary chemical groups linked to very different units, which results in better resolution of peaks of separation.[57] In the current work, a Shimadzu LC-10AS HPLC instrument was used to purify the antimicrobial extract. It was equipped with solvent systems, a detector, a controller, a column, and a sample injection system.

Solvent System. LC-10AS HPLC instrument was equipped with a two-solvent system: solvent A and solvent B. In HPLC, volatile mobile phases provides a good separation of compounds.[58] Two pumps A and B were used to pump the mobile phase to generate a maximum pressure of 6000 psi (lb/in.^{2}) or 414 bar.[59] Formation of air bubbles is a major concern in liquid chromatographic purification; it causes a problem in the solvent delivery and forms specious peaks in the output by the detector.[60] Mobile phase degassing is the best solution to avoid the formation of air bubbles inside the pump.

Vacuum Degassing. Although some modern HPLC instruments are furnished with the degassers,[59] it is necessary to purify and degas the solvents using a convenient method. In the present work, a vacuum method was used. Solvents were placed in a HPLC container with a stir bar, which was connected to a vacuum pump by the stopper. Withdrawing a vacuum using the

pump removes the dissolved air in solvents. Stirring the solvent aids in removing all the air inside the solvents.

Detectors. The current purification analysis was carried out using a Shimadzu LC-10AS HPLC instrument equipped with UV-Vis detector (SPD-10A). UV-Vis detectors are appropriate for a broad range of biological analytes because most organic metabolites show strong absorption in the UV range. These detectors are highly selective and sensitive (10^{-10}-10^{-11}g analyte/s),[59] with low background noise detection with almost all HPLC-grade solvents.[61]

Analytical Columns Used in HPLC. The columns used in liquid chromatography vary in length and inner diameters. Modern columns are available with an inner diameter of 1 to 4.6 mm are packed with a particle size of 3 or 5 µm.[59] A small volume of solvent was required because of a higher number of theoretical plates in the analytical columns.[59] Both preparative and analytical columns were used in the current purification by RP-HPLC. The semi-preparative Hamilton polymeric reversed phase-1 column with inner diameter of 21.2 mm and a length of 250 mm, was packed with poly (styrene-divinyl benzene) (PSDVB) co-polymer with a particle size of 12-20 µm and the pore size of 100 Å. The high hydrophobicity and surface area of the solid phase allow for semi-preparative separation of a bioactive crude extract. Analytical columns such as Kinetex® phenyl hexyl 100 Å with an inner diameter of 4.6 mm and a length of 250 mm was used in a preliminary purification of an extract. Kinetex® 5µm EVO C18 100 Å with an inner diameter of 4.6 mm and the length of 150 mm (from Phenomenex, Torrance, CA, USA) was used to perform multiple rounds of HPLC purification until the extract was purified enough for further analysis. Guard columns from Phenomenex were used to enhance the life of

the column by removing dust particles and contaminants introduced by the solvents.[59] The stationary phase for the phenyl-hexyl column was made from a phenyl hexyl hydrocarbon chain was linked to silica, which enables a greater separation of aromatic compounds. The stationary phase for the C18 column made of a long C18 chain of carbons bonded onto a silica support. (Figure 19)

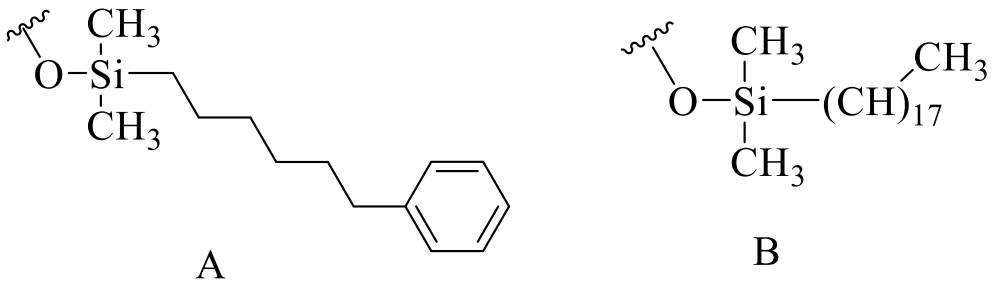


Figure 19. The structure of stationary phases. (A) phenylhexyl stationary phase, (B) C18 stationary phase.

Instrumentation

Shimadzu LC–1OAS, a product of Shimadzu Scientific Instrument Incorporated, was used to analyze crude inhibitory fractions. It is equipped with a SCL–1OAVP system controller, and a SPD–1OA UV – VIS detector. All HPLC experiments were carried out at a wavelength of 254 nm. The temperature maintained at 25 ^{o}C. The HPLC back pressure was maintained within the range of 1200–3800 psi.

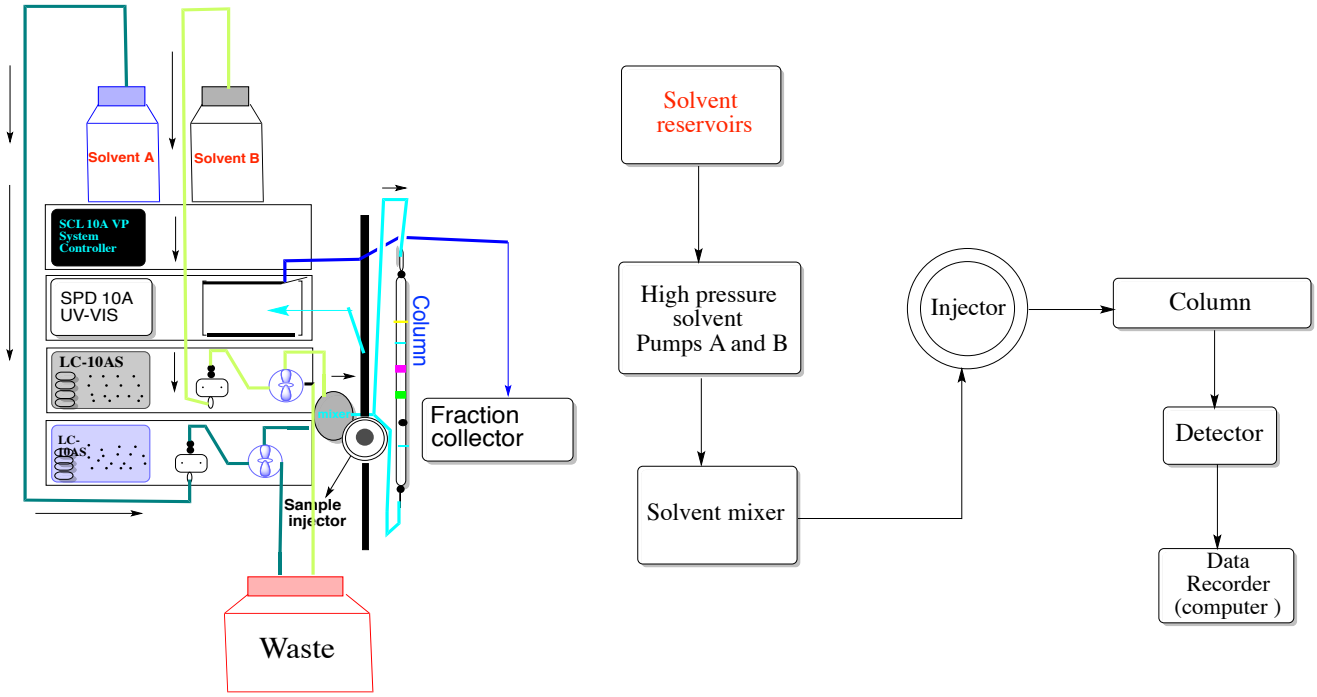


Figure 20. Schematic diagram of the Shimadzu LC–1OAS HPLC system.

Normal and Reversed Phase HPLC

There are two types of partition chromatography, based on the relative polarity, permittivity,[62] also called the dielectric constant[62] of the stationary and the mobile phases.[59] Solute molecules have distinctive interactions with the mobile phase and stationary phase. In normal phase chromatography, a highly polar stationary phase and non-polar solvents as a mobile phase are used to carry out the separation.[63] The less polar compounds are eluted first. The higher the polar mobile phase, the shorter the elution times. In RP-HPLC, the stationary phase is non-polar, polar solvents serve as mobile phase, and highly polar compounds are eluted first.[59] Molecules are eluted typically as a function of their ordinary polarizability.[62] The greater the pressure, the longer retention times for very polarizable compounds and the smaller the retention times for non-polarizable compounds.[62]

Method Development

The development of a method to achieve a better separation of molecules in liquid chromatography is difficult because the analyte interacts with both stationary and mobile phases.[59] Suitable separations by HPLC need to have an appropriate equilibrium of intermolecular forces among the solute, the mobile phase, and the stationary phase.[59] Developing a proper method to obtain a good separation of compounds starts by selecting an appropriate column. Successful separation was obtained when the polarity of stationary phase and analyte match but differ from the mobile phase.[59] An error and trial[59] approach can establish the safest method of separation in RP chromatography. Various trials were made with different mobile phases (different solvent modifiers)[59] until a reasonable resolution was determined. This approach is time-consuming, if the proposed method failed to show a good separation, a different column with different composition of a mobile phase should be tried to determine a better method. Changing the composition of mobile phase and changing the packing material in the column might give better resolution of peaks in the chromatogram.[64]

Isocratic Elution

HPLC elution without changing the composition of the mobile phase is called an isocratic elution.[59] This method involves only one solvent or sometimes a solvent mixture. Larger retention times occur especially with a wide range of polar compounds subjected to HPLC.[59,65] Some sample components might not elute from the column with isocratic flow of the solvent and are retained for a longer time in the column, which causes the contamination of the stationary phase.[65]

Gradient Elution

Gradient elution involves two or more mobile phases, which differ in their polarity and composition.[59] The gradient elution method is programmed using different concentrations of the two solvents. The ratio of solvent A and solvent B is varied in a programmed way, sometimes continuously and sometimes in a series of steps.[59] The solvent gradient method is preferable to elute all the components of the sample from the column by increasing the concentration of solvent, because it decreases the time of separation significantly without losing the resolution.[59] The advantages of gradient elution include a better resolution, quicker separation, and no column contamination.[65] The considerable drawback is longer re-equilibration time to return to initial conditions for the next round of purification.[59]

Optimization of Mobile Phase

One of the aims of this project was to develop a method that would carry out the elution of the active compound efficiently through HPLC. The elution method was optimized by using a combination of water and methanol as solvents. The reason behind choosing them was that water is not toxic, while HPLC-grade methanol is a good polar solvent to use and it is exclusively soluble in water and thought to be a better solvent for the purification procedures.[57] The change in composition of mobile phases led to substantial changes in separation.[66]

HPLC Analysis of Crude Extract. Initially HPLC system was programmed using a different ratio of methanol to water in order to analyze the crude extract. In this elution method, methanol concentration was increasing from 40% to 100% from 3.20-48.4 min; from 48.4 to 63.40 min increased to 100% methanol (Method I, Table 2). The phenyl hexyl column was

equilibrated for 20 min using methanol gradient 40: 60 (methanol: H_2O) before injecting the sample. The injection volume of crude extract was 20 µL.

Elution Method Used in Semi-Preparative HPLC Analysis. The mixture of active compounds eluted from the Sephadex LH-20 column was subjected to semi-preparative HPLC purification. In this elution method acetonitrile (solvent B) and 0.1% KH_2PO_4 (solvent A) were used as mobile phases. Solvent B concentration was from 30% to 100% from 3.20-63.40 min, 100% B maintained from 63.40-78.40 min (Method II, Table 2). The column has been equilibrated with 30: 70 (acetonitrile/ 0.1% KH_2PO_4) for 20 min before injecting the sample.

Gradient Methods Employed in Final HPLC Analysis. The gradient elution method had to be modified using different mobile phases to achieve the highest purity as possible. Acetonitrile (solvent B), H_2O, 0.1 % NH_4OH, and 10 mM ammonium formate as solvent A were used for the final stage purification by Kinetex® 5µm EVO C18 100 Å column. The gradient method used in analytical HPLC purification is graphically represented in Figure 21. The solvent B concentration started from 20% to 70% from 3.20 -63.40 min; was increased to 100% B from 63.40-73.40 min; then 100% B was allowed to wash the column for 20 min from 73.40-93.40 min (Method III, Table 2).

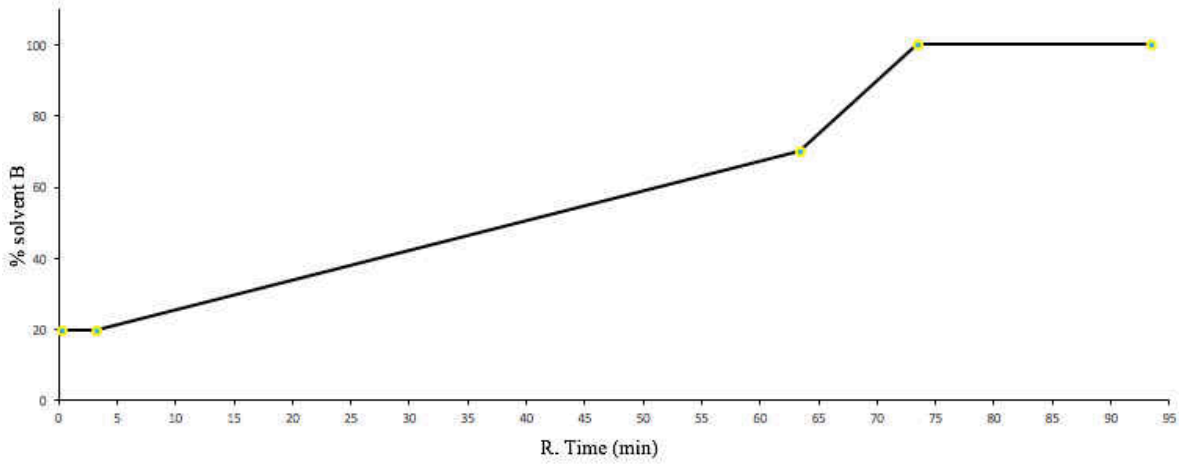


Figure 21. A graphical illustration of the solvent gradient elution method III. The composition of acetonitrile (Solvent B) is charted as a function of retention time (min).

Table 2. Chromatographic Gradient Elution Methods Used in this Work. Methanol in method I, acetonitrile in method II and III was used as solvent B. RP represents reversed-phase mode.

Elution method	HPLC column	HPLC mode	Solvent B%	Flow rate mL/min
Method I	Kinetex® phenyl-hexyl (250 mm × 4.6 mm, I.D, 5 µm)	RP	3.20-48.4 min, 40-100% B 48.4-63.4 min, 100% B	1.0
Method II	Hamilton PRP-1 (250 mm × 21.2 mm, I.D, 12-20 µm)	RP	3.20-63.4 min 30-100% B 63.4-78.4 min 100% B	5.0
Method III	Kinetex® EVO C18 (150 mm × 4.6 mm, I.D, 5µm)	RP	3.20-63.40 min 20-70% B 63.4-73.40 min 70-100% B 73.40-93.40 min 100% B	1.8

Structural Elucidation Methods

Structural determination of natural products is a very laborious process, and typically is the "bottleneck" [67] in the drug discovery.[67] It is easy to elucidate the structure of well-known natural products, but it can absolutely be thought-provoking for new compounds.[67] It involves collecting data from plentiful spectroscopic sources such as UV-Vis spectroscopy, IR spectroscopy, mass spectroscopy, and NMR (1D and 2D).[68, 69]

Ultraviolet-Visible Spectroscopy (UV-Vis)

UV-Spectra were recorded using the Carey 8454 UV-Vis spectrophotometer from Agilent Technologies. 1000 µL quartz cuvettes were used with a path length of 1 cm for UV-Vis absorbance. The UV absorbance was monitored at a wavelength ranging from 280 to 400 nm for all the fractions collected from flash column chromatography, and Sephadex column chromatography. Methanol was used as a blank to record absorbance for fractions collected. The final pure compound obtained from HPLC was dissolved in acetonitrile and its UV-Vis spectrum was recorded at 210 to 400 nm. Acetonitrile was used as a blank.

Infrared Spectroscopy (IR)

IR-spectra were recorded in KBr pellets with a spectral range of 6,000-350 cm^{-1} using a Genesis II FTIR spectrometer.

Liquid Chromatography-Mass Spectroscopy (LC-MS)

LC-MS analysis was performed on crude extracts from the wild strain MTM3W5.2 and mutant strain in Dr. Shawn Compagna laboratory at the University of Tennessee. The extracts

were separated on a Kinetex® 5 µm phenyl-hexyl 100 Å column attached to an UltiMate 3000 autosampler and UHPLC pump, coupled to an Exactive benchtop Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The analyses were done using the gradient elution method with solvents A and B containing 0.1% aqueous formic acid and acetonitrile, respectively. The program started with 30% B which was increased to 40% B from 0-5 min. After 5 to 10 min it was increased to 50% B; from 10 to 15 min it increased to 60% B; from 15 to 20 min it increased to 80% B; from 20 to 25 min increased to 100% B, which was maintained from 25 to 35 min, at a flow rate 1.0 mL/min. From 35 to 35.5 min 100% B was dramatically decreased back to 30% B, which was kept from 35.5 to 40 min, to reconstitute the column before the next run. The column oven temperature was maintained at 25°C, and the temperature of the autosampler was set to 4 °C. An electrospray ionization (ESI) probe was used to ionize the sample at a 3.5 kV spray voltage. The sheath gas flow was set to 50 units and the auxiliary gas was set to 25 units. The S-lens level was set to 50 units. The conditions were kept constant for positive ionization mode acquisition. External mass calibration was performed using the calibration standard mixture and protocol from ThermoFisher. For complete scan profiling experiments, the MS was run with a resolution of 140,000 and with a scan range of 80-1000 m/z, for all ion fragmentation (AIF) scans. The resolution was 140,000 with a scan range of 80-1000 m/z, with a normalized collision energy (NCE) of 20 eV. Xcalibur software was used to examine the results.

High-Resolution Mass Spectrometry (MS)

The high-resolution mass analysis was carried out on Bruker maXis II mass spectrometer. The sample was ionized using electrospray ionization in positive (ESI+) mode. The sample was

dissolved in an acetonitrile and water mixture (50: 50) with 0.1% formic acid.

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H-NMR, ^{13}C-NMR, and 2D-NMR experiments such as Heteronuclear Single-quantum Correlation Spectroscopy (HSQC), Heteronuclear Multiple Bond Coherence (HMBC), Correlation Spectroscopy (COSY) spectrum, Rotating Frame Nuclear Overhauser Effect Spectroscopy (ROESY), and Total Correlated Spectroscopy (TOCSY) were performed on a Bruker Avance II 600 MHz NMR spectrometer (^{1}H 600 MHz; ^{13}C 150 MHz). It was equipped with a 5 mm probe using deuterated methanol (CD_3OD) as a solvent. All NMR experiments were carried out at room temperature. Chemical shift values were measured in parts per million (δ, ppm). The coupling constants value (J) was described in Hz. The splitting patterns of proton signals were also designated as follows: singlet (s), doublet (d), a doublet of doublets (dd), a doublet of the doublet of doublets (ddd), triplet (t), the quartet (q), and the multiplet (m).

CHAPTER 3

RESULTS AND DISCUSSION

Development of Extraction Method

The antimicrobial compound of interest was obtained from a culture extract of *Rhodococcus* sp. MTM3W5.2 grown on RM agar plates and extracted using ethyl acetate. Extraction from RM agar plates was a very tedious and time-consuming process involving the use of large volumes of the extraction solvent to obtain a significant amount of the compound of interest. Thus, a more efficient method was required to give sufficient amount of the active crude metabolites to enable purification and possible characterization of the antimicrobial molecule. To develop a better extraction method, our research team set out to determine first the polarity of the crude ethyl acetate sample by using HPLC. This analysis helped us to determine how polar our compound was, and what the best solvent for extraction would be. 20-100 µL (normal injection volume) of crude agar ethyl acetate extract was separated on phenyl hexyl column using methanol and water as the mobile phase. The resultant chromatogram displayed both the suspected active compound (medium polarity) as well as very polar and non-polar impurities. The fractions collected were tested for their antimicrobial activity, and the fractions with medium polar components showed activity against the sensitive indicator strain. To further increase the efficiency of extraction of the compound of interest as proposed previously, various kinds of solvents were used to extract the compound to ascertain the best extraction solvent.

Effect of Solvents on Extraction of Inhibitory Compound

An extraction method involving the use of two solvents (water and n-pentane) was employed to remove very polar and non-polar impurities thus facilitating the efficient extraction

of the inhibitory compound of interest. The idea was to use water (polar) to remove the polar impurities and n-pentane (nonpolar) for the removal of non-polar impurities. Although this method was efficient in enhancing ease of purification, the research team still aimed for the development of a more effective extraction protocol to scale up the compound by employing the use of RM broth rather than RM agar plates.

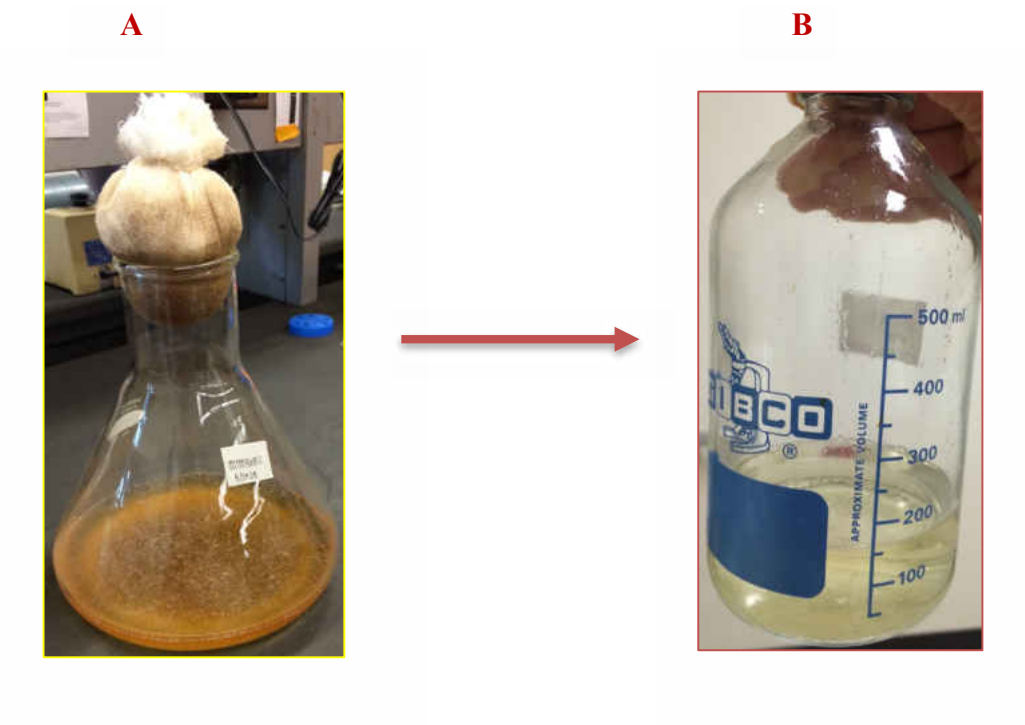


Figure 22. The 1-butanol crude inhibitory extract (B) from 1500 mL RM broth culture (A).

Using RM broth proved to be more effective and easier as the HPLC chromatogram of the crude extract isolated from RM broth was not as complex as the peaks eluted in agar chromatogram. Based on the eluting profile on HPLC, the extraction from RM broth was preferred for scaling up the compound. An efficient extraction method was developed using 1-butanol as a solvent. 1-butanol is not highly soluble in aqueous solution, and it forms an

immiscible layer, which helps to extract the compound more efficiently. Alcohols are the more commonly used solvents for efficient extraction of bioactive compounds from bacterial cultures.[70]

Detection of Activity by Disk Diffusion Assay

Disk diffusion assay was used to detect the antimicrobial compound throughout the purification process. *Rhodococcus erythropolis* IGTS8 was used as an indicator strain as previous research had shown that it is super sensitive to the inhibitor compound produced by MTM3W5.2. [32, 46] The aqueous layer that separated from RM broth culture was tested to ensure that all the active compound(s) was extracted into the organic solvent (1-butanol). The mutant extract was also tested against the indicator strain. During every pre-fractionation process, the fractions were subjected to disk diffusion assay along with UV-Vis spectroscopy to monitor the retention of activity in the fractions. After analysis of pooled bioactive fractions using HPLC, every fraction eluted from the column was also tested for bioactivity.

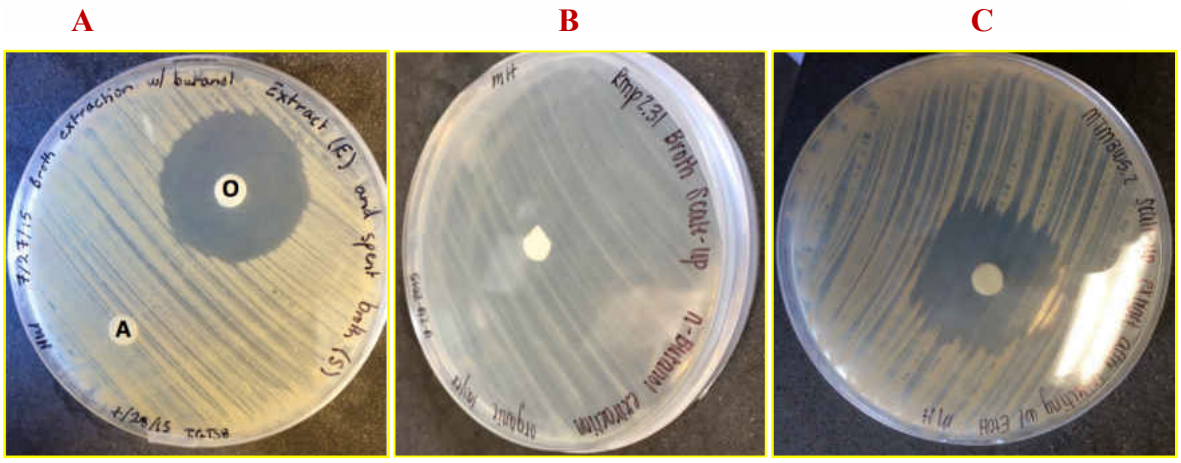


Figure 23. Disk diffusion assay. (A). The organic layer (disk labeled with O) and the aqueous layer (disk marked with A) were tested for antibacterial activity during the extraction process. (B). Disk diffusion assay of mutant strain RMP2.31. (C). Antibacterial activity of ethyl acetate crude extract against *Rhodococcus erythropolis* IGTS8

Preliminary HPLC Analysis of Crude Extract

The crude agar ethyl acetate extract of MTM3W5.2, and RMP2.31 were priliminarily analysed using RP-HPLC. 20 µL of the diluted crude extract was injected into the HPLC loop and eluted through a Kintex 5µm RP Phenyl-Hexyl 100Å column (250 × 4.6 mm), at a 1 mL/min flow rate, and UV absorption wavelength set at 254 nm. A gradient MeOH /H_2O elution [mobile Phase: 40-100% MeOH (3.20-48.40 min) and 100% MeOH (48.40-63.40 min)] was used. The fractions with the peaks of interest were collected and tested for activity against *R. Erythropolis* IGTS8. Fractions 3 to 5 (in Figure 24 with red color) retained antibacterial activity and eluted with retention time of 41.90, 43.43, and 44.54 min, respectively, and were reproducibly observed. All five peaks, which include active peaks, in the range of 40-45 min, were not observed in the mutant chromatogram as shown in Figure 25. These results indicate that the mutant does not produce this active compound. To further confirm this assertion, fractions collected from the range between 40 and 45 min from the mutant extract HPLC chromatogram

were also tested for activity. As shown in Figure 26B, no zone of inhibition was observed thus proving that there was no active compound produced by the mutant RMP2.31.

The major peak at 45.5 min was collected and dried using N_2 gas and tested for solubility using different solvents like acetonitrile, DMSO, methanol, ethanol, and 2-propanol. The compound completely dissolved in methanol, acetonitrile, and DMSO but partially dissolved in ethanol and 2-propanol.

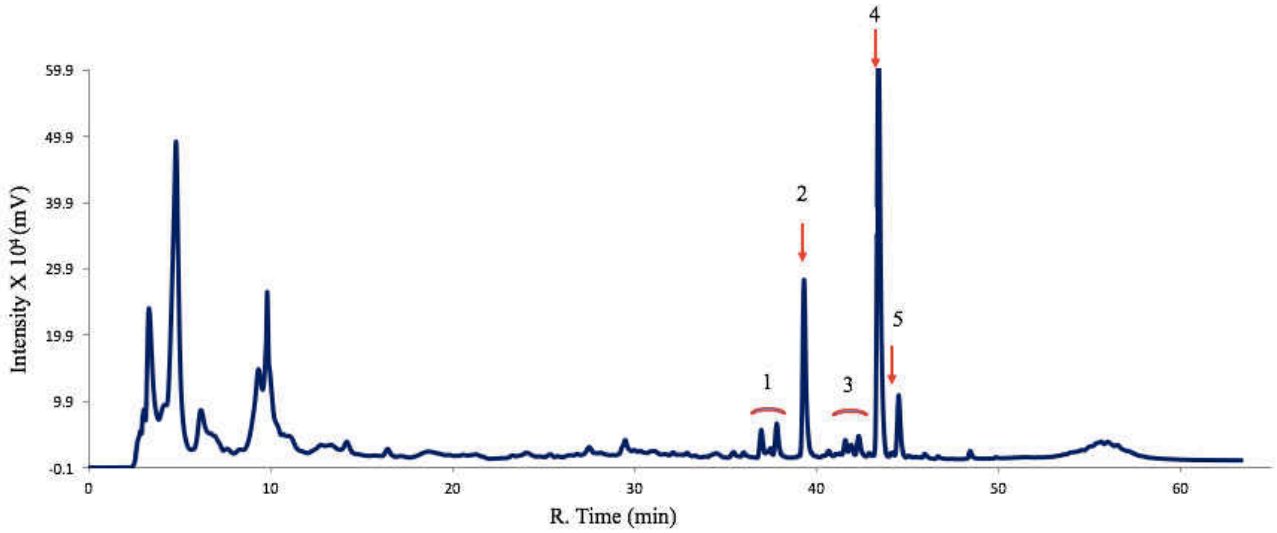


Figure 24. HPLC chromatogram of agar extract of MTM3W5.2 in methanol.

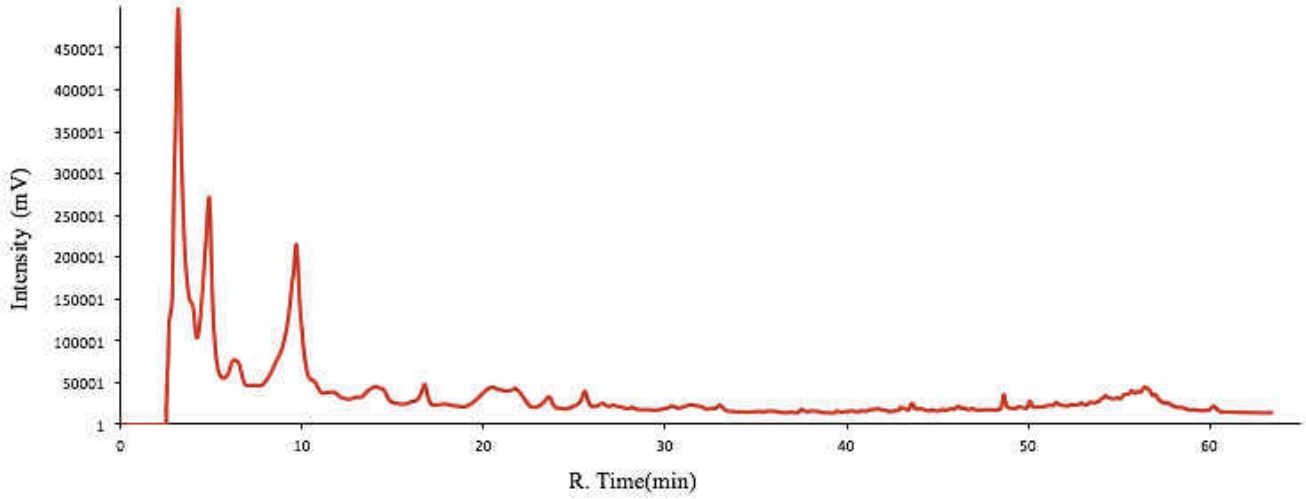


Figure 25. HPLC chromatogram of agar extract of RMP 2.31 in methanol.

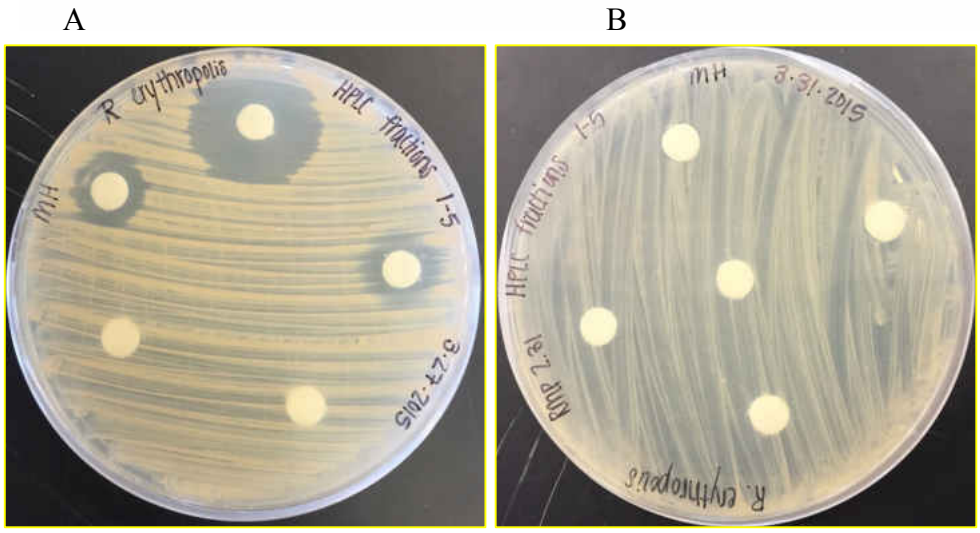


Figure 26. Disk diffusion assays of HPLC fractions.

(A) Among five peaks collected from MTM3W5.2, peaks 3, 4 and 5 were active. The 4th peak showed a large zone of inhibition (disks are placed in a clockwise direction). (B) Five peaks collected from the mutant strain RMP2.31 showed no activity against the indicator strain.

Comparison Between MTM3W5.2 and RMP2.31 HPLC Profile Using RM Broth Extracts

One of the objectives of this research was to compare the HPLC profile of metabolites extracted from the mutant RMP2.31 with the wild type MTM3W5.2. The newly identified mutant strain *R.* sp. RMP2.31 has been reported as a non-producer of the same antimicrobial compound.[46] The wild-type strain MTM3W5.2 is a producer of the antimicrobial compound of interest. To compare differences between both the mutant and wild-type extracts through HPLC, both strains were grown under the same conditions (in RM broth), and their secondary metabolites were extracted in the same fashion as well.

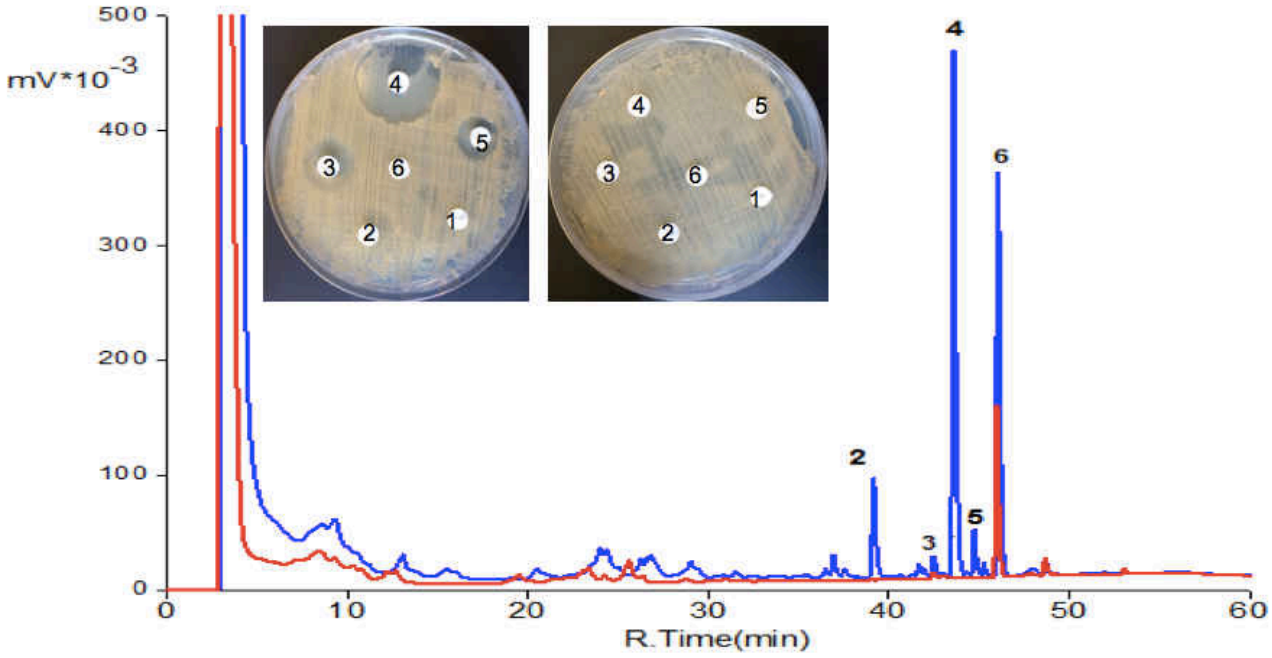


Figure 27. Stacked HPLC chromatogram of mutant RMP 2.31 (red), and wild-type MTM3W5.2 (blue) extract from RM broth cultures. The insert in the chromatogram represent the disk diffusion assay of fractions (1-5) collected from wild-type (left) and from mutant (right).

Remarkably, the HPLC analysis of mutant extract (Figure 27) validated that the mutant RMP2.31 was no longer producing an active compound which is clearly seen in stacked HPLC

chromatograms of wild-type and mutant extracts (Figure 27).

Comparison of the Wild and Mutant Strain Extract LC-MS Profile

The LC-MS results revealed that both strains have different chromatographic and MS characteristics. The HPLC profile had already confirmed that the active compound was not generated in cultures of the mutant strain. In addition, the LC-MS spectrum of wild-type extract showed the active compound had a molecular ion peak at m/z of 911.546 (Figure 28). This molecular ion peak and some significant mass peaks were absent in the mutant broth extract (Figure 29). LC-MS profile also confirmed that the mutant strain is no longer producing the antimicrobial compound.

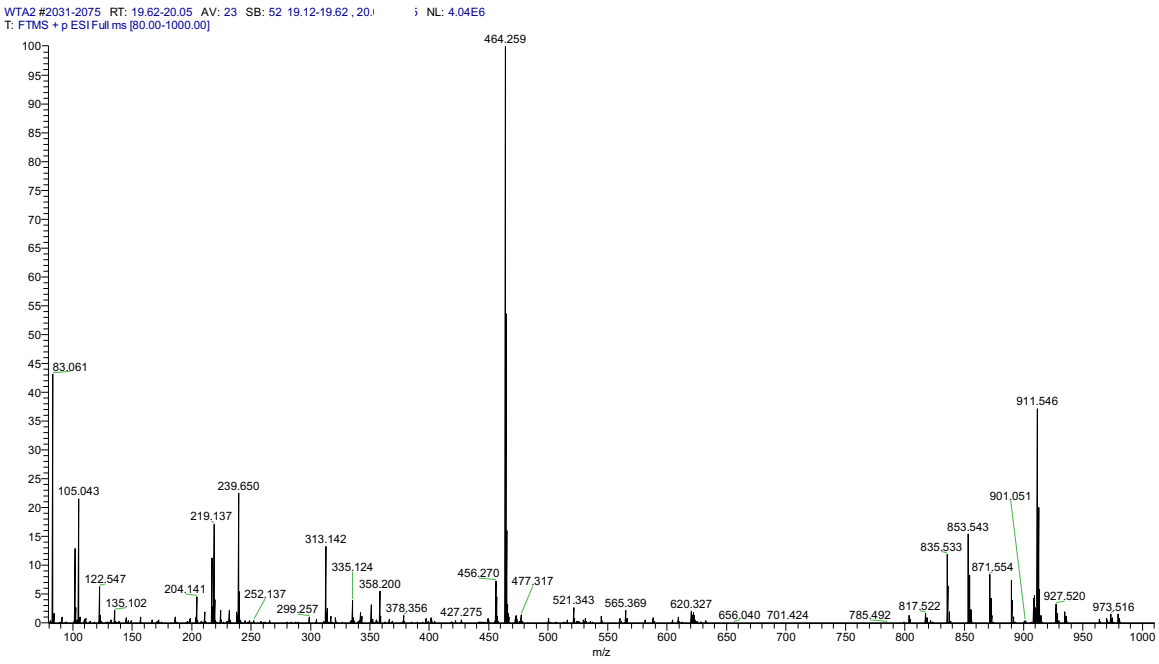


Figure 28. The full mass spectrum of MTM3W5.2 crude extract.

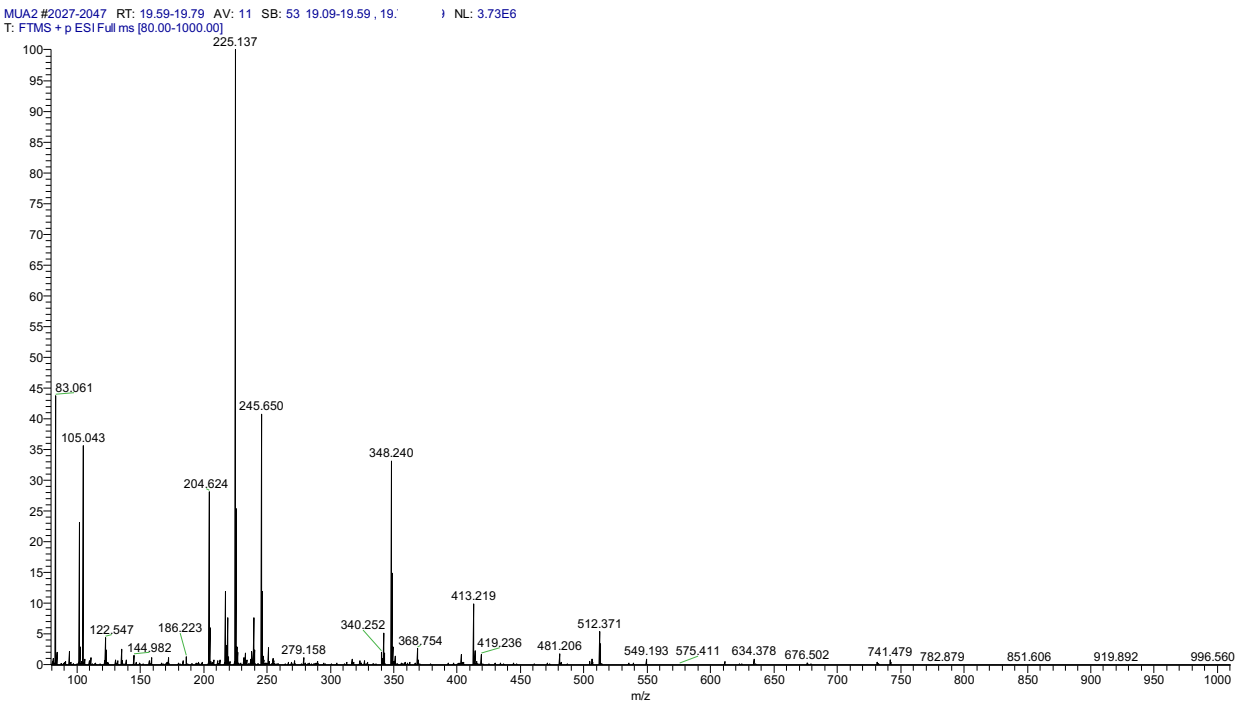


Figure 29. The full mass spectrum of RMP2.31 crude extract.

Primary Fractionation

The HPLC chromatogram of the earlier analysis of the MTM3W5.2 crude extract indicated its polarity based on its retention times. Different chromatographic techniques such as silica gel and size exclusion chromatography were used to purify the inhibitory compound from a large scale broth culture extracts.

Fractionation by Silica Gel Chromatography

Flash column chromatography using silica gel as the stationary phase and a mixture of DCM/methanol (Table 1) was used for the preliminary purification of the dried crude extract.

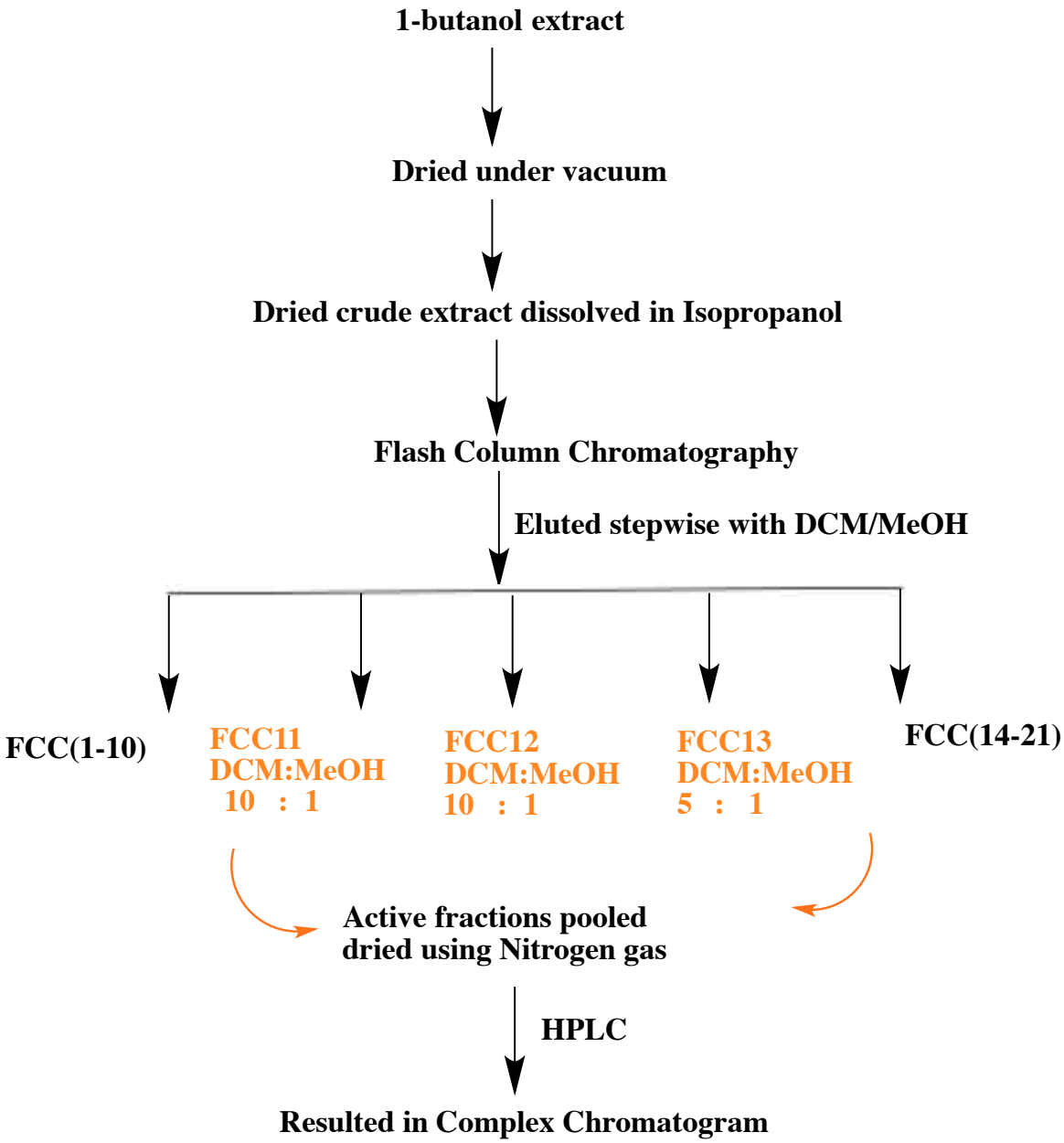


Figure 30. Flow chart of purification process by flash column chromatography.

A total of 21 fractions of (FCC1 to FCC21) were eluted in series with DCM/ CH_3OH [1:0 (total 300 mL), 30:1 (310 mL), 15:1 (320 mL), 10: 1 (330 mL), 5: 1 (300 mL) 1:1 (300 mL) and finally 100% CH_3OH (300 mL). Fractions FCC 11, 12, and 13 were active and were eluted with the 5:1 ratio of the solvent mixture. Active fractions were pooled and dried under nitrogen gas

and further purification was employed with HPLC using different gradient methods to isolate the pure compound. The resulting chromatogram for this dried active compound was more complex than expected. However, the pure compound in a sufficiently high concentration could not successfully be obtained. Several other methods were considered to purify the extract more effectively.

Sephadex LH-20 Fractionation

This size exclusion chromatographic technique gave good separation of the antibacterial compound from crude extracts. Thirty large fractions were eluted through the column with CH_3OH as an eluting mobile phase. The fractions SF10 (Sephadex fraction 10), SF11, SF12, SF13 and SF14 had antimicrobial activity confirmed by the disk diffusion assay. Active portions were combined and evaporated under N_2 gas.

UV profile of Sephadex LH-20 Separation

The absorbance of the fractions eluted with methanol from the Sephadex LH-20 column was measured at wavelengths 210-280 nm. The fraction number 10, 11, 12, 13 and 14 showed antimicrobial activity in a disk diffusion test (Figure 32B).

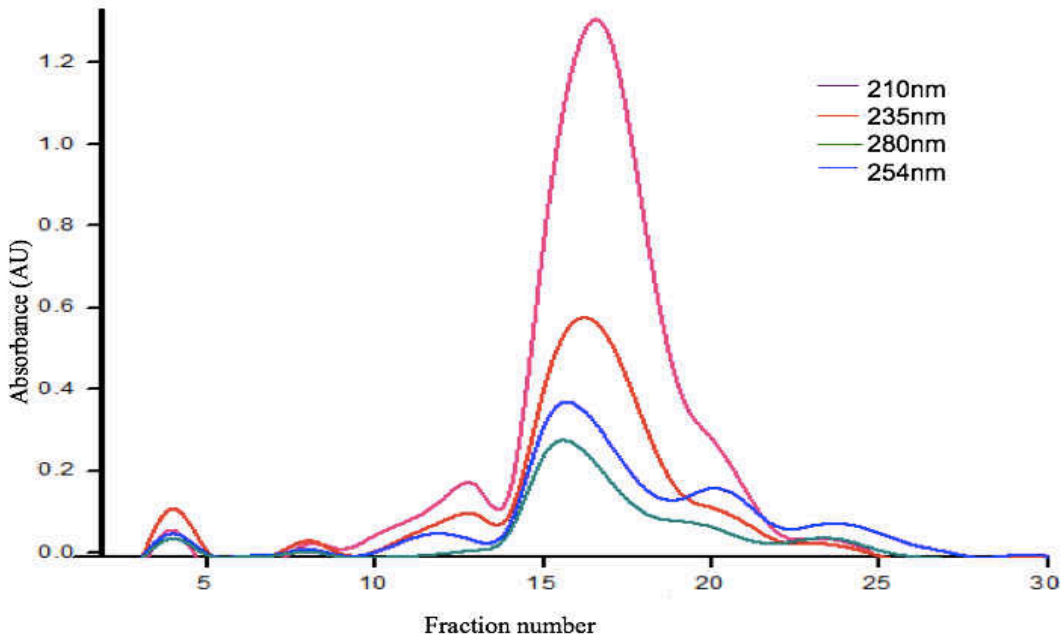


Figure 31. UV spectra of individual fractions separated by Sephadex LH-20 column with MeOH as the mobile phase.

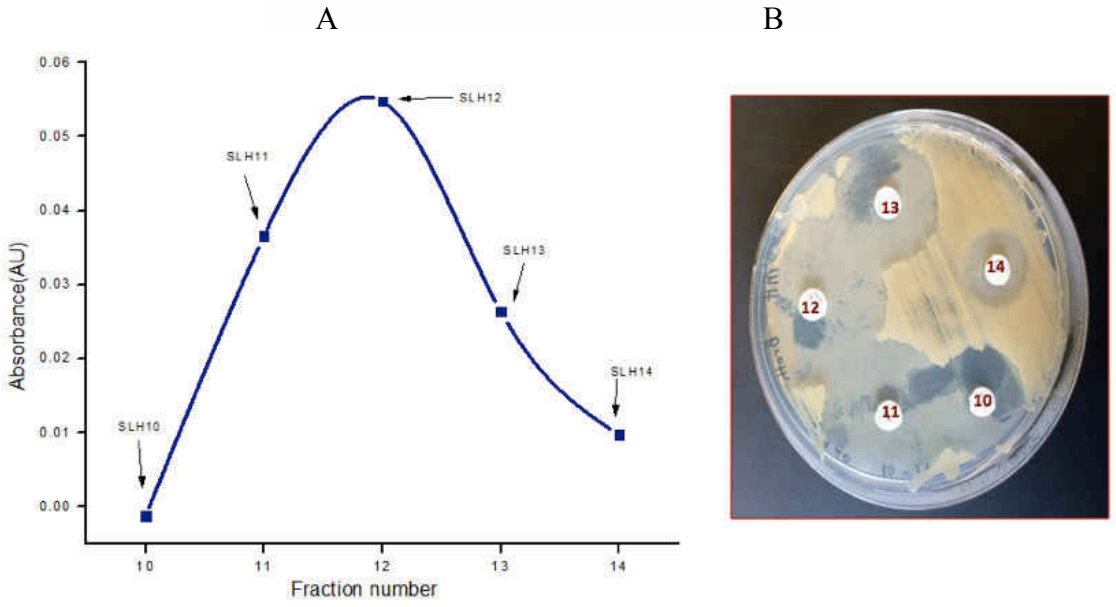


Figure 32. Disk diffusion assay of Sephadex LH-20 fractions. (A) The broad Sephadex LH-20 band at 254 nm which includes all five active fractions marked. (B) The disk diffusion assay of the fraction which showed activity against the indicator strain.

The broad Sephadex LH-20 peak included all the five fractions (SF10-14) showing activity (Figure 32B). This broad peak exhibited higher absorbance at a shorter wavelength (201 nm), moderate absorbance at 254 nm, and the lower absorbance at 280 nm (Figure 31).

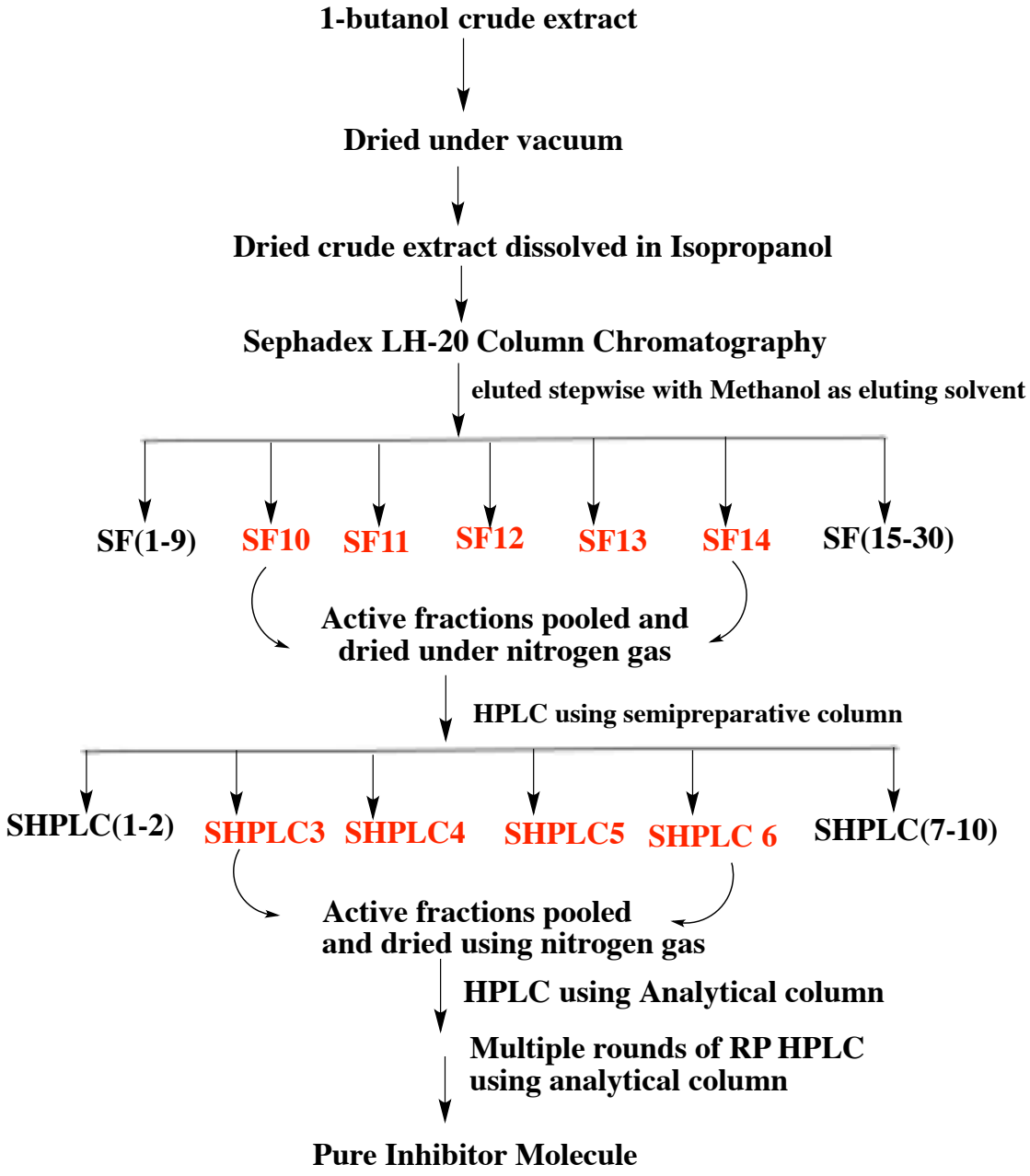


Figure 33. Flow chart of purification process by Sephadex LH-20 column chromatography.

Semi-Preparative HPLC Purification

The second level of purification for the pooled active fractions obtained from the Sephadex chromatography was done using semi-preparative HPLC. The active fractions (approximately 1-2 mL) separated by the Sephadex column were loaded onto a Hamilton PRP-C18 semi-preparative column and eluted with a solvent gradient method with at 5 mL/min flow rate, and UV absorption wavelength set at 254 nm. A gradient acetonitrile/0.1% KH_2PO_4 elution (30-100% B from 3.20-63.40 min and 100% B from 63.40-78.40 min) was used. Four fractions, SHPLC3, SHPLC4, SHPLC5, and SHPLC6 eluted from the column showed antimicrobial activity. The active fractions were combined and dried with nitrogen gas to the next level of purification by analytical HPLC.

Purification Using Analytical HPLC Column

To isolate the pure bioactive compound, analytical column, and various modifiers were employed to finally purify the compound. The active peaks eluted from the semi-preparative C18 column were further purified using a Kinetex® 5µm EVO C18 100 Å column. First, the active compound was eluted with acetonitrile (solvent B) and H_2O (solvent A) used as a mobile phase, at 1 mL/min flow rate, and the gradient method described in Figure 21. As a result, the compound peak was obtained at a retention time of 48.1 min. The chromatogram is shown in Figure 34 and contained a broad peak, which indicated a possible mixture of compounds.

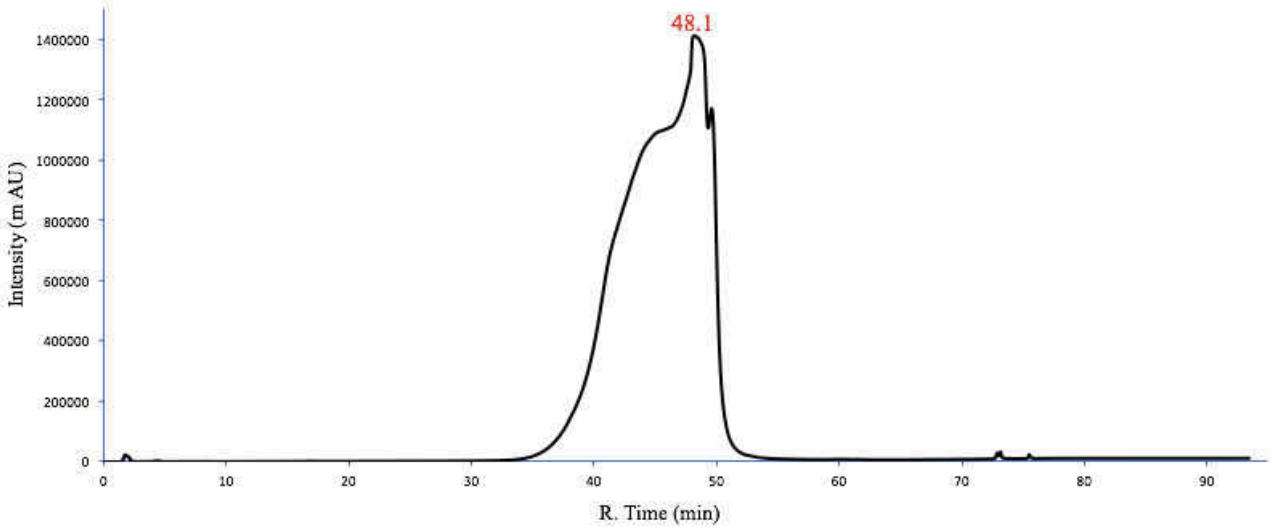


Figure 34. The chromatogram represents the separation by using acetonitrile and water as a mobile phase.

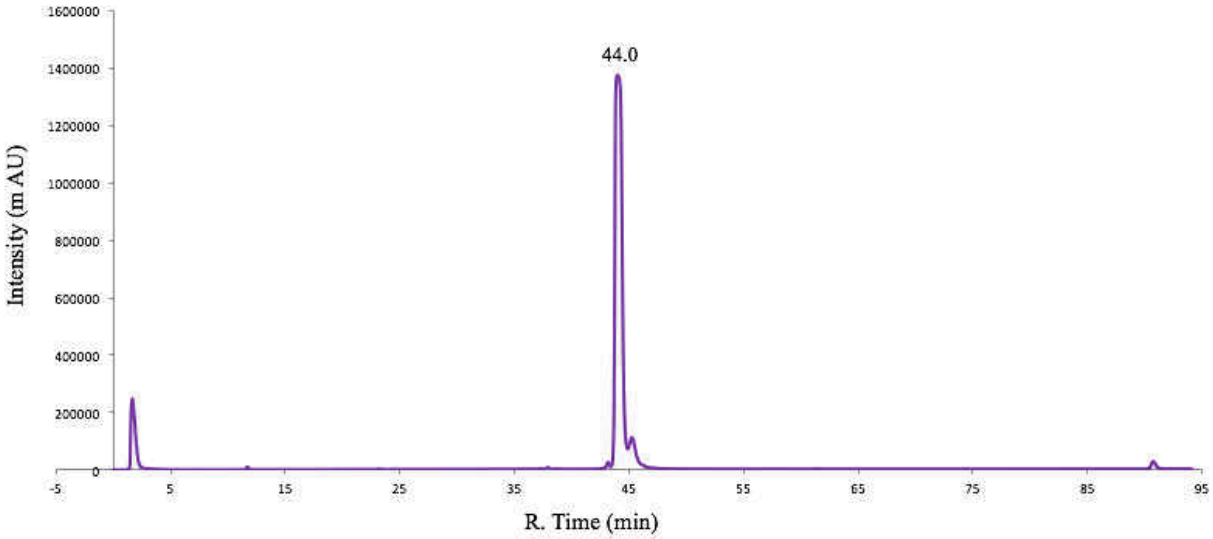


Figure 35. The chromatogram represents the separation by using acetonitrile and 10 mM ammonium formate as a mobile phase.

Further purification was needed to separate this mixture of compounds. A 10 mM ammonium formate solution was tried as solvent to separate the peaks (compounds eluted as a mixture) following the same HPLC conditions. The resulting chromatogram indicated that the

compounds were separated to a limited extent, but the peak eluted with a shoulder still indicative of a mixture of the compounds (Figure 35).

Finally, a pure peak was obtained by using 0.1% NH_4OH as solvent A with the same HPLC conditions as in the first two purification methods (the same column, flow rate, solvent B, and the same elution method described in Table 2) described earlier. The pure compound was scaled up by multiple rounds of HPLC chromatography. Typical injection volumes were 50-100 µL.

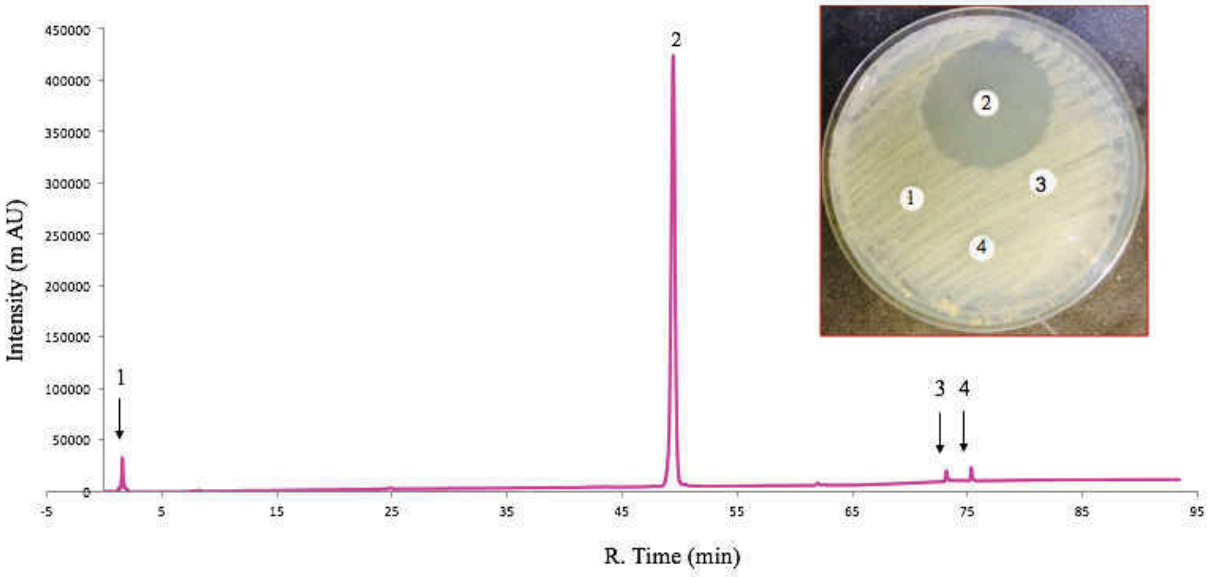


Figure 36. The chromatogram represents the resolution obtained by employing acetonitrile and 0.1% NH_4OH as a mobile phase. The disk diffusion assay image inside the figure indicates the antimicrobial activity of each peak eluted in the chromatogram (fraction 2 at RT 48.9 min is active).

Stability of the Inhibitory Compound

Using the pure active fraction obtained from HPLC, absorptions were measured at room temperature using a UV-Vis spectrophotometer immediately after elution from the column

(within 1 hr), after 3 days, 18 days, and 25 days respectively. UV-Vis spectra (Figure 37) showed that the concentration of the active compound decreased, and this was based on the calculation of the concentration from the absorbance readings using the Beer-Lambert Law equation. The results indicated that the concentration of the compound decreased to 96.3% at day 18th, and 90.6% at day 25th. The compound was injected onto HPLC on the day 25th and the resulting chromatogram indicated that the compound was relatively stable with a slight possibility of decomposition occurring, as shown in the peak in Figure 38B.

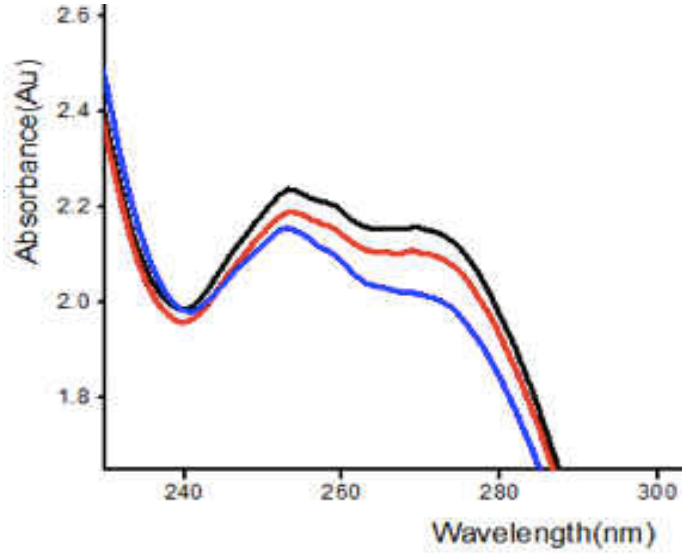


Figure 37. UV-Vis absorbance of the inhibitory compound**:** The stability of the active HPLC fraction was monitored by UV-Vis absorbance. Spectra recorded after 1 hr (black line), 18 days (red line), and 25 days (blue line).

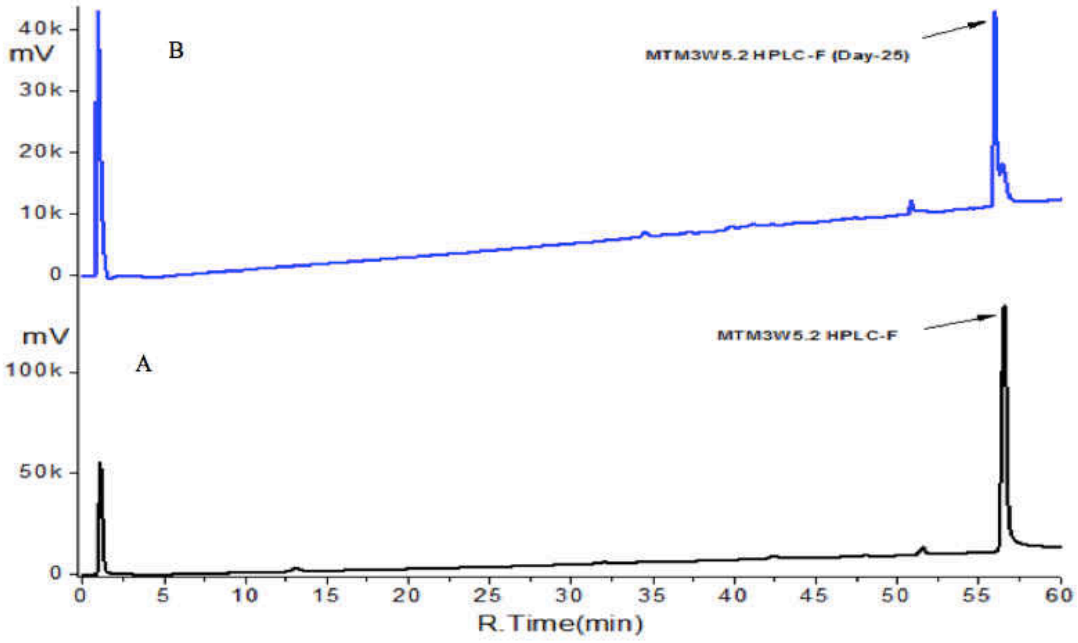


Figure 38. HPLC chromatograms representing the stability of the compound. (A) The chromatogram obtained at day 1. (B) The chromatogram for the same compound obtained after 25 days.

Spectroscopic Characterization of the Compound

UV-Vis Spectra Results

The UV spectrum showed intense broad absorption with maxima at 277 nm, and 327 nm (Figure 39). This indicates the presence of conjugated systems in the chemical structure of the compound. Most type I PKS secondary metabolites, excluding polyenes, are known to have reasonably small conjugated systems and exhibited UV absorption bands approximately at 230-300 nm.[71] These characteristic absorption bands at similar wavelengths, indicated that the purified compound could be the product of a type I PKS.

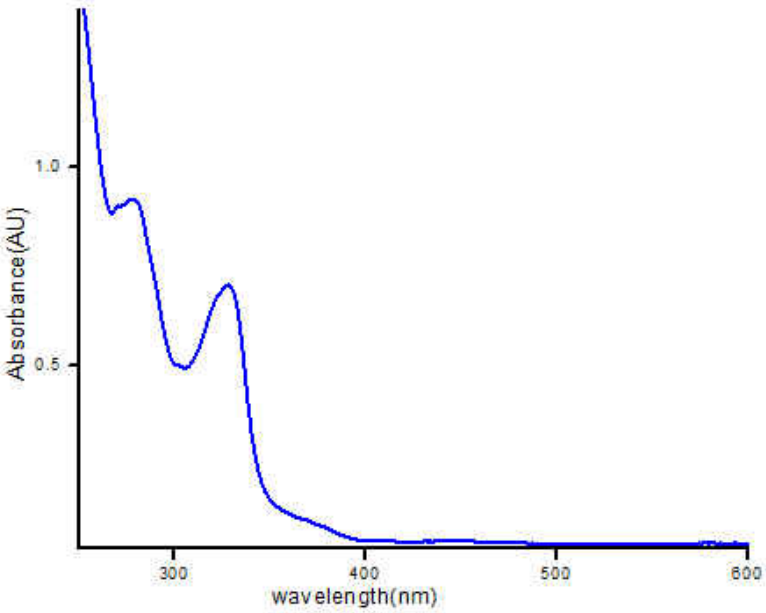


Figure 39. UV-Visible spectrum of the pure compound.

The IR Spectroscopy Result

The IR spectrum showed a broad absorption at 3338 cm^{-1} that is characteristic of a hydroxyl group, an absorption at 3079 cm^{-1} associated with an alkene, and the absorption bands around 3000 cm^{-1} that indicated the -C-H stretches of alkane groups. Absorption at 1728 cm^{-1} confirms the presence of a carbonyl functional group in the molecule (Appendix A).

High-Resolution Mass Spectrometry

A molecular formula of $C_{52}H_{78}O_{13}$ was determined by high-resolution mass spectrum analysis of the compound. The molecular ion peak found at m/z 911.5490 $[M+H]^+$ in the mass spectrum was derived from the ESI-Time of Flight Mass Spectrometer (Appendix B1). The degree of unsaturation was estimated to be 14 by using the following formula.[72]

Degree of unsaturation (U) = C+1-[1/2(H+X-N)] = 14

Structure Elucidation Using NMR Spectroscopy

In antimicrobial metabolite isolation from natural sources, the major challenge is the determination of its structure. After purification, the purified metabolite was shipped to Bruker BioSpin, Billerica MA for 1D (^{1}H NMR, ^{13}C NMR) and 2D (HSQC, COSY, TOCSY, and HMBC) extensive NMR spectral analysis. Partial structure determination of an unknown compound was performed using the results obtained from Bruker BioSpin. Among them, the HSQC spectrum provided valuable information regarding the total number of "C→H" correlations (single carbon directly attached to its proton(s)) in a different chemical environment. Structure determination of an unknown compound using homonuclear ^{1}H-^{1}H COSY depends on the ability to detect couplings between neighboring protons "H↔H." The correlations between protons and neighboring carbons can be detected using the heteronuclear HMBC spectrum.

Based on the characteristic UV-Vis absorption maxima at 277 nm, and 327 nm; strong IR absorptions at 3338 cm^{-1} (-OH), 3079 cm^{-1} (=CH-); large molecular weight 911.5490 Da with a higher number of carbon, hydrogen, and oxygen atoms from HR-MS and proton signals (olefinic, and aliphatic) from ^{1}H NMR, the compound of interest would most likely be a polyketide. Close inspection of spectral data revealed the presence of minor peaks in NMR spectra, which could be related to the decomposition of the natural product during the purification process, as well as distinctive tautomers or conformers. Apart from minor signals, the structure analysis of the unknown polyketide was started with major peaks present in CD_3OD.

^{1}H and ^{13}C NMR data in combination with the HSQC analysis showed the presence of 52 carbons attributable to seven (7) sp^3 methyl ($-CH_3$) groups among which one was an oxygenated sp^3 methyl carbon. There are eleven carbons corresponded to sp^2 methine (-CH=C), ten carbons

sp^3 methylene ($-CH_2-$) (eight are diastereotopic carbons), eighteen carbons are sp^3 methine (eight oxygenated), and six carbons are quaternary carbons (three oxygenated, two quarternary sp^2 carbons).

^{13}C-NMR interpretation disclosed three sets of distinct chemical shifts: i) Saturated methyl ($-CH_3$), methylene ($-CH_2-$), methine (sp^3 CH), and quaternary carbons were observed in the range of δ 12- 58 ppm, ii) In the range of δ 60-99 ppm, signals arising from sp^3 oxygenated carbons were detected; iii) Signals resulting from olefinic methines (-CH=C) and quaternary olefinic carbons were detected at δ 115-166 ppm. Table 3 shows the chemical shift NMR spectroscopic data for the polyketide compound (600 MHz, methanol-d_4).

A number of small "spin systems" were generated based on ^{1}H-^{1}H COSY correlations.

Table 3. NMR Spectroscopic Data for the Antimicrobial Compound (600 MHz, Methanol-d_4).

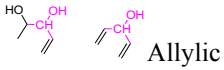
Proton #	δ_H, mult (*J* in Hz)	δ_C	Carbon	COSY (H↔H)	HMBC(H→C)
1	6.752, dd (*J*=11.2, 16.2 Hz)	**152.66**	CH=C	5.86 (**6**), 5.58 (**27**)	165.93 (**52**), 119.76 (**6**), 80.16, 17.01 (**41**) 41.02 (**27**)
2	6.358, dd (*J*=9.8, 14.9 Hz)	**128.68**	CH=C	5.98 (**5**), 5.36 (**10**)	137.14 (**50**), 128.40 (**5**), 46.03 (**32**)
3	6.058, t	**132.38**	CH=C	5.42 (**8**)	132.23 (w), 40.60 (**30**)
4	6.051, d	**130.41**	CH=C	5.419 (**9**)	
5	5.998, d	**128.38**	CH=C	6.35 (**2**)	9.49 (**36**), 82.16 (**21**), 133.57 (**10**)
6	5.860, d (*J*=15.5 Hz)	**119.76**	CH=C	6.72 (**1**)	165.99 (**52**) 41.14 (**27**), 17.01 (**41**)
7	5.590, dd	**65.60**	 Allylic	2.09 (**33a**)	165.93 (**52**), 61.76 (**48**), 43.78 (**25**)
8	5.421, dd	**138.28**	CH=C	6.058 (**3**), 2.29 (**30**)	132.38 (**3**), 130.40 (**4**)
9	5.420, dd	**133.29**	CH=C	6.051 (**4**)	130.40 (**4**)
10	5.363, td	**133.56**	CH=C	6.35 (**2**), 2.20 (**32**)	128. 36 (**5**), 70.48 (**22**), 46.19 (**32**)
11	5.210, dd	**126.29**	CH=C	2.45 (**28a**), 2.35 (**28b**)	86.41 (**15**)
12	4.069, t	**73.02**	CH–O		
13	4.063, dd	**70.13**	CH–O		
14	4.010, d	**75.53**	CH–O	1.33 (**40**)	
15	3.877, d	**86.45**	CH–O		137.88 (**51**), 126.26 (**11**), 77.11 (**17**)
16	3.776, d	**80.31**	CH–O	2.51 (**27**)	75.55 (**14**), 3.79 (**44**)
17	3.716, t	**77.12**	CH–O		
18	3.692, t	**60.90**	CH_2–O	3.58 (**20a**)	
19	3.588, m	**77.59**	CH–O		17.65 (**42**), 17.81 (**43**),

Table 3. (continued)

20a,/20b	3.579, t / 3.352, m	**72.15**	CH_2–O	2.42 (**29a**), 2.26 (**29b**)	60.99 (**18**), 26.65 (**29a,b**)
21	3.524, d	**82.13**	CH–O		128.3 (**5**), 40.60 (**30**), 9.49 (**36**)
22	3.412, m	**70.50**	CH_2–O		46.16 (**32**), 57.16 (**24**), 31.13 (**31a,b**)
23	3.335, m	**57.12**	CH–C		
24	3.315, m	**57.14**	CH–C		
25	2.928, dd	**43.78**	CH–C		98.64 (**49**), 61.76 (**48**), 65.65 (**7**)
26	2.855, dd	**43.79**	CH–C		98.64 (**49**), 61.76 (**48**)
27	2.510, d (*J*=9.9 Hz)	**41.12**	CH–C	6.75 (**1**), 3.77 (**16**), 1.12 (**41**)	119.75 (**6**), 80.17 (**16**)
28a/28b	2.456, m / 2.372, m	**36.12**	CH_2–C	4.063 (**13**)	
29a/29b	2.421, m /2.262, m	**26.62**	CH_2–C		86.45 (**15**)
30	2.298 m	**40.66**	CH–C		
31a/31b	2.216, m / 1.422, m	**31.09**	CH_2–C		
32	2.208, m	**46.20**	CH–C	5.36 (**10**), 3.41(**22**), 1.41 (**34b**)	70.49 (**22**)
33a/33b	2.098 dd / 1.288, m	**37.69**	CH_2–C		61.76 (**48**),
34a/34b	1.794, m /1.412, m	**28.30**	CH_2–C		61.76 (**48**), 98.62 (**49**)
35a/35b	1.789, m / 1.684, m	**17.57**	CH_2–C		98.62 (**49**)
36	1.728, s	**9.50**	CH_3–O		137.14 (**50**)
37	1.683, dd	**33.44**	CH–C		
38a/38b	1.638, m / 1.380, m	**26.12**	CH_2–C		
39	1.497, m	**40.84**	CH–C	3.87 (**15**), 3.71 (**17**)	77.14 (**17**)
40	1.330, m	**39.05**	CH–C	0.8874 (**44**), 3.77 (**16**)	
41	1.119, t	**17.00**	–CH_3	2.51 (**27**)	41.17 (**27**), 80.27 (**16**), 152.68 (**1**)

Table 3. (continued)

42	1.050, d	**17.63**	—CH_3	1.68 (**37**)	17.83 (**43**), 33.41 (**37**), 77.56 (**19**)
43	0.954, d	**17.85**	—CH_3	1.68 (**37**)	17.80 (**43**), 33.41 (**37**), 77.55 (**19**)
44	0.887, d	**3.78**	—CH_3	39.05 (**40**)	39.13 (**40**), 75.49 (**14**), 77.36, 80.27 (**16**)
45	0.804, t	**16.20**	—CH_3	2.29 (**30**)	40.64 (**30**), 82.04 (**21**), 138.20 (**8**)
46	0.450, d	**12.70**	—CH_3	1.49 (**39**), 3.88 (**15**)	86.45 (**15**), 77.14 (**17**), 39.09 (**40**)
47	q	**53.20**			
48	q	**61.76**			
49	q	**98.61**			
50	q	**137.15**			
51	q	**137.88**			
52	q	**165.92**			

In the COSY spectrum (Appendix F) of the inhibitory compound, the methine doublet of doublets at δ 6.752 H(1, J=11.2, 16.2) correlates with the doublet at δ 5.86 H(6), and the doublet δ 2.51 H(27). The latter proton, H(27) further correlates with the triplet at δ 1.119 H(41). This linkage of COSY correlation indicates an H(6)-H(1)-H(27)-H (41) spin system **1** (Table 4). The HMBC correlations of H(1), H(6), and H(41) with C-27, the protons H(1), and H(6) with C-41 supported this fragment structure. The proton H(6) did not show further correlation with any other proton. The aliphatic methine multiplet H(32) at δ 2.20 coupled with an olefinic methine proton H(10) at δ 133.55. The proton H(10) showed COSY correlation with a doublet of doublets at δ 6.358 H(2), and H(32) at δ 2.208. Further COSY correlation of H(2) with a doublet at δ 5.99 H(5), and H(10) give the correlation pattern H(32)-H(10)-H(2)-H(5), which suggests the possible spin system **2** represented in Table 4. This pattern was supported by HMBC correlations of H(2) with C(5, and 32) the protons H(10) with C(5, and 32), and H(5) with C-10.

The triplet H(3) at δ 6.05 has a COSY correlation with olefinic methine at δ 5.42 H(8). The proton H(8) is further coupled to aliphatic methine multiplet H(30) at δ 2.29. The COSY cross-peaks of H(30) with doublet H(21) at δ 3.52 and triplet methyl H(45) at δ 0.804 ppm. The correlation pattern, H(3)-H(8)-H(30)-H(21, 45) suggests a spin system **3** as represented in Table 4. This fragment structure is supported by HMBC correlations of H(21, and 3) with C-30, and H(45) with C-(21, 30, and 8).

The proton doublet of doublets at H(7) δ 5.59 ppm was correlated with diastereotopic methylene CH_2(33a,b) δ 5.59 ppm. This COSY correlation with downfield H(7) suggests an extra electronegative atom, and the HMBC cross-peaks of H(7) with ester carbonyl (C-52) suggested the fragment **4** (Table 4). Diastereotopic methylene pairs were identified from the HSQC spectrum.

A three-carbon spin system **5** was deduced from a chain of COSY correlations of a diastereotopic methylene protons H(35a,b δ 1.78, 1.68) with another diastereotopic protons H(31a,b δ 2.21, 1.42). The latter proton further coupled to methylene multiplet protons H(22) at δ 3.41 ppm. The link of COSY correlations suggests a CH_2(35a, b)-CH_2(31a, b)-CH_2(22) connectivity outline. The diastereotopic methylene protons (35a/35b, 31a/31b), and non diastereotopic protons (22) were confirmed by the HSQC spectrum and HMBC correlations of H(22) with H(31a,b) with carbon chemical shift 31.13 ppm) that supported the connectivity.

The COSY cross peak was detected between H(32) δ 2.20 and diastereotopic methylene H(34a, b). This methylene again coupled to another diastereotopic multiplet protons H(38a, b). The latter protons H(38a, b) correlated to multiplet H(19 δ 3.58), which was further coupled to a doublet of doublets H(37) at δ 1.68. H(37) that has a COSY cross-peaks with two methyl protons doublet H(42) at δ 1.05 and doublet H(43) δ 0.95 ppm. This network of COSY connections suggest an H(32)-H(34a,b)-H(38a,b)-H(19)-H(37)-H(42, 43) connectivity pattern. This pattern with H(19) at δ 3.588 ppm suggests the fragment structure **6**, and which possibly could be supported by HMBC correlations of H(42) with C(19, δ 77.58), C(37, δ 33.44), and C(43, δ 17.85), H(43) with C(19, δ 77.59), C(37, δ 33.44), and C(42, δ 17.63), and H(19) with C(43, δ 17.85) and C(42, δ 17.63).

A four carbon fragment is observed from a series of COSY correlations of methyl doublet H(46) at δ 0.45 with sp^3 methine multiplet H(39) δ 1.49 ppm. The latter proton is coupled to oxy methine doublet H(15) at δ 3.87 and another oxy methine triplet H(17) at δ 3.71. These network COSY correlations make spin system **7** represented in Table 4. The HMBC supported this structure by the correlations of H(46) with C-15, C-17, & C-39, and H(39, 15) with C-17.

The COSY correlations of an oxygenated methine doublet of doublets H(14) chemical shift 4.01 ppm with methine multiplet H(40) at δ 1.33 ppm. This methine proton H(40) further showed cross-peaks with methyl doublet H(44) at δ 0.88, and oxy methine doublet H(16) at δ 3.77 ppm. This series of COSY correlations suggest the spin system **8**. This connectivity is supported by HMBC correlations H(44) to C-14, C-40, & C-16, H(16) to C-44, & C14, and H(40) to C-16, & C-44. The ^{1}H spin system **9** was deduced from the doublet of doublets sp^2 methine proton H(11) δ 5.21 correlated with methylene pairs H(28a,b) at δ 2.45, 2.37. The methylene protons again coupled to sp^3 methine doublet of doublets H(13) at δ 4.06. The HMBC correlations of H(15) with C-11, quaternary carbon (C-51) and the correlation of H(11) with C-15 suggests the fragment structure **9** shown in Table 4.

Three carbon ^{1}H spin system **10** was determined from the COSY correlations from triplet methylene protons H(18) δ 3.69 to diastereotopic methylene protons H(20a,b). The cross-peaks between methylene pairs H(20a,b) and other methylene diastereotopic H(29a,b) at δ 2.42, 2.26 ppm. HMBC correlations H(20) with C-18 and C-29 demonstrated the spin connectivity.

Spin system **11** is suggested based on the HMBC correlations of doublets of doublet H(26) δ 2.85 with carbons (C-48, C-49), the correlations of H(25) δ 2.92 with carbons (C-49, C-48, and C-7), the HMBC cross-peaks of H(7) with carbon (C-48, C-25) and additional HMBC correlations of methylene protons H(34a,b) with carbons (C-48, C-49). The proton cross-peaks of H(26) and H(25) on the HSQC spectrum indicate that they were possibly symmetric protons. It could be possible that they are both linked to the oxygen atom shown in structure **11.** (Table 4)

Table 4. Different Spin Systems Were Deduced from ^{1}H-^{1}H COSY and Corresponding HMBC Correlations.

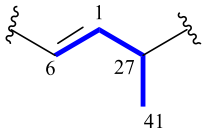
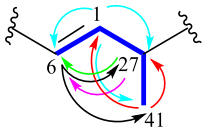
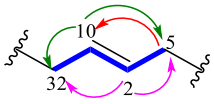
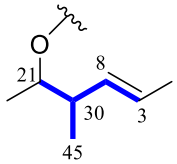
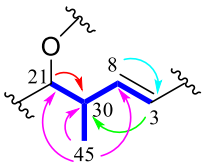
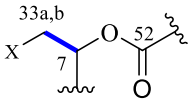
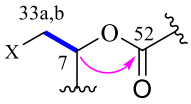
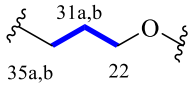
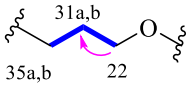
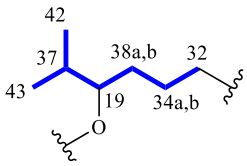
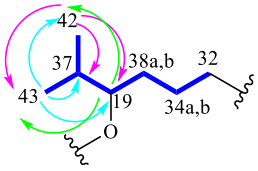
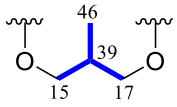
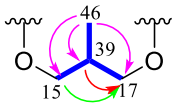
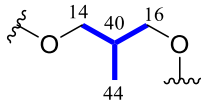
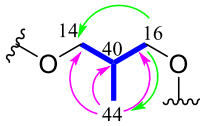
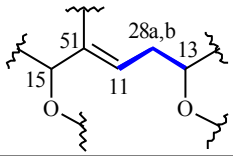
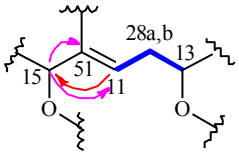
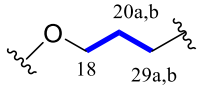
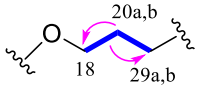
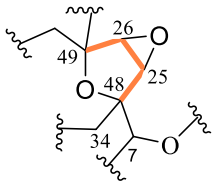
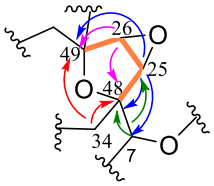
Key fragment structures	HMBC correlations
1 	
2 	
3 	
4 	
5 	
6 	

Table. 4 (continued)

Key fragment structures	HMBC correlations
7 	
8 	
9 	
10 	
11 	

The HMBC correlation of protons H(6) with the ester carbonyl carbon C-52 at δ 165.92, and olefinic carbon C-1 (δ152.66) establishes an α-β unsaturated ester moiety. The chemical shift of H(1) is an unusually high δ 6.75 ppm, because proton H(1) is located at the β position of an α-β unsaturated system. H(6) has no further COSY correlations except with H(1) suggesting no extra neighboring protons.The HMBC correlation of H(7) and H(6) to the ester carbonyl carbon C(165.92) allowed fragments **1** to be connected to spin system **4**. The additional HMBC correlations of protons attached to C-41 with C(16, δ 80.17), C(27, δ 41.17), and C(1, δ 152.68)

updated fragment **A** could be generated. The HMBC correlation of allylic methine proton H(27) with C-6 supported structure **A.**

As stated earlier, H(5) has no additional COSY correlations but has HMBC correlations to C(50, δ 137.15), C(21, δ 82.13), and C(36, δ , 9.5). In addition to these correlations, the HMBC correlations of H(9) with C-3, and C-4 intercepts the spin systems **2** and **3** (Table 4) and generate fragment **B** as shown in Figure 40**.** The HMBC correlations of H(2 and 5) with quaternary carbon C-50 and H(21) with C-36 may support the structure of fragment **B**.

The HMBC correlations of H(32) with C(22, δ 70.50) and H(22) with C(32, δ 46.20) allowed spin systems **5**, and **6** to be connected with fragment **B**, so updated fragment **C** can be proposed (Figure 40). The proton H(2) showed HMBC correlation to allylic methine carbon C-32 at δ 46.20 ppm.

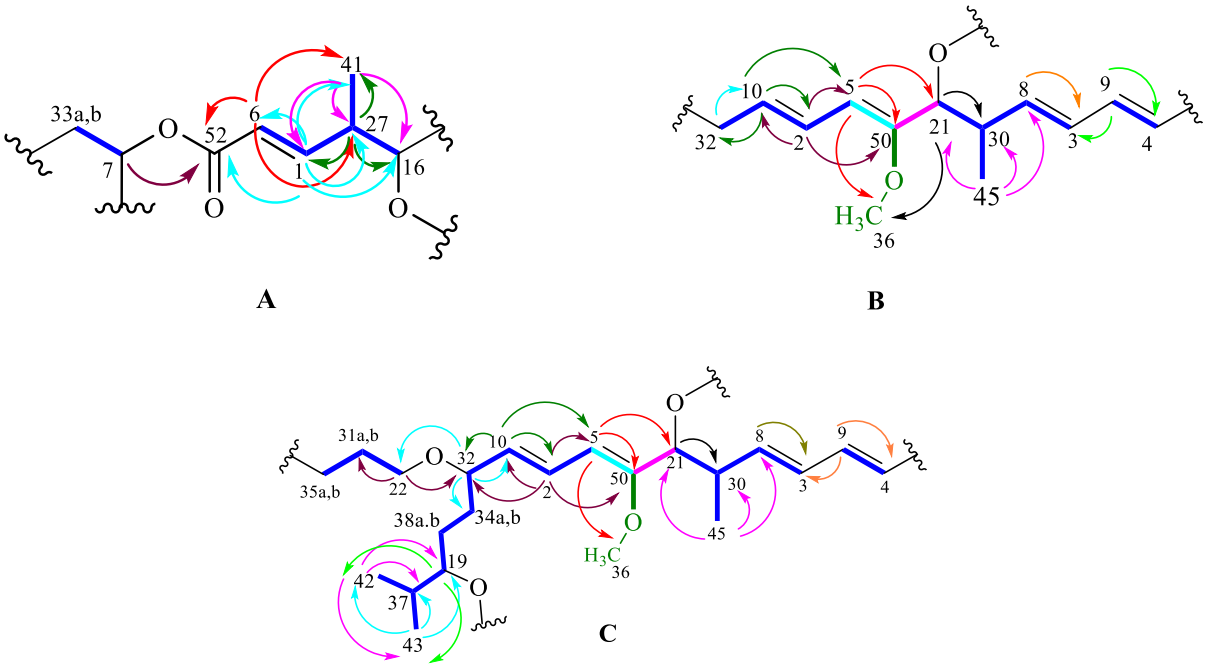


Figure 40. Partial spin systems assembly based on HMBC correlations for polyketide.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

Conclusions

There is an essential need for new classes of antibiotics to fight against ever growing antibiotic resistance emerging in various pathogenic bacterial species. The increase in discovery of numerous gene clusters in *Rhodococcus* with unknown activity motivates scientists to find the function of those genes that may produce novel antibiotics. Recently, during the process of finding new antimicrobial compounds from soil bacteria, *Rhodococcus* sp. MTM3W5.2 was identified as a novel strain that produces an inhibitory compound against most related *Rhodococcus* microbes. This study focused on the development of purification methods for the inhibitory compound produced by *Rhodococcus* sp. MTM3W5.2, as well as stability studies and the characterization of the compound.

A crude mixture of metabolites containing the active compound was extracted from broth culture by using 1-butanol and was purified using Sephadex LH-20 column chromatography before further purification using semi-preparative HPLC chromatography. Sephadex LH-20 purification was employed as the best method for efficient first stage purification of the compound of interest from the crude extract as compared to other purification techniques also explored during this research. It enabled suitable fractionation of 1-butanol crude extract containing a mixture of different compounds. The semi-preparative HPLC method using a Hamilton PRP-1 column accomplished satisfactory removal of all highly polar impurities (RT $\leq$ 5 min). Different solvent elution systems using acetonitrile/$HCOONH_4$ and acetonitrile/NH_4OH were effectively developed and applied for the final step purification of the inhibitory compound.

1.2 mg of pure compound was obtained by multiple rounds of analytical HPLC using reversed-phase C18 column as a stationary phase.

The molecular composition of the inhibitory compound ($C_{52}H_{78}O_{13}$) was determined by HR-TOFMS analysis of the compound. Tests demonstrated that the active compound undergoes slight degradation during the purification process. In addition, using some modifiers such as formic acid and TFA may have facilitated this perceived decomposition. Consequently, the presence of slight impurities perhaps due to this decomposition in the final compound or the presence of distinctive isomers caused difficulties in confirming the structure of the compound regarding a total number of carbons and hydrogens. Apart from minor signals in the NMR spectra, the structure analysis of the unknown molecule was started with major signals present in the 1D and 2D NMR spectra. The possible fragments of the structure have been proposed based on the NMR spectra in CD_3OD.

Future Work

Due to time limits, this research study only determined the partial chemical structure of the compound. For this type of research, purification should be optimized in analytical scale followed by scaling up the compound for further characterization. Two strategies to elucidate the complete structure of this compound are proposed below.

1. The compound should be extracted on a large scale and purified using the established method. After purification, the compound should be taken to Murdock Research Institute, North Carolina for determining HR-MS, 1D, and 2D NMR spectroscopy. The compound should not undergo any decomposition.

2. The stability tests should be performed in different reagents such as trifluoroacetic acid (TFA), formic acid, and ammonia. Based on the stability of the compound in the presence of these reagents, the polyketide will undergo hydrolysis in one of them. The hydrolyzed extract will be subjected to bioactivity-guided column chromatography. The pure active fragments will be further characterized using NMR spectroscopy. Hydrolysis of the whole compound into small fragments will simplify the structural determination of individual fragments by 2D NMR and consenquently will facilitate the final characterization of the entire molecule.

Crystals of the compound could be grown and submitted to X-ray crystallographic analysis to confirm the absolute configuration of the chemical structure. Once the structure is elucidated, the possible genes responsible for the production of this antimicrobial compound can be identified. Only a few antimicrobial compounds have been reported from the genus *Rhodococcus* to date; and thus, the discovery of the entire structure of this compound and genome would supplement ongoing research by scientists in exploring the genus *Rhodococcus* for novel antimicrobial compounds. The type of PKS, which could be responsible for the biosynthesis of the molecule, can also be identified by the retrosynthetic pathway. The research could also propose synthetic routes for the production of this compound. The bioactivity profile of this compound was reported as a narrow spectrum antibiotic. A bioactivity profile against a huge library of bacteria could determine its antibiotic nature and specificity.

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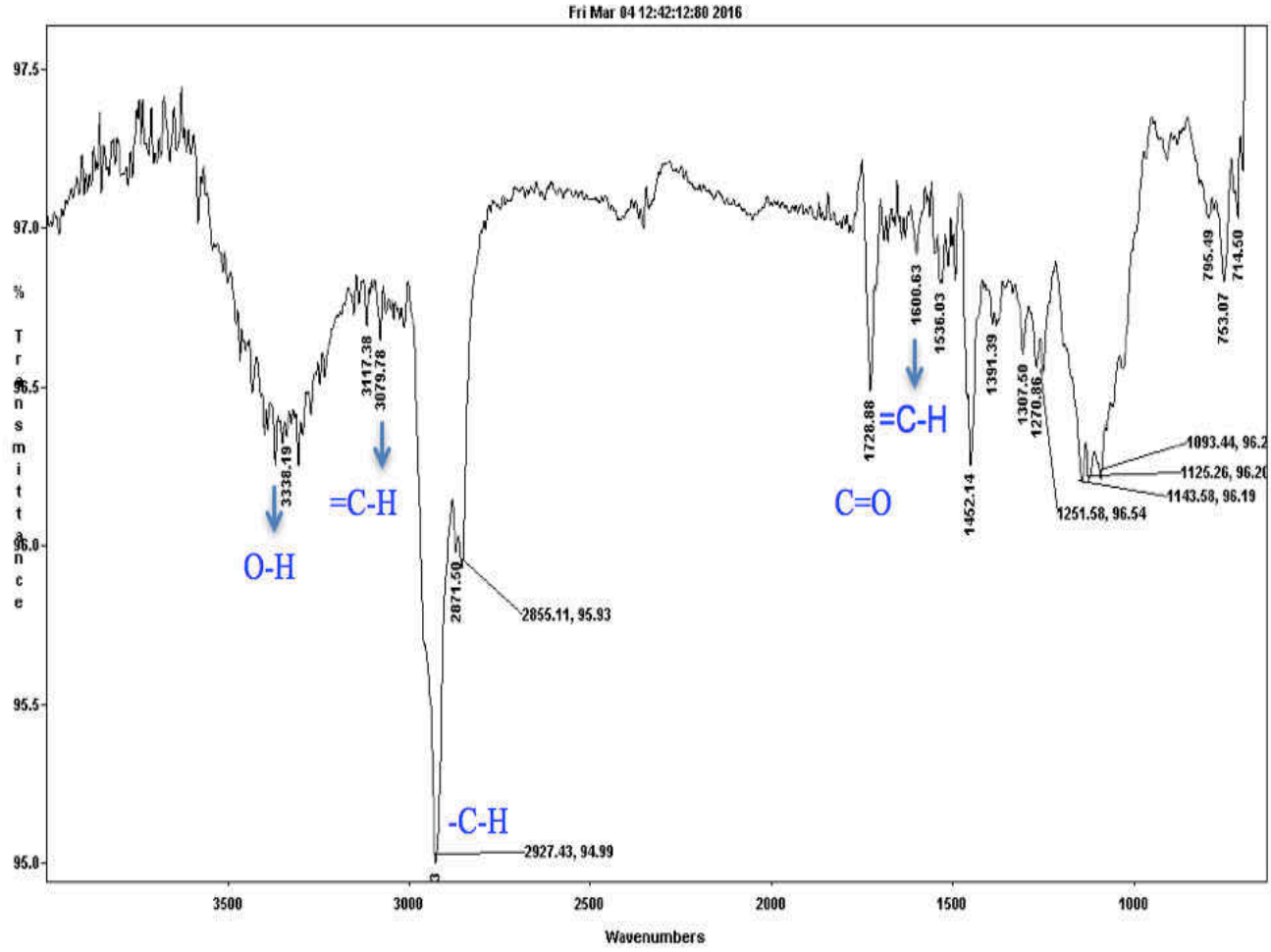
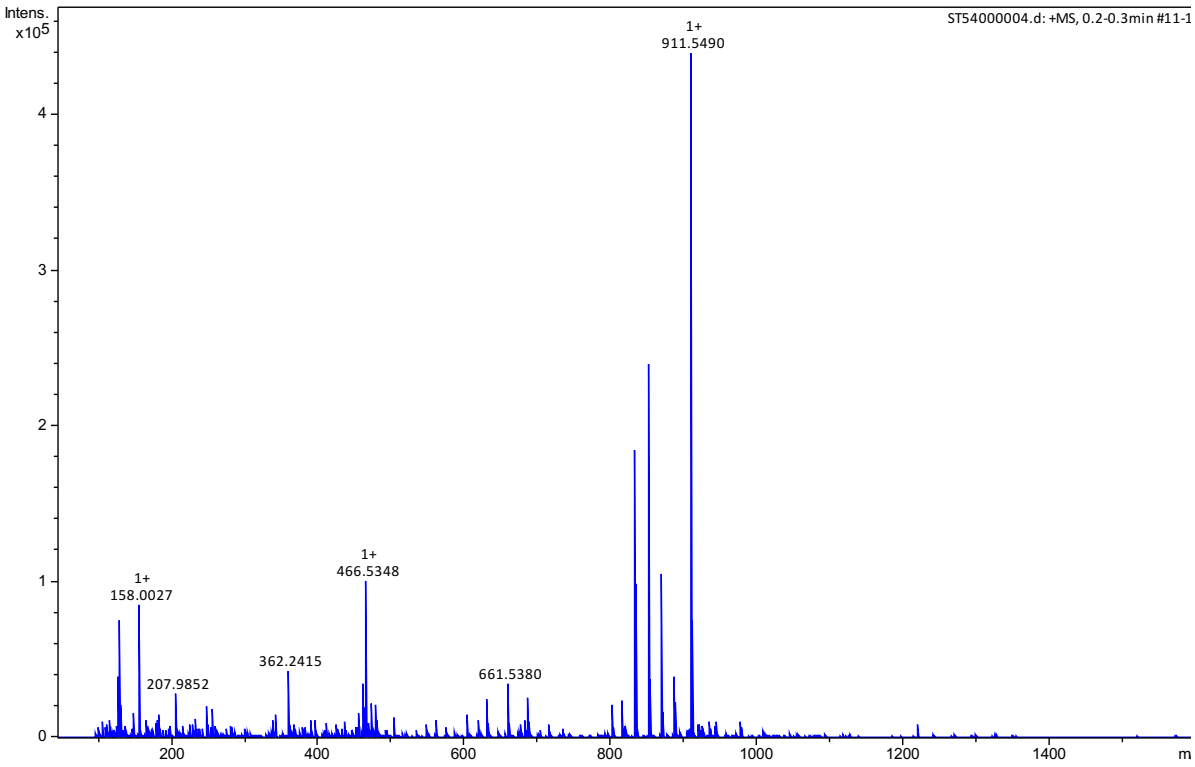
APPENDICES

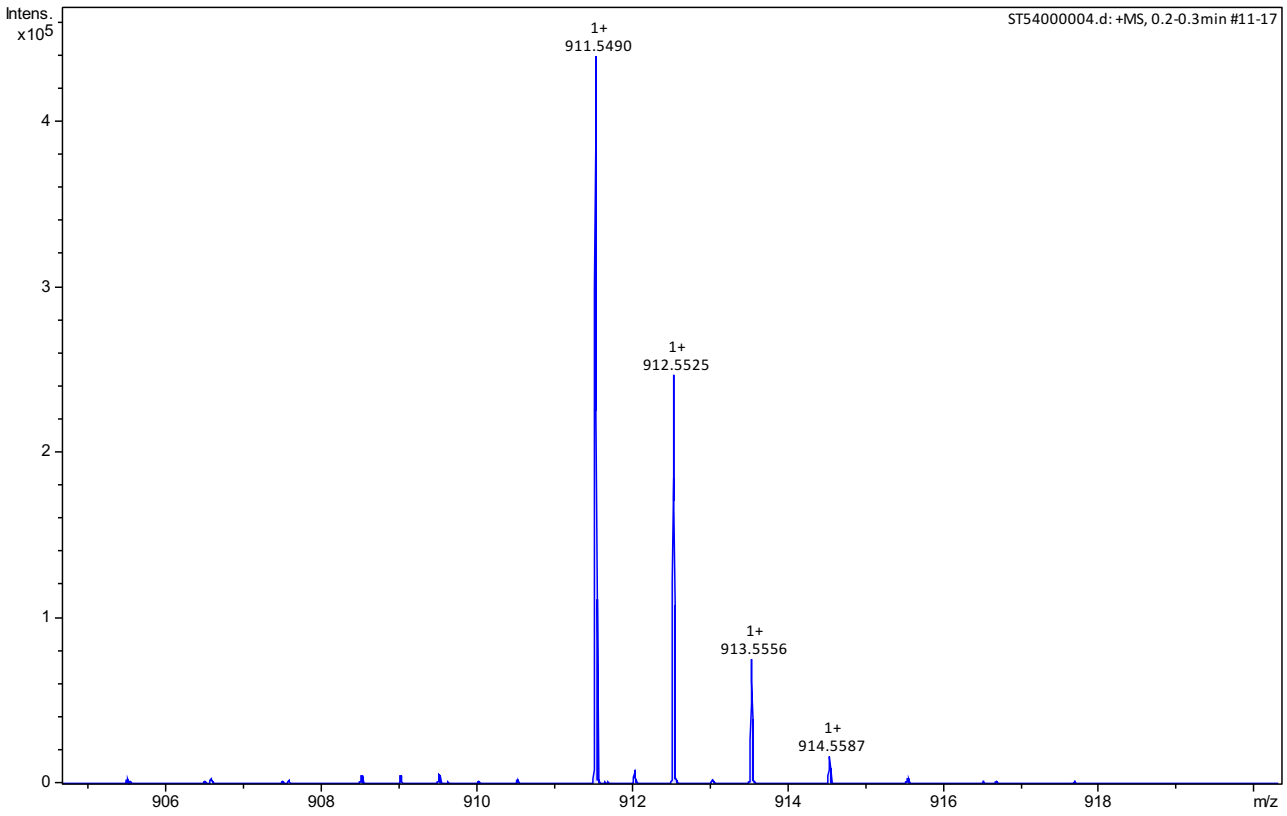
Appendix A: IR Spectrum of Inhibitory Compound



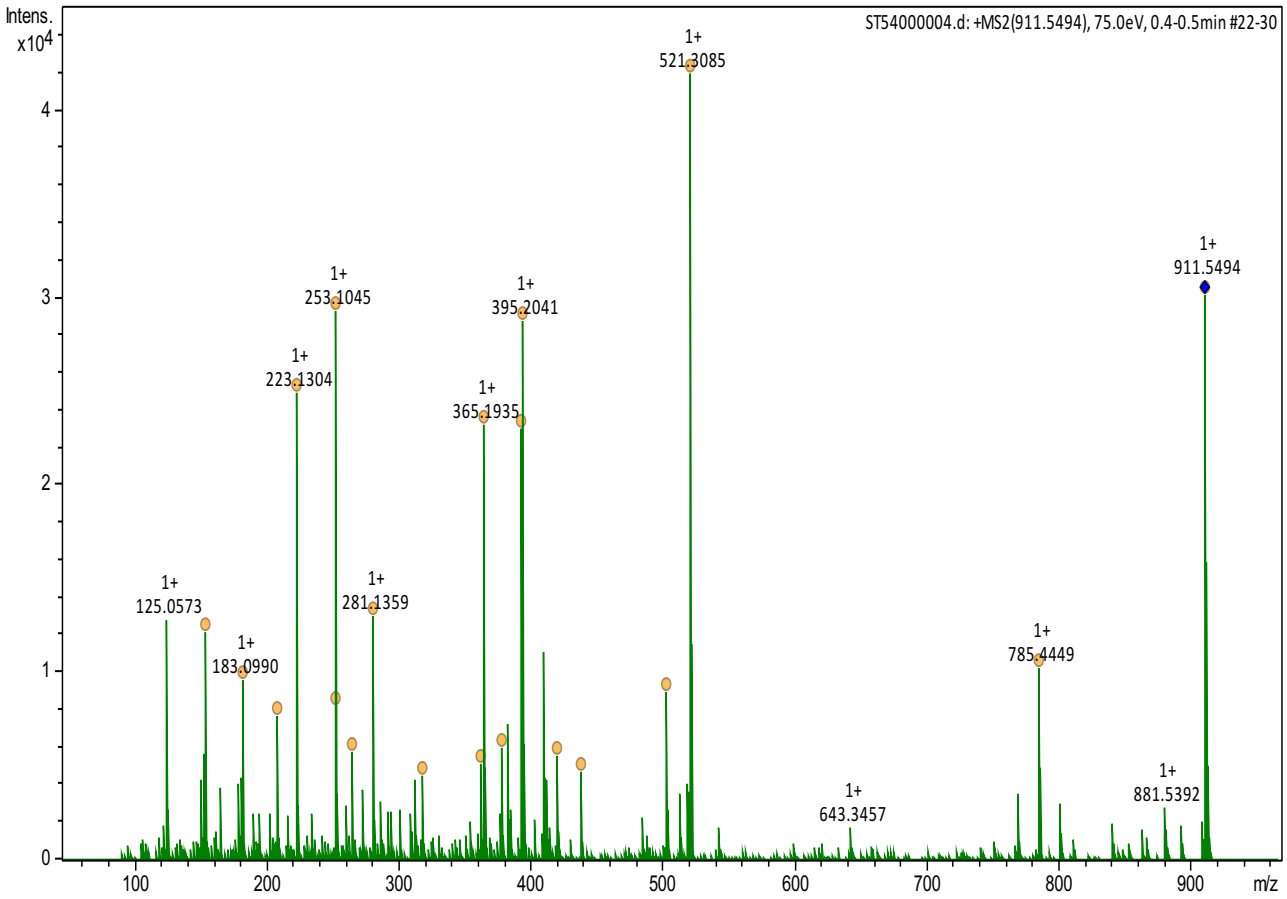
Appendix B1: MS Spectrum for The Compound



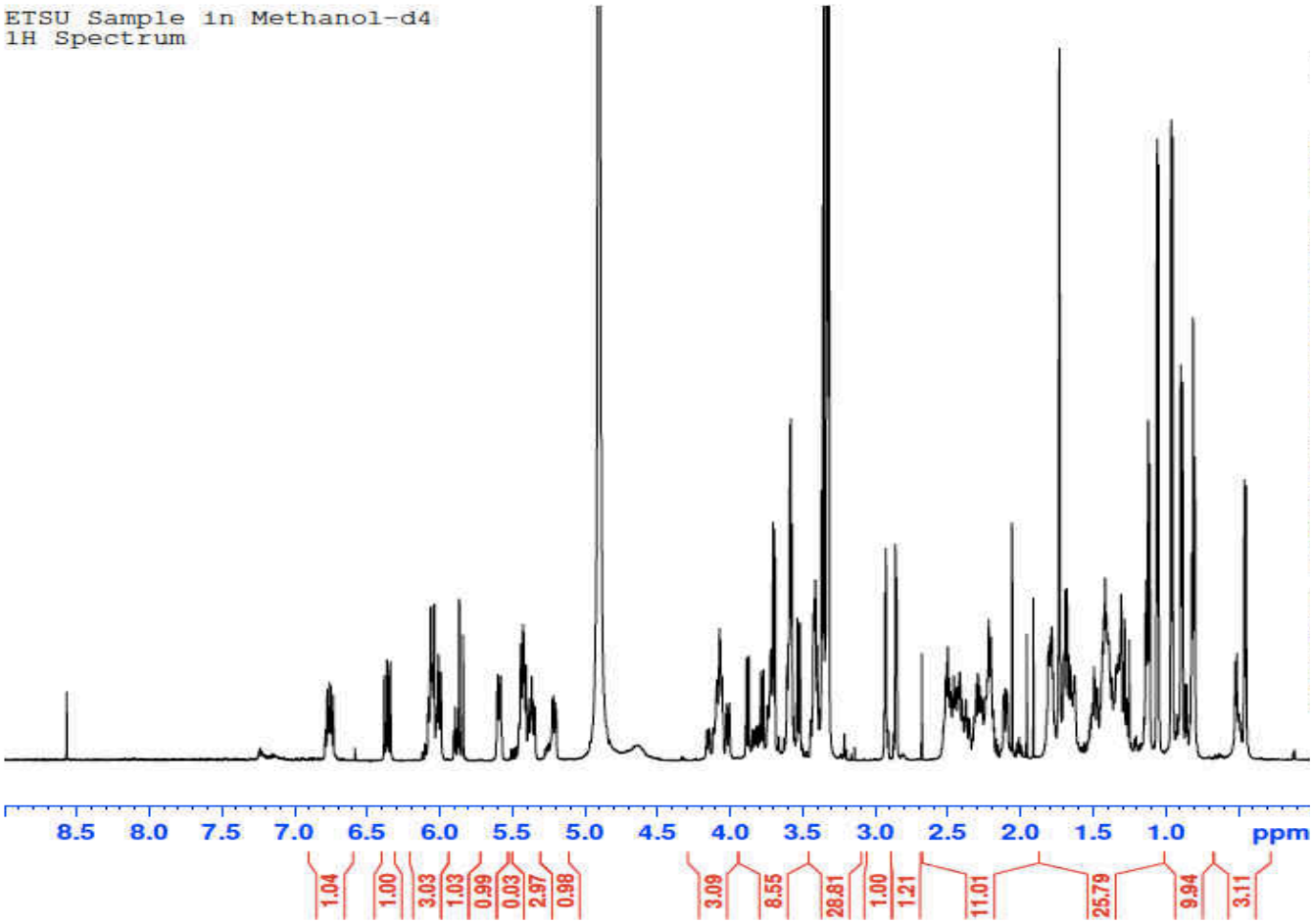
Appendix B2: MS Spectrum Zoomed Around Molecular Ion Peak at 911.5490 m/z



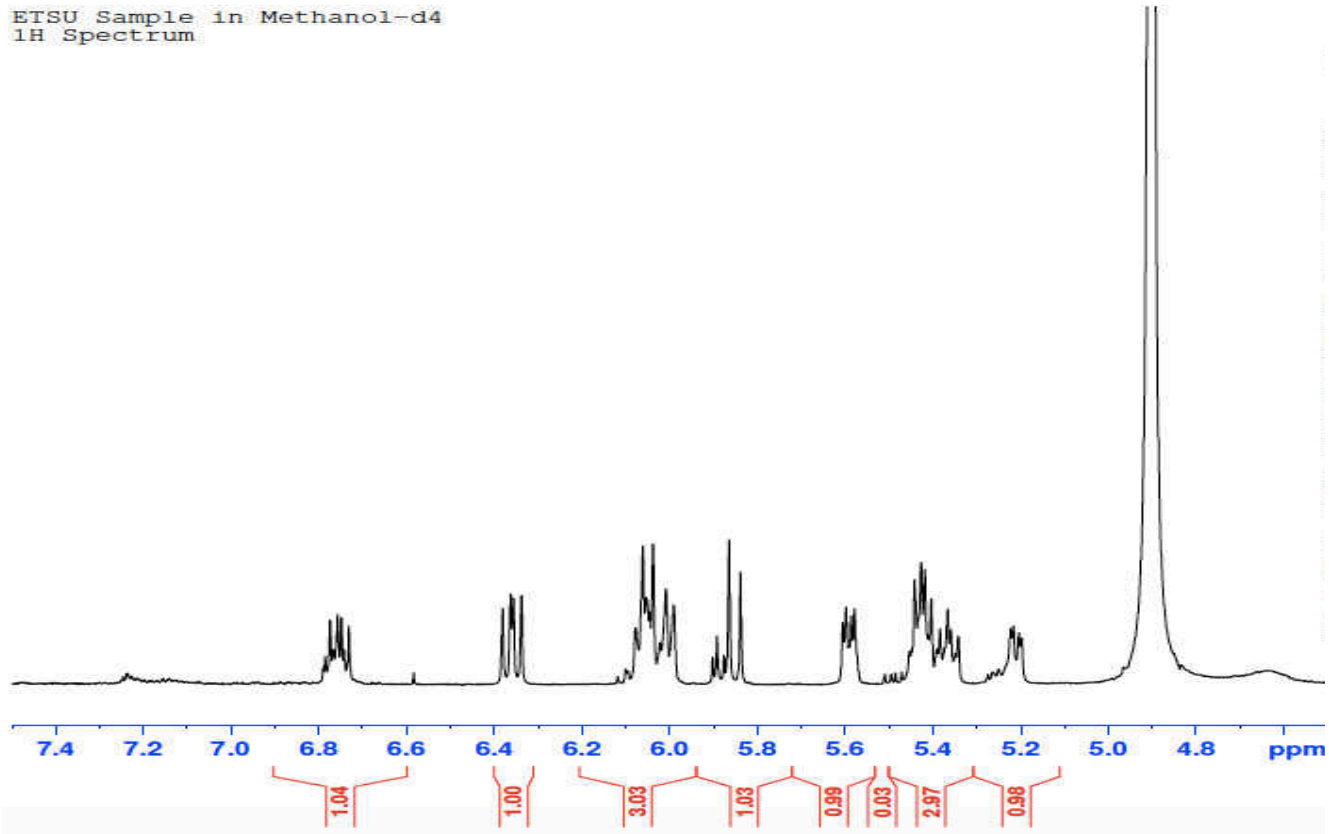
Appendix B3: MS/MS Spectrum for The Compound



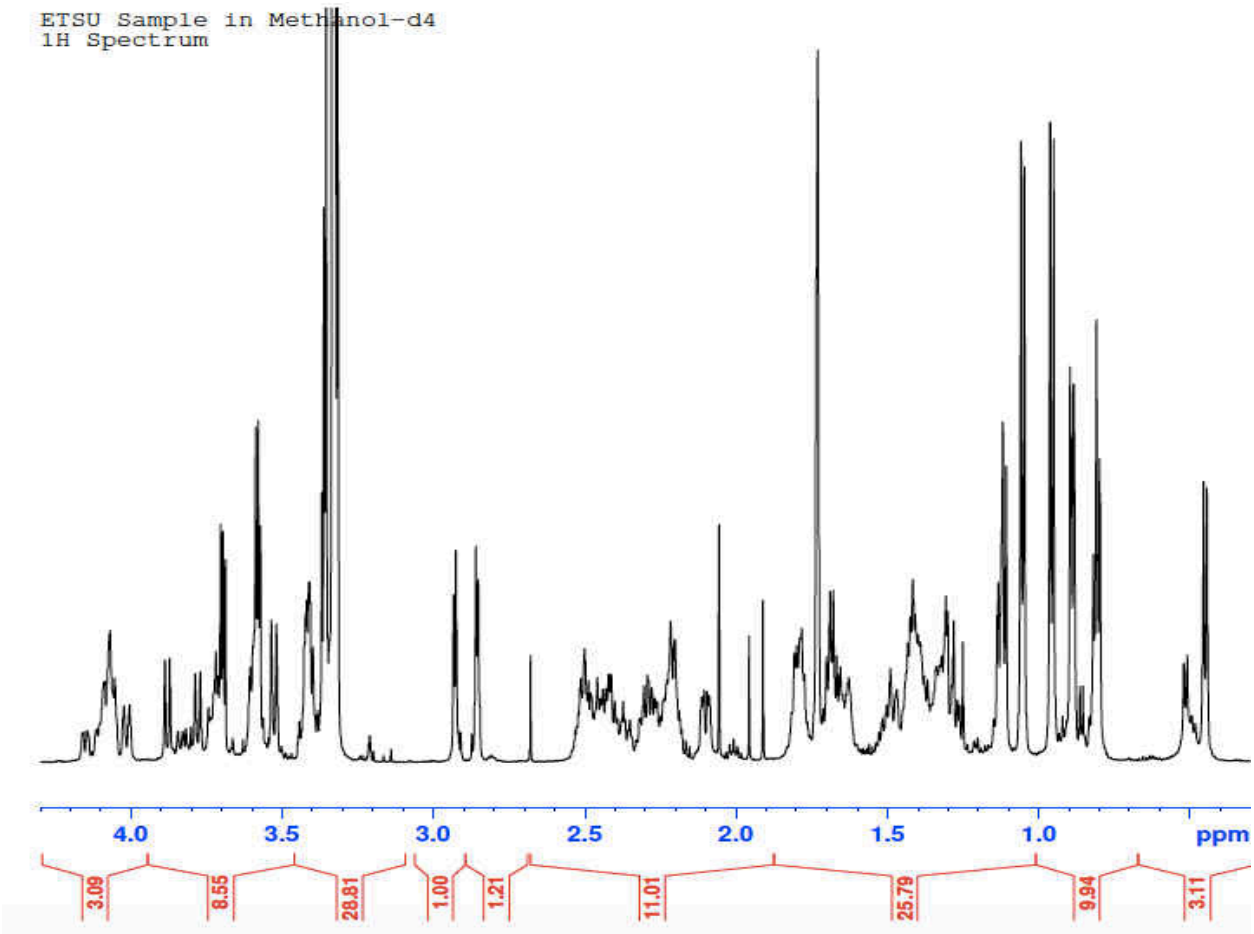
Appendix C1: ^{1}H NMR Spectrum for The Compound



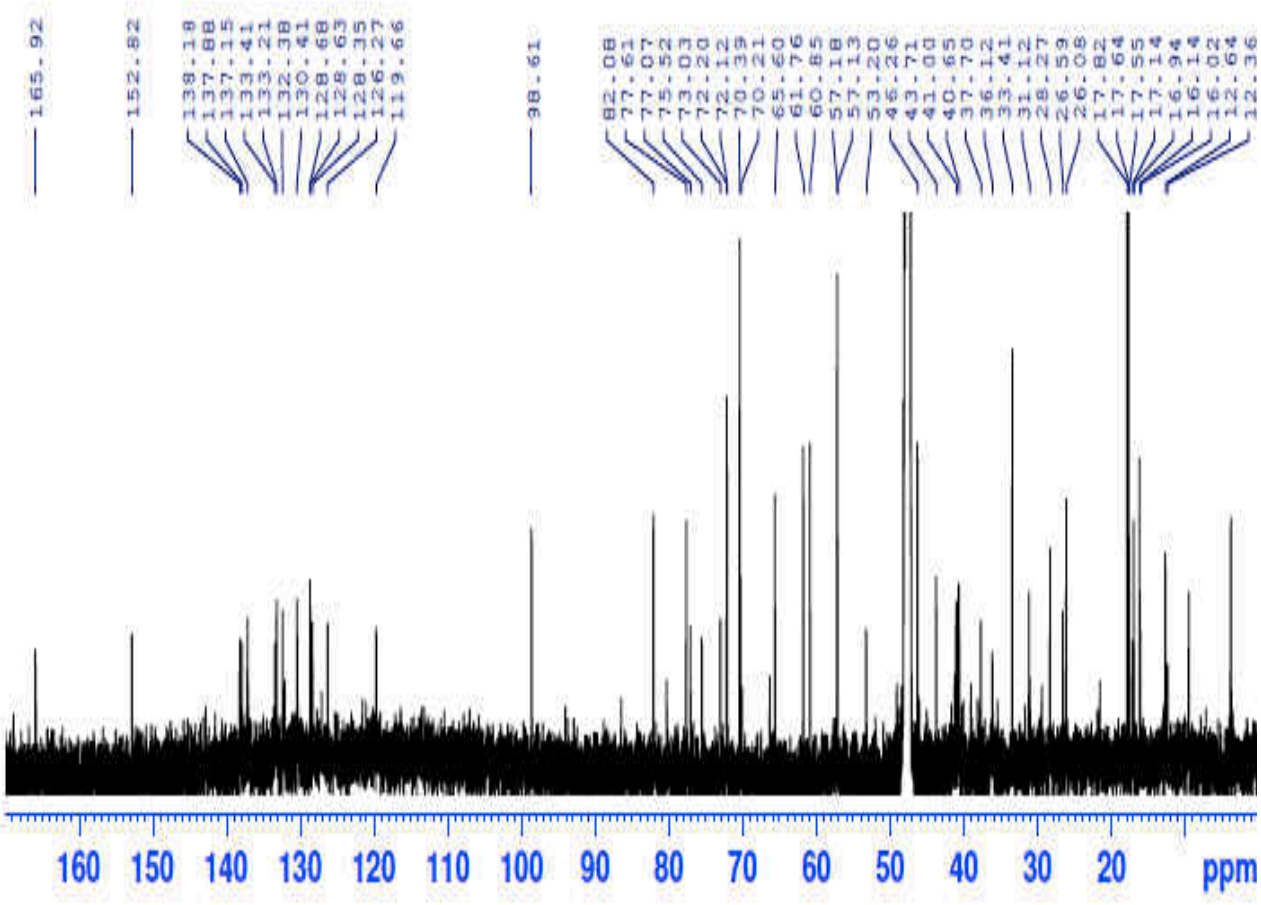
Appendix C2: ^{1}H NMR Spectrum for The Compound



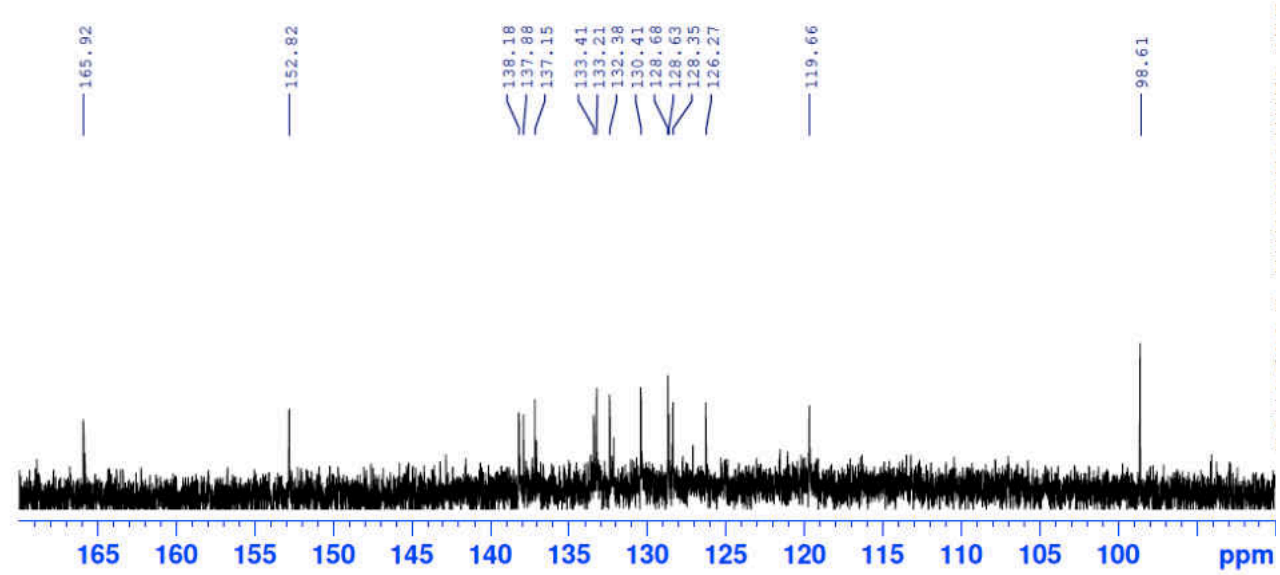
Appendix C3: ^{1}H NMR Spectrum for The Compound

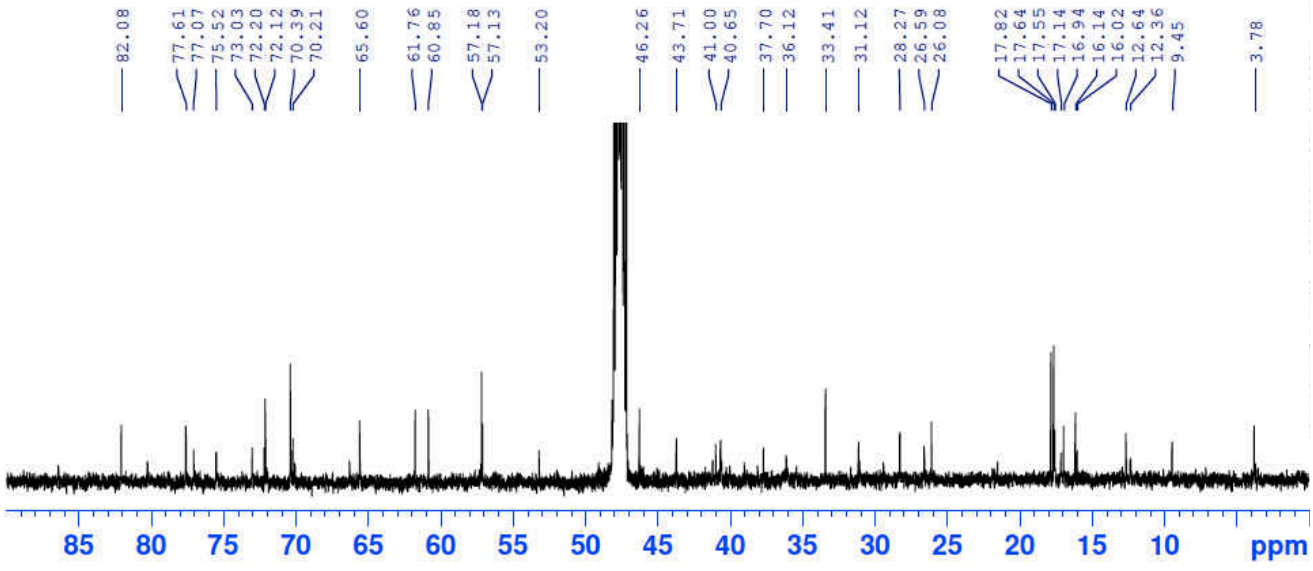


Appendix D1: ^{13}C NMR Spectrum for The Compound

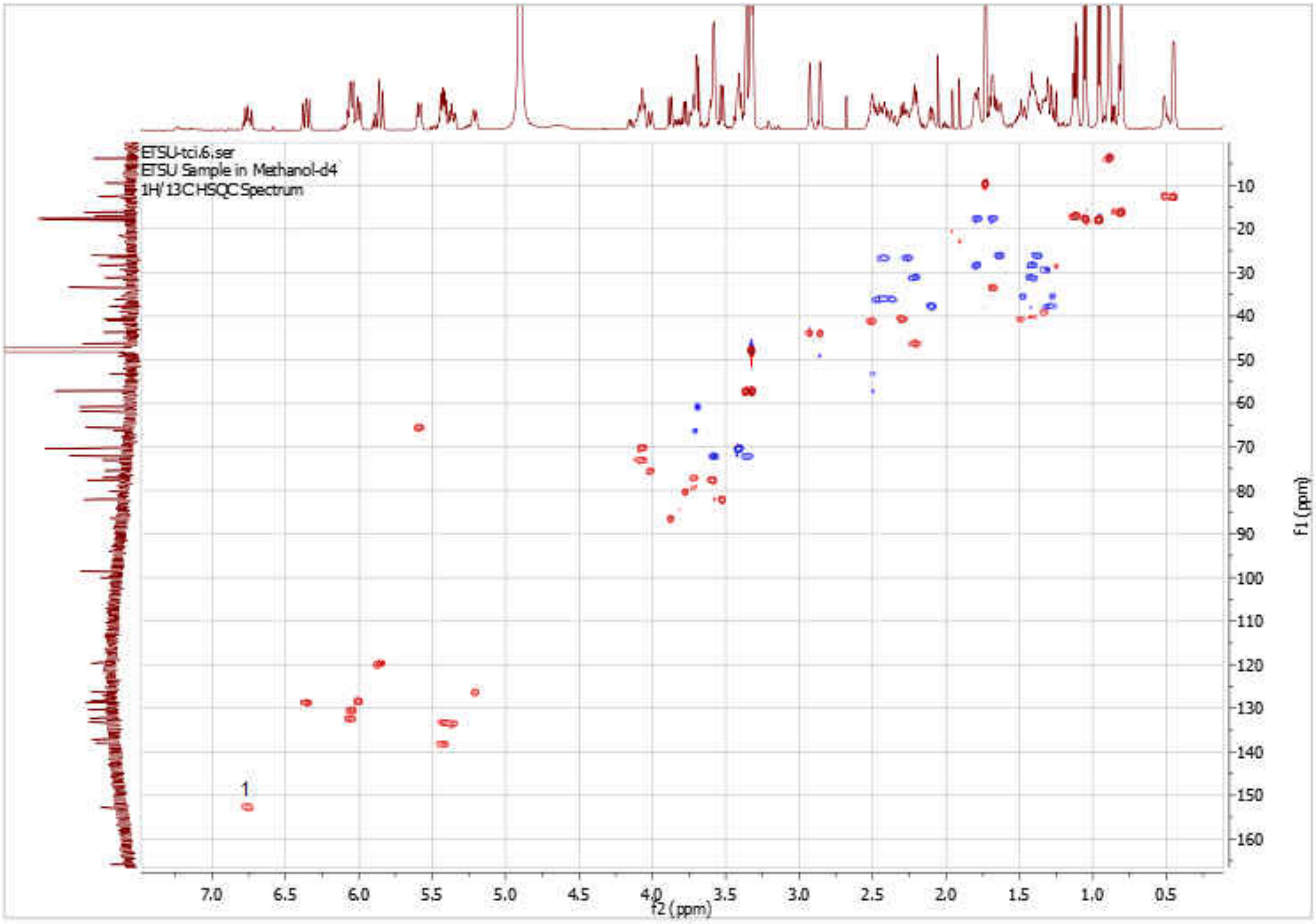


Appendix D2: ^{13}C NMR Spectrum for The Compound

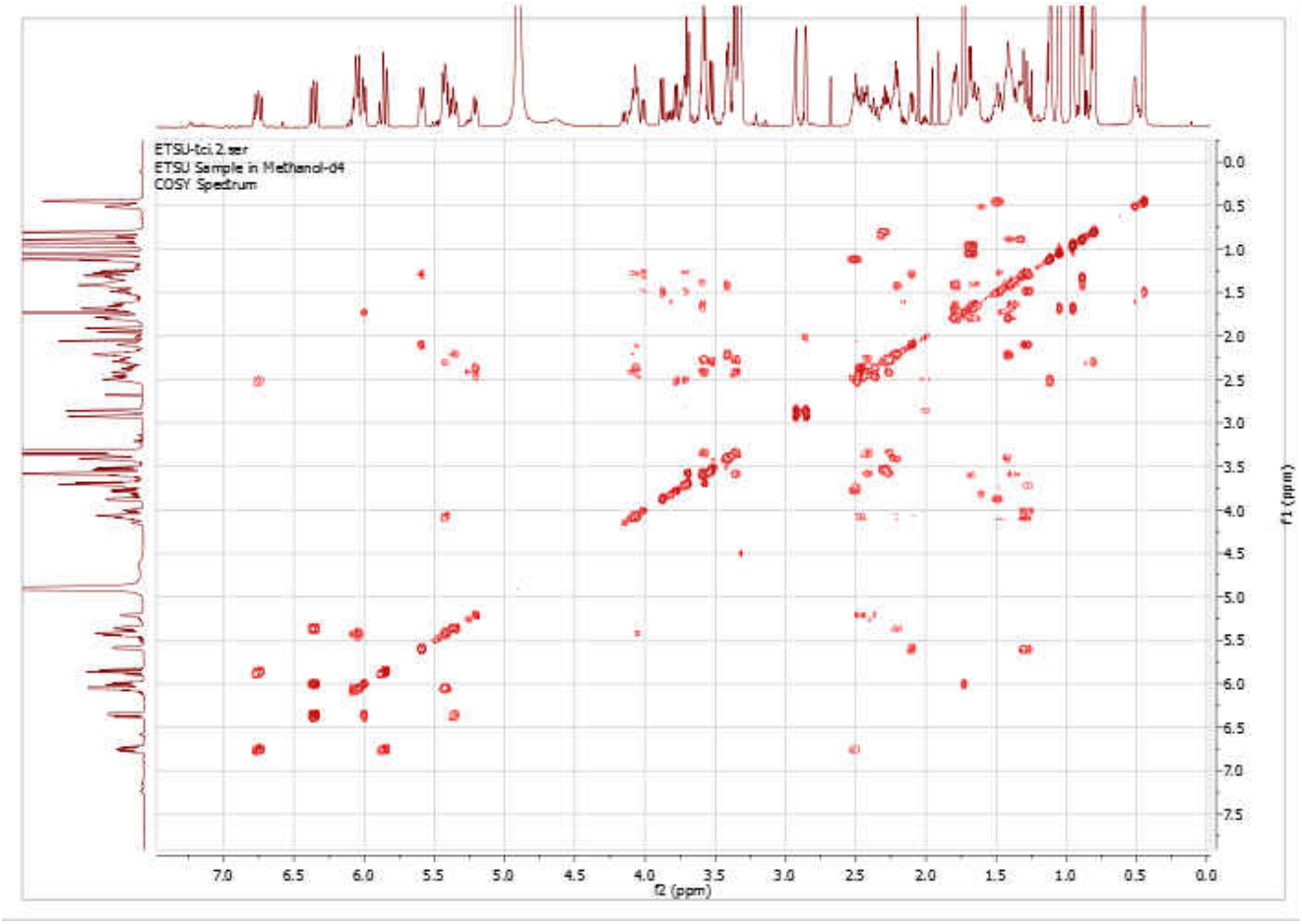




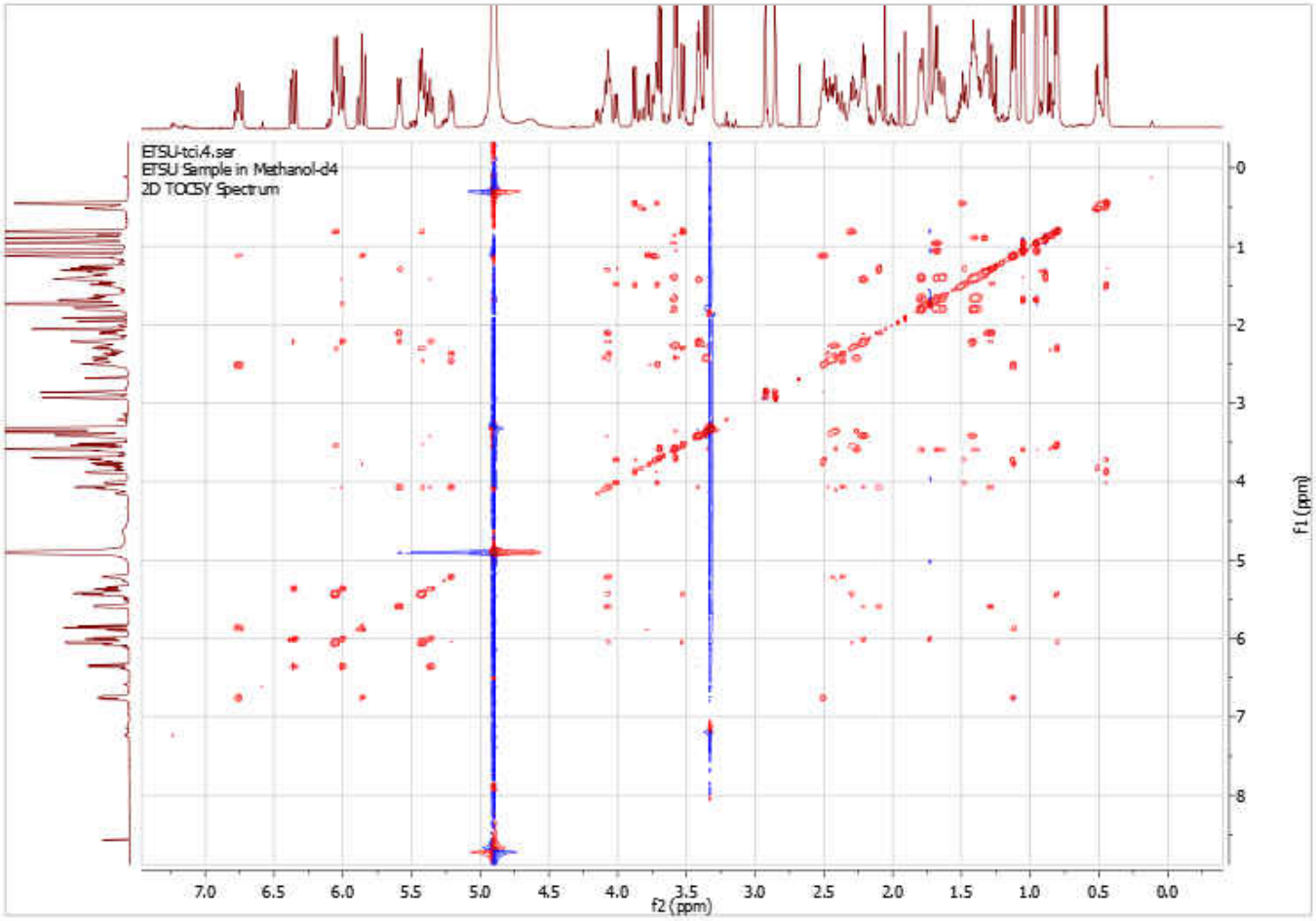
Appendix E: HSQC Spectrum for The Compound



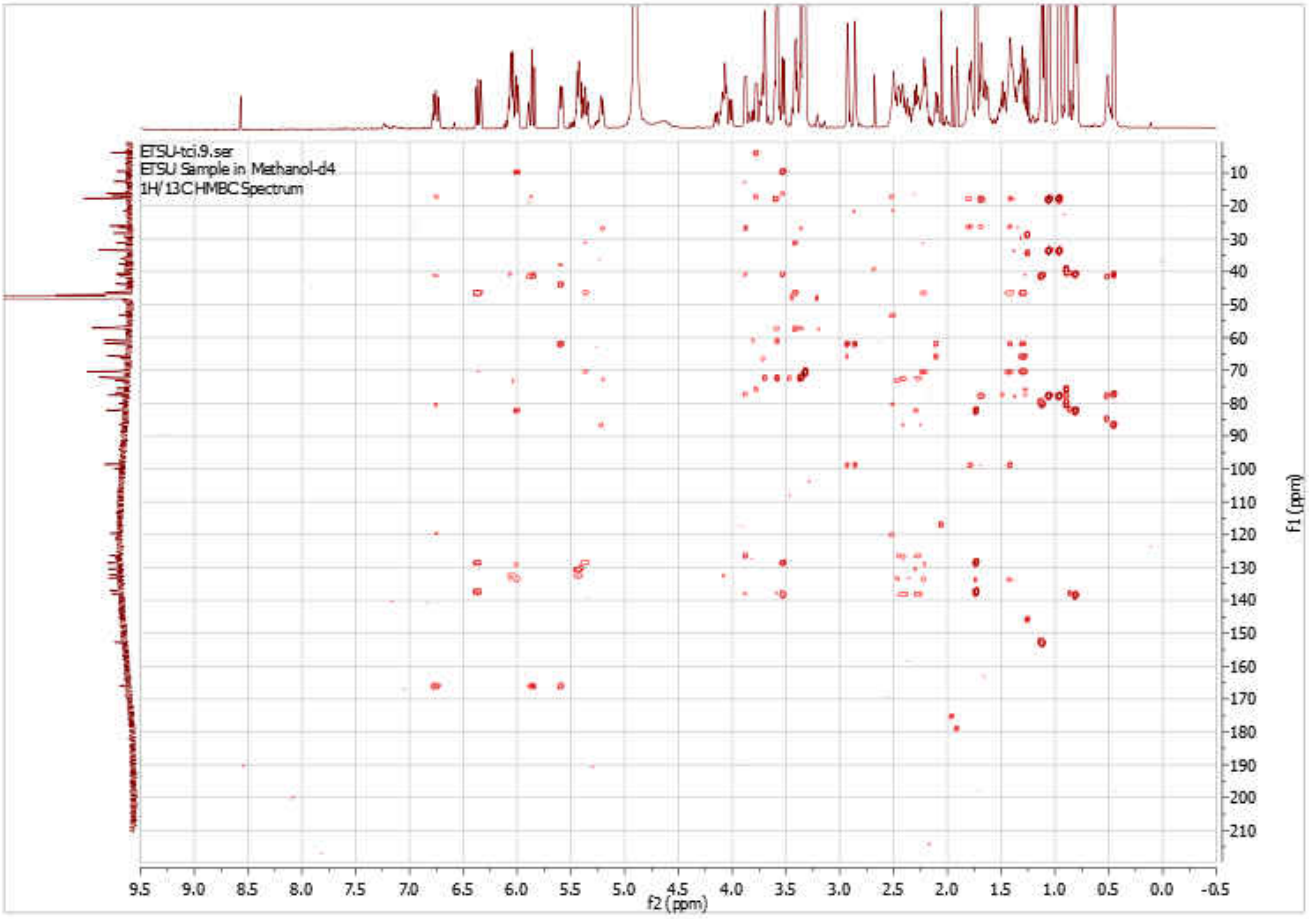
Appendix F: COSY Spectrum for The Compound



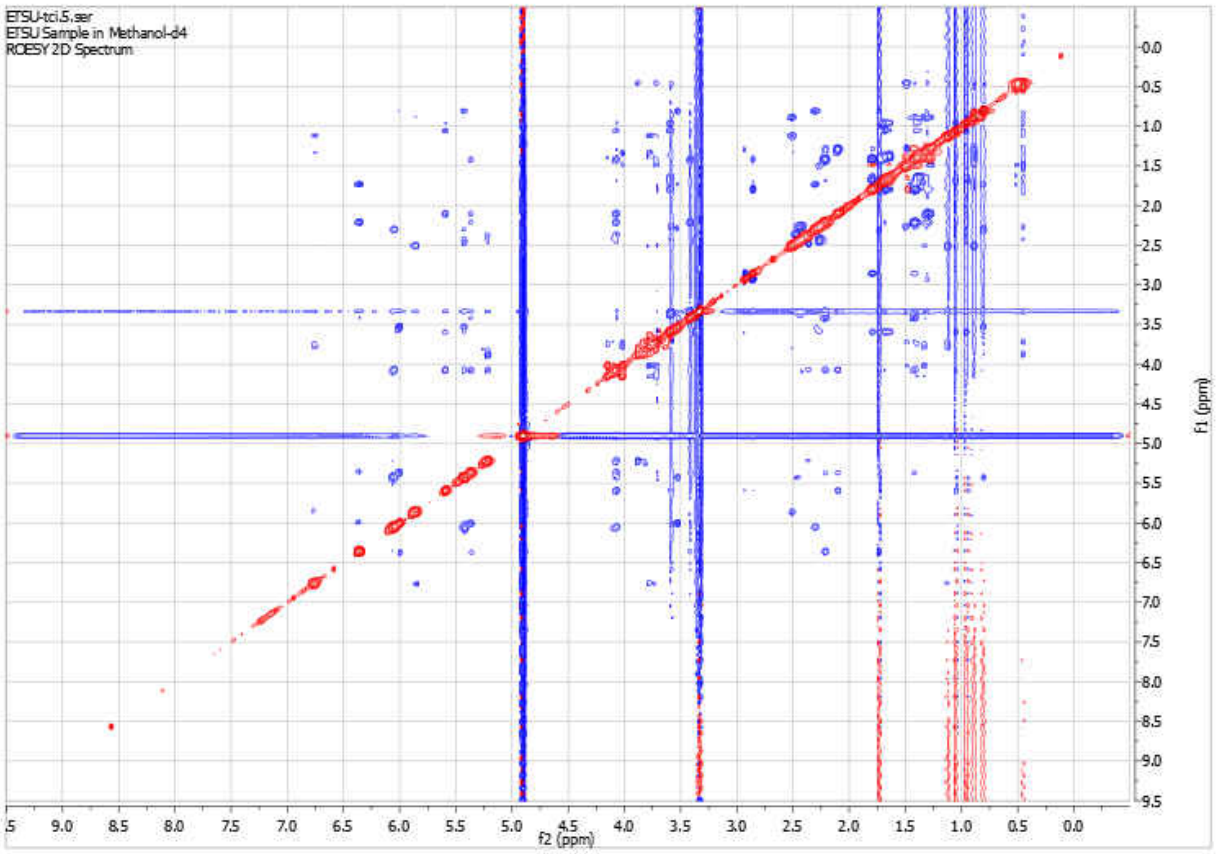
Appendix G: TOCSY Spectrum for The Compound



Appendix H: HMBC Spectrum for The Compound



Appendix I: ROESY Spectrum for The Compound



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Presentations:

Reddyvari Manikindi, P., Ward, L. Amber., Lampson, C. B., Shilabin, G. Abbas; "Extraction and Characterization of an antibiotic- like molecule produced by *Rhodococcus* sp. MTM3W5.2" 67th Joint Southwest Regional Meeting/Southeast Regional Meeting of the American Chemical Society, Memphis, TN, November 4-7, 2015, Abstract: 503

Ward, L. Amber., Reddyvari Manikindi, P., Shilabin, G. Abbas., Lampson, C. Bert; "Using Genomics to Infer The Identity of an Inhibitory Compound Produced by *Rhodococcus sp.* MTM3W5.2" Appalachian Student Research Forum, Johnson City, TN, April 8-9, 2015.

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