New Methods to Induce and Detect Microbial Secondary Metabolite Production

By

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To all of my friends and family,

especially my mother,

this would not have been possible without you

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LIST OF ABBREVIATIONS

ACP	Acylcarrier protein
A-Domain	Adenylation
AT-Domain	Acyltransferase
BIMS	Bio-autography imaging mass spectrometry
CDC	Center for disease control
C-Domain	Condensation
COSY	Correlation spectroscopy
Cy-Domain	Cyclization
DESI	Desorption electrospray ionization
DH-Domain	Dehydratase
DIOS	Desorption ionization on silicon
DNA	Deoxyribonucleic acid
E-Domain	Eperimerization
ER-Domain	Enoylreductase
FDA	Federal drug administration
HDX	Hydrogen-deuterium exchange
HMBC	Heteronuclear multi-bond correlation spectroscopy
HR-MS	High resolution mass spectrometry
HSQC	Hereronuclear single quantum correlation
IM-MS	Ion mobility mass spectrometry
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride
KR-Domain	Ketoreductase

KS-Domain	Ketosynthase
LCMS	Liquid chromatography mass-spectrometry
LOD	Limit of detection
m/z	Mass to charge ratio
MALDI	Matri-assisted laser ablation/desorption ionization
MRSA	Multidrug resistant *Staphulococcus aureaus*
MSI	Mass spectrometry imaging
nESI	Nano-electrospray ionization
NIMS	Nano-initiated mass spectrometry
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhouser effect spectroscopy
NRPS	Non-ribosomal peptide synthetase
OPLS-DA	Orthoganol partial least squares differential analysis
PCA	Principal component analysis
PKS	Polyketide synthase
ROI	Region of interest
RP-HPLC	Reverse phase high pressure liquid chromatography
SIMS	Secondary ion mass spectrometry
T-Domain	Thiolation
TLC	Thin layer chromatography
UV	Ultra-violet

CHAPTER 1

INTRODUCTION

Clinical Relevance of Microbial Secondary Metabolites

Natural products, or secondary metabolites, derived from microbial sources have had a significant impact on the development of society and human health for centuries. These compounds, and those from other biological sources, have been credited for the increase in life expectance from around 40 years at the turn of the 20th century to slightly less than 80 at the turn of the 21st century. The first isolated and purified natural product was morphine in 1804, while the first antibiotic, penicillin, was discovered in the 1940's.[1] Since the serendipitous discovery of penicillin from the fungus *Penicillium notatum*, microorganisms have been targeted for production of secondary metabolites with pharmacopia capabilities relevant to preventing and treating a multitude of human diseases.[2] The result of which was the focused efforts of pharmaceutical companies to focus on discovery of new bioactive secondary metabolites from microorganisms.[3] The "golden age of discovery" for secondary metabolic compounds was characterized by an impressive rate of discovery of unreported compounds and occurred between the 1940s and mid-1960s. Many of the compounds discovered during this era are still viable chemical entities in the clinical setting for treating a variety of medical ailments.[4]

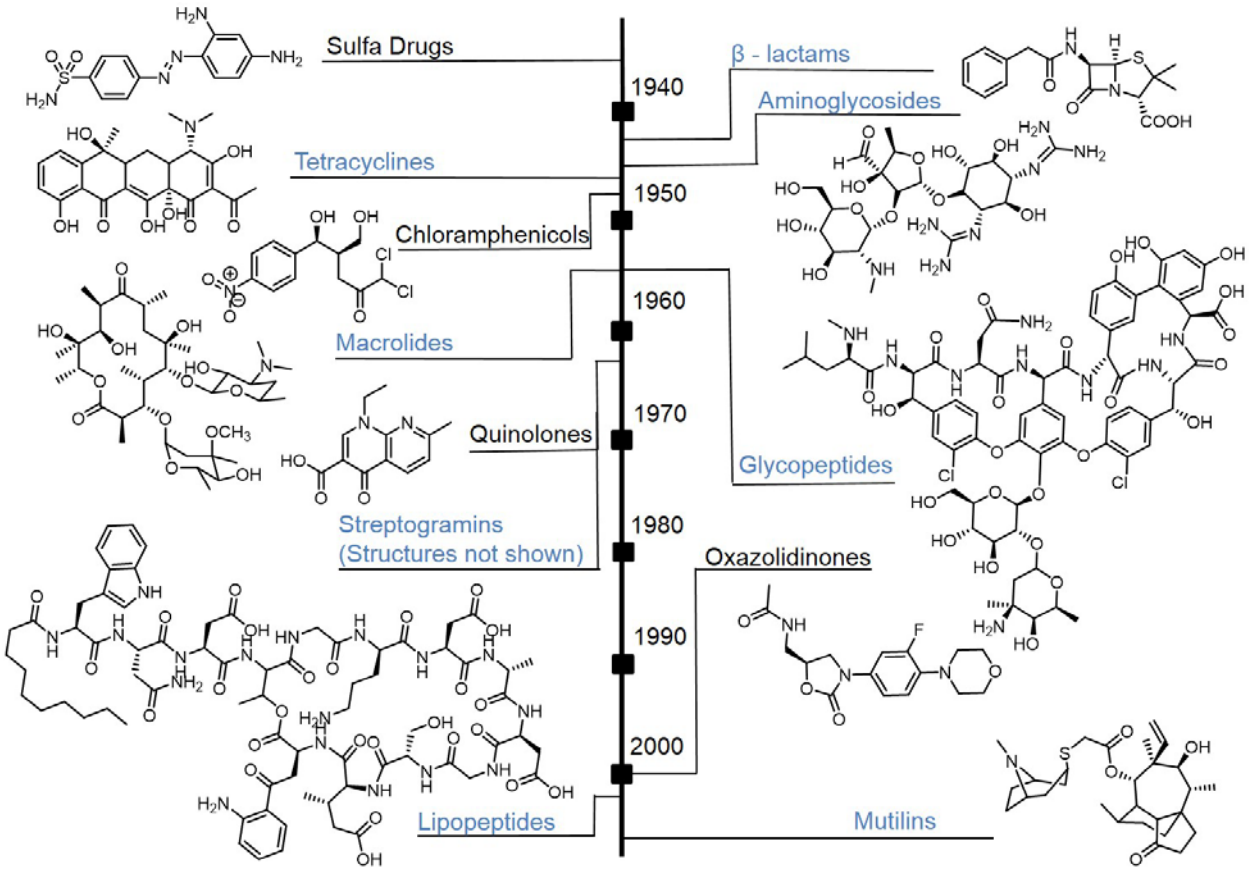

Figure 1-1. Timeline of FDA approval classes of antibacterial drugs with example molecules. Sulfa drugs, Prontosil, 1930s; ß-lactams, Penicillin, 1941, Aminoglycosides, Streptomycin, 1943, Tetracyclines, Tetracycline, 1945, Chloramphenicol, 1949, Glycopeptides, Vancomycin, 1953, Macrolides, erythromycin, 1953,Quinolones, Nalidixic acid, 1962, Oxozolidinones, Linezolid, 2000, Lipopeptides, Daptomycin, 2003. Class name is color coded to identify natural products (blue) and synthetic compounds (black).

The broad range of chemical structures, combined with the impressive stereochemical control of secondary metabolite production, make these compounds attractive targets for pharmaceutical development.[5] Secondary metabolites generally contain a large number of stereocenters and complexity and a greater relative amount of carbon, hydrogen, and oxygen while incorporating less nitrogen than molecules typically designed by medicinal chemists. Furthermore, many of the secondary metabolites with clinical relevance exceed the 500 Dalton limit generally thought of as the cutoff for

medicinal compounds and higher polarities.[6] In addition to violating molecular weight cutoff of 500 Daltons according to “the rule of 5” established by Lipinski, bioactive secondary metabolites tend to exceed the limit of 5 hydrogen bond donors and 10 hydrogen bond receivers.[7]

While the bioactivity of penicillin was observed in 1929, the compound was not approved as an anti-infective until 1942.[8] The timeline showed in Figure 1-1 details the FDA approval rate for new chemical scaffolds, both synthetic compounds and secondary metabolites, which are effective at treating a range of microbial infections. The first aminoglycoside discovered, streptomycin, was approved in 1944 for treatment against gram-positive and gram-negative bacteria which were resistant to penicillin.[9] The first tetracycline scaffold was approved in 1945, the discovery of which was the result of an observed yellow pigment produced by a fungus which inhibited the growth of other microorganisms in close proximity to the producing organism.[10] Vancomycin, the first glycopeptide identified, was approved in 1953 as an intravenous treatment for penicillin resistant *S. aureus*.[11] The natural compound class of macrolides were approved in 1953 with the discovery of erythromycin.[12] The streptogramin class of antibiotics represent the first class of an anti-infective consisting of multiple compounds used in a polypharmacy method to treat infections were also first reported in 1953.[13]

Within the last 50 years, more than 25% of the FDA approved compounds were either secondary metabolites or secondary metabolite derivatives and more than half of all approved cancer drugs fell into these two categories.[14] Unfortunately, the discovery and reporting of natural products containing novel chemical scaffolds following the “golden age of discovery” was virtually nonexistent.[15] As a consequence, only three new

classes of antibiotics since the mid - 1960s, two of which are natural products, the lipopeptides and mutilins.[16, 17] During this same timeframe, only two new synthetic classes of compound were approved for treatment against bacterial infections.

One possible explanation for the gap in discovering new chemical scaffolds of secondary metabolites could be the use of biosynthetic modules which are used for production of secondary metabolites and shared by many bacteria. The capacity of various microbes to produce the same or similar compounds has resulted in isolation and identification of known compounds.[5] The time and cost associated with isolating and identifying a compound are significant, and the high rate of isolation of previously reported compounds may be one of the reasons why pharmaceutical companies abandoned efforts *en masse* to exploit microbial organisms as producers of novel therapeutics.[18] The process of dereplication, or identifying a compound based on a collection of analytical data as a chemical entity that had already been reported, remains a significant hindrance in natural product discovery.[14] The development of new methods to identify lead compounds and expedite the process of dereplication will improve the efficiency of isolation for novel secondary metabolites. New analytical techniques with improved sensitivity for detection of low relative abundance and methods for determination of lead compounds within a complex mixture are needed, as current methodologies have resulted in only a single new class of FDA approved antibiotic secondary metabolite.[19]

Microorganism	Estimated cases for 2013	Estimated deaths for 2013
Methicillin resistant *Staphylococcus aureus*	80,000	11,000
Streptococcus pneumonia	1,200,000	7,000
ß-lactamase producing Enterobacteriaceae	26,000	1,700
Vancomycin resistant *Enterococcus*	20,000	1,300
Carbapenem resistant Enterobacteriaceae	9,300	610
Multidrug resistant *Acinetobacter*	7,300	500
Multidrug resistant *Pseudomonas aeruginosa*	6,700	440
Drug resistant typhoidal *salmonella*	100,000	40
Drug resistant *Campylobacter*	310,000	28
Drug resistant *Neisseria gonorrhoeae*	246,000	<5
Vancomycin resistant *Staphylococcus aureus*	<5	<5

Table 1-1. Reported cases of antibiotic resistant microorganisms and the number of cases and deaths caused by these infections. Selected resistant pathogens were extracted from data reported by the Centers for Disease Control (CDC) .[23]

Even with approximately $25 billion per year spent for antibiotics in 2002[20], pharmaceutical development in recent years has focused on long term drugs, such as those prescribed for high blood pressure, rather than those used for a limited duration, such as antibiotics.[21] An increase in antibiotic resistant pathogenic organisms, particularly methicillin resistant *Staphylococcus aureus* (MRSA), is changing this focus. Due to the increase in the occurrence of antibiotic resistant pathogens, the lack of new

chemical scaffolds for antibiotics represents a significant health hazard.[22] In a report published by the CDC on MRSA in the United States, 94,360 people developed an invasive infection in 2005, and approximately 18,650 died as a result of the infection.[23] While the number of cases and fatalities associated with MRSA were reduced in 2013, presumably as a result of understanding and education about practices to prevent infection. A dramatic increase in the number of resistant microbes is causing significant alarm within the health care community. Of particular concern, is the development of resistance by *S. aureus* to vancomycin, the drug of last resort for treatment of antibiotic resistant microbes,.[24] A selection of resistant microbes and the number of cases and deaths reported for each are listed in Table 1-1. Without new medications to address these pathogens, the development of lifesaving treatments within the hospital setting may be negated as hospital acquired infections represent a significant number of the cases reported.

Understanding the resistant mechanisms of these microbes has led to the development of polypharmacy, the use of drug combinations to combat resistance, as with the use of ß-lactamase inhibitors for microbes that are capable of deactivating ß-lactam containing drugs such as the penicillin class.[25] The generation of chemically modified derivatives of an already reported chemical scaffold, such as the development of piperacillin to improve the efficacy of penicillin compounds that are no longer effective at treating infection, have extended the bioactivity capacity of certain known chemical scaffolds.[22] While this method has improved the pharmaceutical longevity of many compound classes, resistance to the modified structure frequently occurs resulting in the need for new scaffolds to which microbes have not developed resistance.[22]

There are several approaches to exploit microorganisms, especially actinomycetes, to combat the increase in antibiotic resistant microbes. Exploitation of novel environments, such as marine environments, for isolation of microbes capable of secondary metabolite production has resulted in the discovery of pharmaceutically relevant compounds.[26] Analysis of environments associated with deep-sea hydrothermal vents which were originally thought to be prohibitive to life have been investigated and revealed a microbial population of 10^5 microorganisms mL^{-1}.[27] Evaluation of microbes from such hostile environments have resulted in the discovery of a variety of secondary metabolites.[28]

Sources of Secondary Metabolites

Secondary metabolites come from a range of producers including plants, animals, fungi, and bacteria. Among the bacteria, the actinomycete genus is the most exploited microbe for secondary metabolites, with the streptomycete species, as the most prolific producers.[29] Actinomycetes have been discovered in almost every environment around the globe, though secondary metabolite isolation efforts have historically focused on those isolated from terrestrial environments. This is not surprising given that the rhizosphere is teeming with microbes at a concentration of 10^8 individual microorganisms per gram of soil.[30] Isolation and evaluation of the microbes from environments previously thought to be uninhabitable by microorganisms have been explored in recent endeavors, resulting in the identification of a variety of new chemical entities (NCE).[31]

The production of secondary metabolites within these microorganisms is often closely regulated by a series of complex biological events, some of which are still not fully

understood. The introduction of genomics to identify genes responsible for regulation and production of secondary metabolites revealed that only a handful of compounds that are genetically encoded are produced in detectable and isolatable quantities.[32] The first streptomycete genome that was annotated and published was for *Streptomyces coelicolor* A3 (2), which has become a model actinomycete known to produce of a variety of pigmented and biologically active compounds.[33] Completing the analysis of *S. coelicolor* resulted in the knowledge that 29 gene clusters were capable of producing secondary metabolites. As many of the identified genes do have a corresponding isolated and characterized secondary metabolite, it is hypothesized that these genes are not expressed utilizing current culture methods practiced in the laboratory setting.[34]

The collective number of genomes, and characterized genes capable of producing secondary metabolites, has increased the understanding of how secondary metabolism is controlled.[35] Evaluation of streptomycete genomes that have been sequenced revealed that these microbes generally possess more than twenty gene clusters capable of producing secondary metabolites.[34] The genomes of these organisms are approximately 8 Mb, indicating that genes responsible for secondary metabolism represent 5% of the total genome.[34] Genetic analysis of micro-organisms possessing smaller genomes revealed that these microbes also incorporate biosynthetic genes for secondary metabolites though at a lower frequency.[34] To further complicate the understanding of secondary metabolism from microbes, there remain a number of identified molecules for which the biosynthesis still remains unknown as no gene(s) have been identified that would be hypothesized to produce the compounds. Genomic analysis to determine if actinomycetes, and more specifically streptomycetes, were capable of

producing secondary metabolites resulted in renewed efforts to exploit these microbes for isolation of potentially relevant pharmaceutical compounds.[36]

Many of the molecules capable of being produced based on genetic analysis remain elusive, potentially as a result of failure to activate the genes responsible for production at detectable levels, isolation methods which result in degradation of the compounds produced, or inability to detect the molecules due to limit of detection (LOD) analytical capabilities. As a result, new methods have been developed to elicit the expression of genes that are considered silent, cryptic, or unexpressed.[37-40] In general, secondary metabolic gene clusters are grouped together on the chromosome along with any regulatory genes that control activation.[41] Genes responsible for secondary metabolism within streptomycetes are classified into several categories, the most extensively studied of which are the nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS).[42] The modular organization of these classes of genes facilitate putative structure determination through genetic analysis which has led to the development of rationally guided approaches for culture conditions and isolation methods.[43] Significant efforts are under way worldwide to improve the number and variety of secondary metabolites which can be isolated and characterized with biological relevance.

How Secondary Metabolites are Produced

The most commonly identified classes of modular genes responsible for production of microbial secondary metabolites are PKS and NRPS.[42] Compounds produced by PKS genes are FDA approved for treatment of a variety of medical ailments resulting in the

generation of $15 billion in 1999.[20] Analysis of the genomes of *S. ceolicolor* and *S. griseus,* the most extensively studied streptomycetes, has revealed the conserved nature of secondary metabolite production. The conserved nature of the chemical reactions performed for secondary metabolite production by these genes has led to the development of multiple computer algorithms which can be utilized to predict, with high levels of accuracy, the secondary metabolite produced if expression of the genes is initiated. [42] This information has facilitated the relational design of culture and analytical methods to elicit and confirm production of secondary metabolites.

Three classes of PKS genes have been identified; the classification of each is determined by the organization and utilization of domains. PKS type I genes organized in an assembly line manner where each module consists of various domains, each of which perform a specific chemical modification. The well conserved chemical modification produced by each domain and the order in which the modules are assemble facilitate a fairly accurate *de novo* structure prediction.[42] Initiation of production of a secondary metabolite assembled by PKS type I genes begins with the activation of the ketosynthase$_Q$ (KS_Q) domain, which incorporates glutamine instead of cysteine for bonding with the initial starter unit. The identification of this domain within the gene indicates the starting point for the production of a secondary metabolite and can potentially aid in the identification of regulatory genes. As with most enzymes, the substrate, or in this case the extender unit, utilized in PKS type 1 modules is well conserved, facilitating reasonably accurate structure predictions. The most common extender unit is methylmalonate which incorporates two carbons into the extension of the secondary metabolite for each domain within the gene cluster.[42] A variety of other

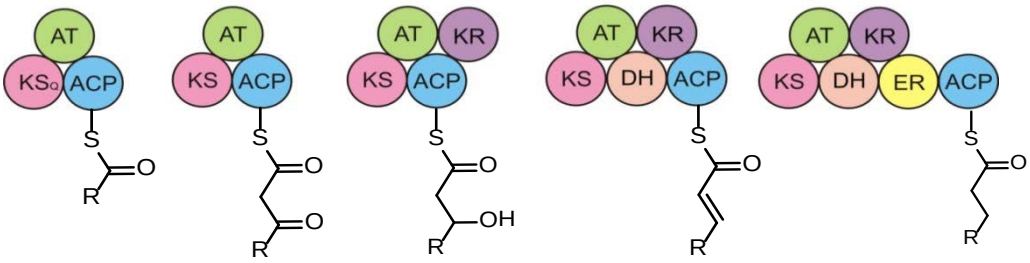

Figure 1-2. PKS module. Organization of the most common domains within PKS type I and the expected extension of a secondary metabolite utilizing methylmalonate as the extending unit. KS = ketosynthase, AT = acyltransferase, ACP = acyl carrier, KR = ketoreductase, DH = dehydratase, ER = enoylreductase.

uncommon extension units have been observed including hexyl-, propyl, chloroethyl- and isobutylmalonate.[44]

Following enzymatic attachment of the extender unit to the KS domain, the acyltransferase (AT) domain attaches the extender unit and transfers the entire molecule to the acylcarrier protein (ACP). The KS, AT, and ACP domains are the minimum requirements for a functional PKS I module though additional domains may be incorporated into the modules to perform specific chemical transformations. Ketoreductase (KR) modules reduce the carbonyl to an alcohol, dehydratase (DH) eliminates the alcohol to produce an alkene, and the enoylreductase (ER) reduces the alkene to produce two methylene carbons. The organization of and chemical transformations performed by each domain is illustrated in Figure 1-2.

PKS type II genes are aggregates of modular proteins resulting in the iterative use for production of carboxylated methylene groups.[34] PKS type III genes do not incorporate the acyl carrier (ACP) domain like PKS I and PKS II genes, though the iterative use of enzymes for production of a secondary metabolite are similar to those of PKS II.[34] Regardless of the type of PKS gene responsible for production of the secondary

metabolite core structure, further modification of the compound can occur. Enzymes have been identified which are capable of incorporating a hydroxyl group via oxidation, a sugar molecule via glycosylation, or a methyl group via methylation. The additional modifications can occur as the molecule is being produced or as post translational modifications after the compound is completed. While structure prediction based on the arrangement of the domains can be very accurate, several examples exist in which one domain within a module is not expressed, an entire module is not expressed, or a module is used successively in the production of the secondary metabolite.

Similar to the enzymatic synthesis of PKS molecules, biosynthesis of secondary metabolites produced by NRPS encoded genes is controlled through the arrangement of multiple modules containing domains responsible for specific chemical modification. The minimal module required for NRPS is made up of three domains: 1) the condensation (C) domain which incorporates specific amino acids, 2) the adenylation (A) domain, which joins the amino acid to the extending secondary metabolite, and 3) the thiolation (T) domain, which holds the molecule during the extension through the addition of amino acids. Other domains that can be incorporated into the modules include the epimerization (E) domain which inverses the stereochemistry at the α-position, and cyclization (Cy) domain, which cyclizes a serine, threonine or cysteine into a oxazolidines or thiazolidine (Figure 1-3).[42] Just as with PKS produced molecules can undergo secondary

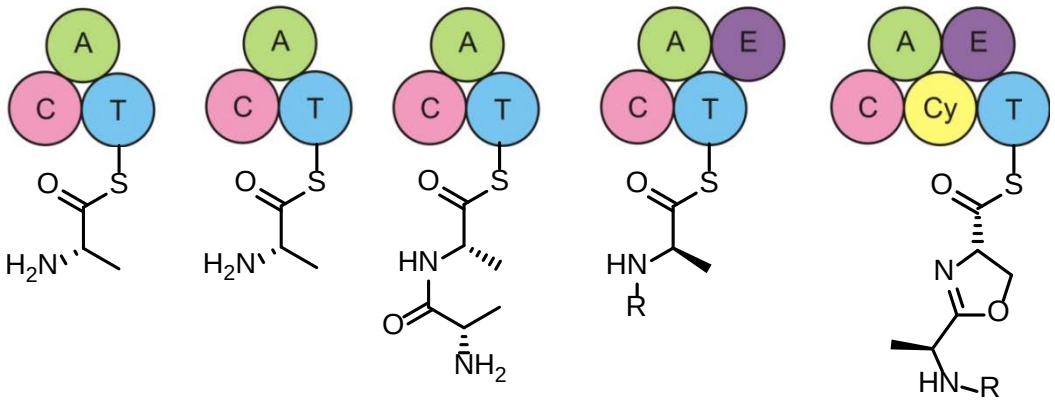

Figure 1-3. NRPS module. Organization of the most common domains within NRPS and the expected extension of a secondary metabolite utilizing amino acids as the extending unit. C = condensation, A = adenylation, T = thiolation, E = epimerization, Cy = cyclization.

modifications NRPS molecules can be modified through the use of methylating, oxidizing, or glycosylating enzymes. The elucidation of the number and type of amino acids within this type of secondary metabolite is fairly straightforward, though it can be complicated due to the iterative use of a single domain or the lack of utilization or expression of domains within the modules or entire modules.[45]

Following the putative identification of amino acids incorporated into the secondary metabolite, mass spectrometry analysis of the culture broth can be used to determine if the molecule of interest is produced. Supplementing the culture media with heavy labelled amino acids can also be used to identify the compound of interest produced by a NRPS gene cluster.[46] Unlike traditional isotope feeding experiments that seek to determine the biosynthetic route for secondary metabolite production, this method is used to identify a metabolite within a complex extract to guide compound isolation.[46] One disadvantage to this approach is that the amino acid used for feeding could be incorporated into other biosynthetic processes resulting in low levels of incorporation in

the compound of interest, making this method potentially cost prohibitive. One method to address this limitation is the use of tandem mass spectrometry (MS-MS) techniques in which fragmentation of the compounds facilitate the determination of the other amino acids within the compound. Comparison of the amino acids and the order in which they are incorporated in the molecule can be used for comparison to the gene of interest to determine if the molecule detected is the desired compound.[46]

An additional MS approach to aid in determining if a desired secondary metabolite is produced by NRPS genes is the use of hydrogen-deuterium exchange (HDX) to

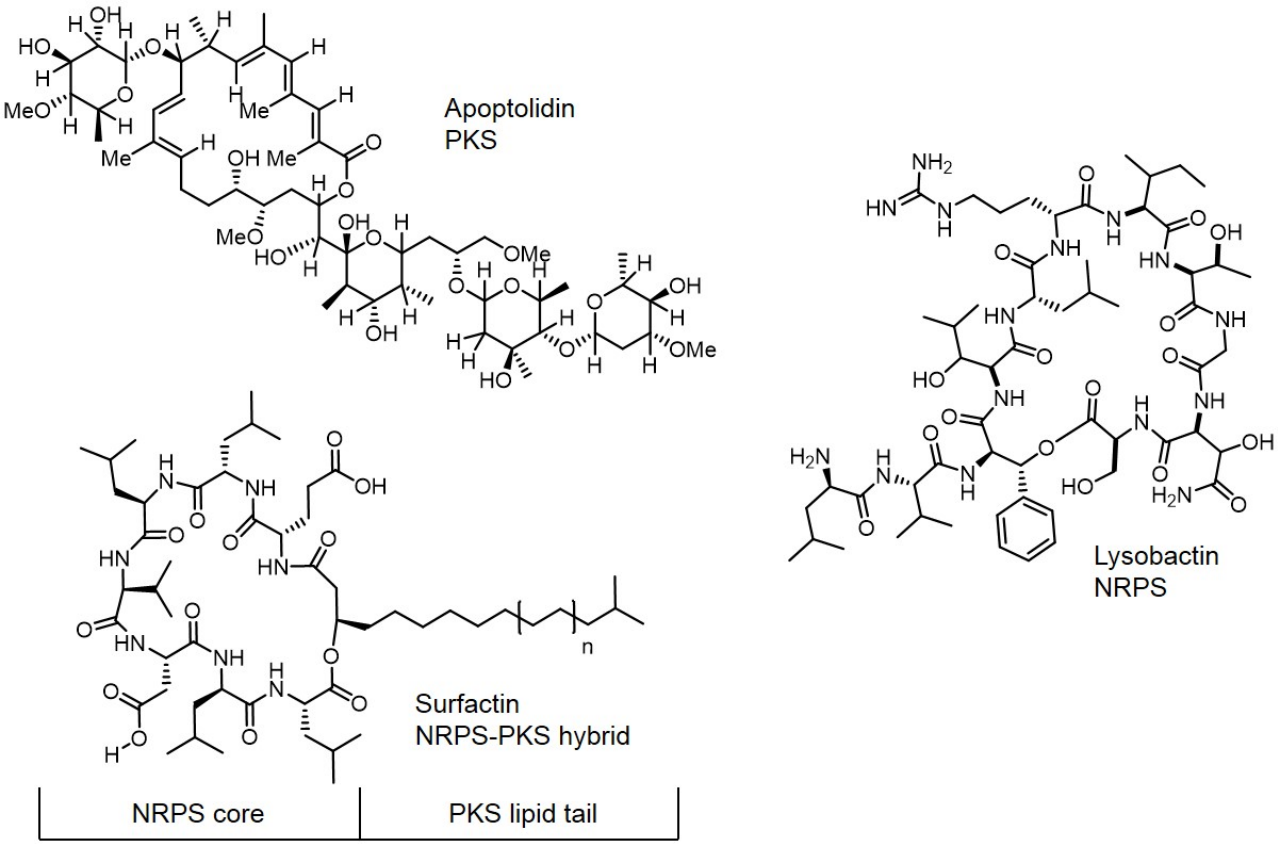

Figure 1-4. Representative molecules produced by PKS (apoptolidin), NRPS (lysobactin), and NRPS-PKS hybrid gene clusters.

determine the number of amino acids within a compound.[47] This analysis is achieved by suspension of a small amount of crude extract in a deuterated solvent to allow all labile protons to be exchanged for deuterium. Drying and suspending the sample in protonated solvent at low pH facilitates the back-exchange of deuterium for a proton in all non-amide bond labile deuterium. Comparison of the LCMS data for samples exposed to HDX and the non-deuterated sample will reveal the mass difference between the compounds of interest to facilitate the determination of the number of amide bonds within the compound. Unfortunately, the degradation of acid sensitive compounds, which are not amenable to the HDX conditions, make this method slightly limited.

An additional class of genes that are observed in streptomycete microbes is the hybrid NRPS-PKS genes, which encode modules from both NRPS and PKS classes.[39] Among the compounds produced by this class of secondary metabolic gene clusters is the most recently FDA approved natural product class of lipopeptides.[39] The amino acid core structure is produced through the use of NRPS modules while the lipid portion of the molecule is produced through the use of PKS modules. Examples of chemical strucutres for secondary metabolites produced by each type of gene cluster are shown and identified in Figure 1-4.

Turning on Silent Gene Clusters

Genes responsible for secondary metabolite production that are not expressed under laboratory conditions have been classified as either cryptic, silent, or orphan, the activation of which could induce production of secondary metabolites.[41] A variety of methods have been developed to induce expression of these genes resulting in

identification and correlation of secondary metabolites to novel biosynthetic pathways.[48] Heterologous and homologous expression methods for the production of a secondary metabolite by an identified gene of interest are effective under specific culture conditions.

Heterologous expression is particularly useful for determining the structure of compounds produced by an identified gene cluster, characterizing biosynthetic pathways and modifying them if desired to produce secondary metabolic derivatives, and potentially lead to increased yield of secondary metabolites.[49] While there is no single approach that will work for all genes of interest, a general outline of the approach for heterologous expression is outlined in Figure 1-5. Initial steps include the isolation of the DNA from the microbe containing the gene of interest, followed by the mobilization of the gene of interest into the heterologous host.[49] Further development of these techniques has led to the biosynthetic production of secondary metabolites.[20] One disadvantage of this method is the time consuming process of extracting gene clusters of interest followed by incorporation into the genome of another microbe with no guarantee of success. Furthermore, many gene clusters require regulators which would have to be cloned with the gene of interest and, both regulator and gene, would have to be incorporated and expressed in the host organism. Further complicating the process is the fact that many of the precursors used in secondary metabolism are incorporated into a variety of metabolic pathways, including primary metabolism, making precursor concentration during cultivation an experimental parameter which can be optimized following successful expression of the secondary metabolite of interest.

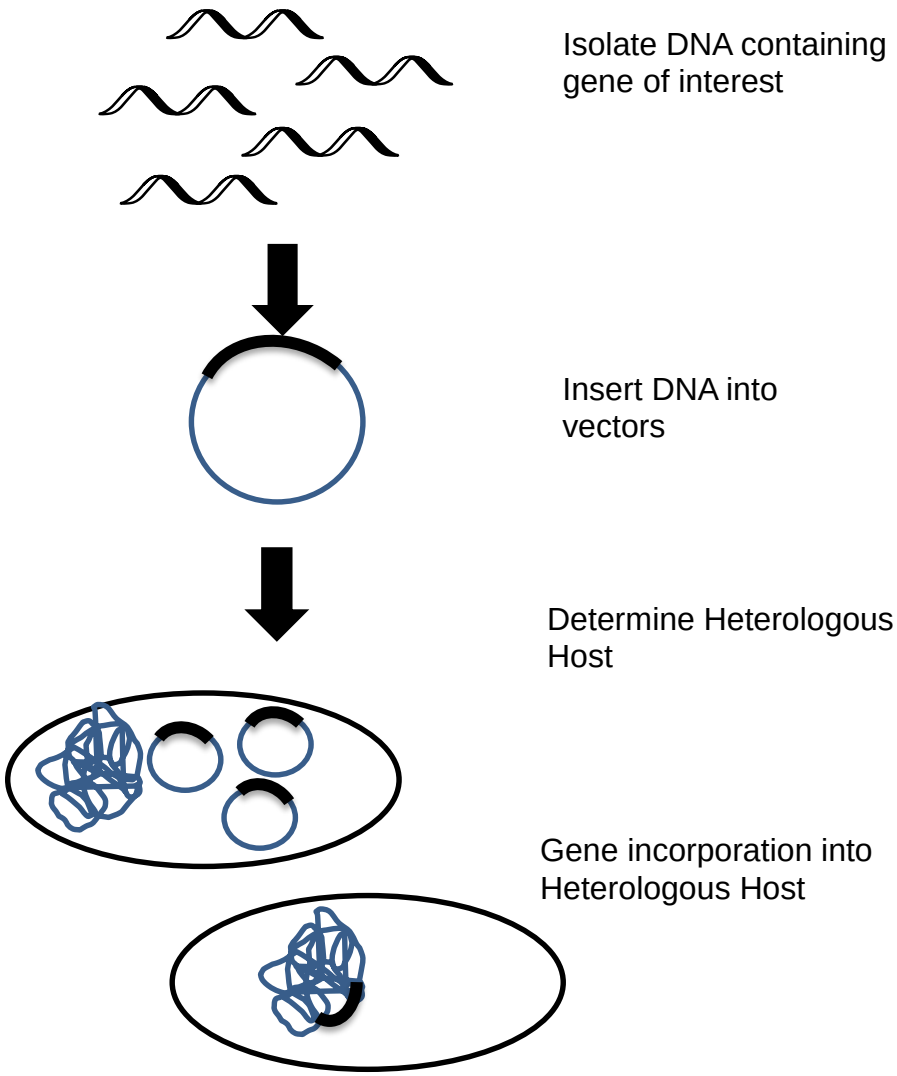

Figure 1-5. General approach to heterologous expression of identified gene clusters for improved production of secondary metabolites. First, DNA must be isolated from the producing organism, followed by isolation and incorporation of the gene of interest in a vector or plasmid. Once a heterologous host has been identified, the gene must then be incorporated into the host genome.

It has been speculated that a number of gene clusters require some trigger which exists within the genome and is responsible for inducing gene expression. As a consequence, several approaches have been developed to induce gene expression without removing the gene of interest from the host microorganism. By blocking the production of the most abundant secondary metabolite produced by the microbe, accumulation of starter units which would be utilized in the pathway could accumulate and initiate expression of genes that are silent due to unavailability of starter units at low concentrations as they are consumed by the production of the abundant metabolite.[50] Generation of antibiotic resistant actinomycetes to chemical entities known to target the ribosome has a significant impact on the translation proteins associated with secondary metabolic pathways.[51] Alteration of the ribosome, results in the expression of genes and production of different secondary metabolites resulting in an altered metabolic profile. The combination of blocking the production of the most abundant secondary metabolite and incorporating antibiotic resistance has resulted in the reporting of a previously unreported metabolite, exemplifying the need to use multiple approaches to maximize the number of secondary metabolites isolated from a single organism.[52]

Just as the introduction of antibiotic resistance influences the metabolic profile of an organism, chemical perturbations have been evaluated for ability to improve the chances of finding secondary metabolites. These methods rely on the use of chemical additives that are incorporated into the final structure of the secondary metabolite or resulting in the expression of genes associated with secondary metabolism as a result of possible toxicity, cell wall permeability, or improved survivability.[53] An extension of this technique is the development of co-culture methods in which microbes of interest are

cultured with other organisms that have been shown to influence the metabolic profile, presumably as the result of chemical signaling molecules that allow microbes to determine population density or invasion by competing organisms.[54]

New Methods to Detect Low Abundance Metabolites

Secondary metabolite isolation and identification from microbial species have focused primarily on abundant metabolites. It is possible that genes believed to be cryptic or silent, are in fact expressed at low levels relative to the cultivation volume making detection, isolation, and structure elucidation difficult to achieve. New analytical methods have been developed to aid in the detection and identification of compounds produced at low levels by incorporation of nano-scale analysis.[21, 55, 56] Ion mobility mass spectrometry (IM-MS) relies on the separation of ions separated based on the biomolecular class of the molecule, which is determined by the drift time of the ion when influenced by an electric field resulting in specific bands observed in a 2-D plot for lipids, polysaccharides, or peptides.[57] The result of the separation allows the detection of molecules that would otherwise not have been observed using conventional liquid chromatography mass spectrometry (LCMS) given the additional dimension of separation, especially for compounds with similar chromatographic profiles. This method has the ability to detect and preliminarily dereplicate chemical entities at low concentrations within a complex mixture in an effort to rationally guide isolation attempts of lead compounds is essential for guiding isolation efforts focused on discovery of unknown chemical scaffolds.

The incorporation of new statistical methods to identify lead compounds within a complex mixture has also proven effective at identifying lead compounds for isolation

through the use of principal component analysis (PCA).[58] The combination of statistical analysis with NMR analysis of partially purified extracts from plants as a means of preliminary dereplication to aid the process of isolation resulted in the identification of bioactive compounds contained within multiple extracts.[59]

A common problem that occurs during microbial secondary metabolites identification is the lack of sufficient pure compound to complete the necessary analysis for definitive structure elucidation via nuclear magnetic resonance (NMR) analysis. This problem has been addressed through the development of nano-NMR techniques which facilitate structure elucidation of minimal amounts of samples.[60, 61] Expanding the analytical techniques for the detection and identification of molecules at extremely low concentration has the potential to increase the rate of reporting secondary metabolites that have evaded natural product chemists for decades. Once a new chemical entity (NCE) has been identified at low levels of abundance, strain improvement methods may be employed to increase production of the compound of interest.

Determining Microbes to Evaluate for Secondary Metabolites

Biological novelty of microorganisms usually leads to chemical novelty of secondary metabolites, therefore, by exploring uncultured microorganisms from underexploited environments, it is possible to discover new chemical entities.[62] Marine environments were historically discarded as an environment capable of supporting diverse microbial communities due to the salinity and lack of nutrients available.[63] Current bacterial counts of marine samples are similar to terrestrial samples, with one million cells per mL, and one billion per mL in ocean-bottom sediments.[63] As with

terrestrial microbes, the biological diversity and secondary metabolites produced cover a broad range. Between 1997 and 2008, 659 natural products were isolated from marine bacteria, with 256 produced by actinomycetes, 57 % of which were produced by streptomyces.[63] In an early pharmacological study of the *Salinispora sp.* cultivable organisms, over 100 microbes were tested against human tumor cell growth and antibacterial activity against drug-resistant human pathogens, 80 % and 35 % showed activity respectively.[63] A unique feature of compounds isolated from marine samples is the incorporation of chlorine in the final structure. Salinosporamide A, a highly bioactive

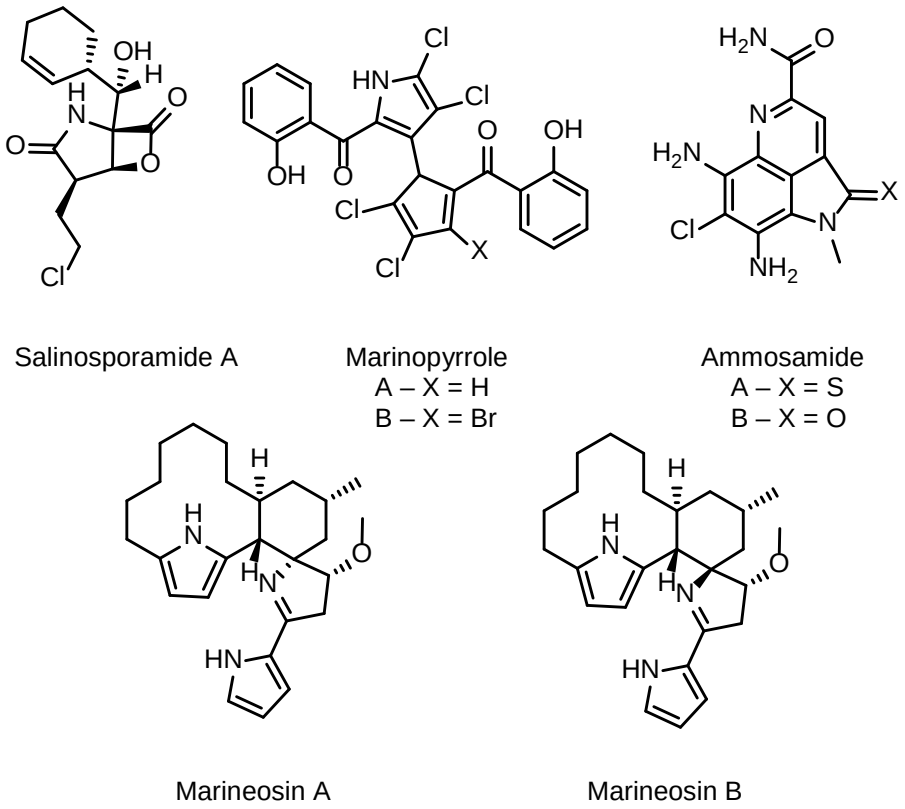

Figure 1-6. Compounds isolated from actinomycetes isolated from unique ecological environments.

compound that entered clinical trials three years after its discovery, is the most notable natural product isolated from a marine actinomycetes, *Salinispora*.[26] The marinopyrols, two compounds that are highly halogenated bispyrroles containing a unique N,C-biaryl axis, are highly active against *Staphylococcus aureus*, were produced by a marine streptomycete isolated from sediment collected of the coast of California.[26] The chlorinated tricyclic pyrroloquinoneline alkaloids, ammosamides, were produced by a Streptomycete strain isolated from deep-sea sediment.[26] Not all of the compounds isolated from marine microorganisms have contain halogen atoms in the final structure, despite the high concentration of chlorine and bromine. A unique spiro-tetrahydropyran-dihydropyrrole aminal moiety was discovered in the marineosin compounds isolated from a marine streptomycete.[26] The chemical structures of these unique compounds produced by microbes isolated from a marine environment are shown in Figure 1-6.

Unlike all other environments that have been explored for new natural product producers, hypogean, or cave, environments maintain static conditions year round. Most notable about these environments is the limited nutrients available for the microorganisms, leading to a greater competition for survival.[64] Since photosynthesis is only carried out at the entrance of caves, microorganisms must adapt to chemoautotrophy and ammonium-, nitrate-, sulphur-, manganese- or iron-oxidizing chemolithoautotrophy[65], with most of the organisms living an oligotrophic lifestyle.[66] Due to the nutrient poor environment within cave systems, production of antibiotics by one organism may assist in the competition for nutrients.[67] Actinomycetes are known to survive on minimal nutrients and play an active ecological role by selectively degrading lignocelluloses and humic materials, the most abundant dissolved organic material within caves, making

these environments capable of supporting a variety of actinomycetes.[64] Another food source for hypogean actinomycetes may the decomposition of other organisms, a possible adaptation to nutrient deprived environments.[66]

Phylogenetic analysis of the microbiome within hypogean environments has revealed that these environments are home to a variety of microorganisms. Bacteria within hypogean environments are classified into two different categories depending the duration of their lifecycle within the caves: those that are accidentally introduced to the cave environment and those that spend their entire life cycle within this environment.[67] Isolation of actinomycetes if readily achieved from these environments as they can been seen as colonies on the cave walls. Limited studies have focused on evaluating the microbes isolated from these environments for their ability to produce novel secondary metabolites.[64]

Dissertation Statement

Extensive research efforts in secondary metabolite isolation have focused on actinomycetes and revealed that the chemical diversity these microbes are capable of producing has not been fully realized. Many compounds remain elusive either as a result of insufficient analytical methods for detection of low abundant compounds, cultivation methods in which compounds of interest are not produced, or isolation methods which result in the degradation of compounds of interest. The most likely approach to discover new chemical scaffolds from actinomycetes is the development of new analytical approaches to detect compounds within a complex mixture or the use of unconventional

culture conditions to induce production of secondary metabolites when applied to actinomycetes isolated from a novel environmental niche.

Current culture conditions rely of the use of chemical additives for single species cultivation and have been shown to have a significant impact on the metabolic profiles resulting in the production of NCE. The process of determining metabolic differences between samples produced by a single species exposed to multiple culture conditions in which a single medium component is altered is a daunting task. Chapter 2 will discuss the application of chemical additives and how to determine their impact on metabolic alterations of secondary metabolites produced by actinomycetes. Determination of metabolic impacts was achieved through the use of statistical analysis to rapidly identify the compounds produced by various chemical challenges in an effort to facilitate rapid determination of lead compounds within a complex mixture.

Chemical perturbations as a result of exposure to specific molecules could be the result of an evolutionary process in which microbes develop specific defensive responses to the compounds produced by a neighboring organism. Bacterial communication has been shown to alter the metabolic profile of neighboring microorganisms, and several new chemical entities have been isolatied as a result of these efforts. Many of these methods have focused on the application of co-culture in liquid medium. Chapter 3 will focus on the development of a co-culture method to determine the metabolic impact of microbial communication when the microbes are geometrically fixed to a solid medium.

Antibiotics can act as chemical perturbations at low concentrations, though toxic effects are apparent as concentration increases. Chapter 4 will focus on the development of a new analytical technique to identify the metabolic plumes produced by an assay

organism as a result of exposure to biologically active secondary metabolites. The application of imaging mass spectrometry (IMS) in conjunction with 2-D TLC and bioautography has the ability to identify lead compounds and biological responses to these compounds simultaneously within a complex mixture.

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CHAPTER 2

INDUCTION OF MICROBIAL SECONDARY METABOLISM VIA CHEMICAL PERTURBATIONS

Investigating the Impact of Chemical Perturbations on Streptomycetes

In the natural environment, bacteria are exposed to a variety of biological diversity, which exposes them to a vast amount of chemical diversity as a result of chemical plumes produced by neighboring microorganisms. General investigation of the metabolic output for isolated microbes has traditionally been performed through variation of the medium recipes used for cultivation. A more specific approach is the exposure to various chemicals at concentrations which do not completely inhibit the growth of all microorganisms during cultivation and stresses the survivors. Applying statistical analysis to evaluate alterations in the metabolic output as a result of exposure to various chemical perturbations can potentially identify correlations to the chosen perturbations, for example, organic compounds, rare earth element salts, and anti-infective agents.

What are Chemical Perturbations?

As with all living organisms, basic requirements for primary metabolism and survival depend on the availability and utilization of essential nutrients. Chemical perturbations can be thought of as manipulation of the nutrients and essential elements available during cultivation by controlling the medium composition. Based on evaluation of environmental DNA (eDNA), the biological diversity present in the natural environment

is poorly represented by that capable of being cultured in the laboratory setting.[1] This could be the result of an imbalance of essential nutrients, including trace minerals, required for primary metabolism and homeostasis. Essential nutritional components, and those most frequently adjusted for optimization of culture medium, are nitrogen, carbon, and phosphorous, while trace elements which can be used as enzyme co-factors such as iron and magnesium can also be adjusted.[2]

Carbon catabolite repression (CCR) is the process by which bacteria selectively catabolize carbohydrates to ensure that carbon is economically utilized so that a limited number of genes are expressed in an effort to conserve energy.[3] In the model streptomycete, *S. coelicolor,* 53 systems have been identified which are available for microorganisms to utilize for primary metabolism.[4] Manipulation of the availability of the nitrogen source during cultivation of *S. clavuligerus* revealed that many were capable of supporting primary metabolism and influencing secondary metabolism resulting is a significant decrease of cephalosporin production.[5] While inorganic phosphate is essential for maintaining viable microbes, an excess can also result in decreased production of cephalosporin. For maximum cephalosporin production by *S. clavuligerus,* the optimum concentration was determined to be 25 mM phosphate and doubling the concentration resulted in an 85% decrease in production.[6] The most commonly manipulated medium additives are amino acids, for the nitrogen source, and sugars, for the carbon source.[7] The term “OSMAC” (One Strain Many Compounds) has been used to describe the deliberate manipulation of fermentation ingredients to maximize the number of compounds produced by a single organism.[8] Microbes that are cultured under laboratory settings are limited in their ability to alter the chemical and nutritional composition of their

environment, though some organisms have developed methods of adjusting the pH, neutralizing potentially toxic compounds, and biosynthesis of compounds for chelation of metals from the surrounding environment.[9]

Low levels of bioavailable Fe (II) have resulted in many organisms development of siderophore production to chelate the insoluble Fe (III) widely available in the environment for uptake into the cells.[10] Genomic analysis and gene annotation identified the potential production of molecules capable of chelating metals in *S. coelicolor*. The rationally guided search for this molecule required cultivating *S. coelicolor* in iron depleted media to induce expression of the gene cluster and specific compound isolation methods successfully led to the identification of coelichilin[11]. Siderophores have been shown to induce growth of previously uncultivable microorganisms when added to the chemically defined medium employed for cultivation.[12] Through the practice of 'siderophore piracy', many organisms have the ability to utilize these compounds produced by other microorganisms to facility the uptake of Fe(II) with most of these organisms containing more siderophore uptake genes than synthesis genes based on genomic sequencing.[12] Studies have shown that the addition of ferrous sulfate, $FeSO_4$, to culture medium have been able to promote the growth of previously uncultured microorganisms.[12] Through varying these, and other, media components, hundreds of media recipes have been developed around the world, many of which have been optimized for production of specific compounds or compound classes.

As a result of how the environment and nutritional availability impact the secondary metabolic profiles of microbes, it has been proposed that the central dogma of biology be redefined to include how chemical compounds influence cellular biology at all stages of gene expression (Figure 2-1).[13] The production of metabolites are the result of not only gene expression, but also the interaction between the environment and the organisms that inhabit that environment in an integrated fashion.[14] Secondary metabolites have been shown to have an impact on the transcriptome through the use of siderophores and antibiotics that target the ribosome, such as streptomycin, which result in an altered secondary metabolic output.[7] It has also been shown that the use of enzyme inhibitors have the ability to influence biosynthetic pathways altering the metabolic profile and resulting in the identification of new compounds.[7]

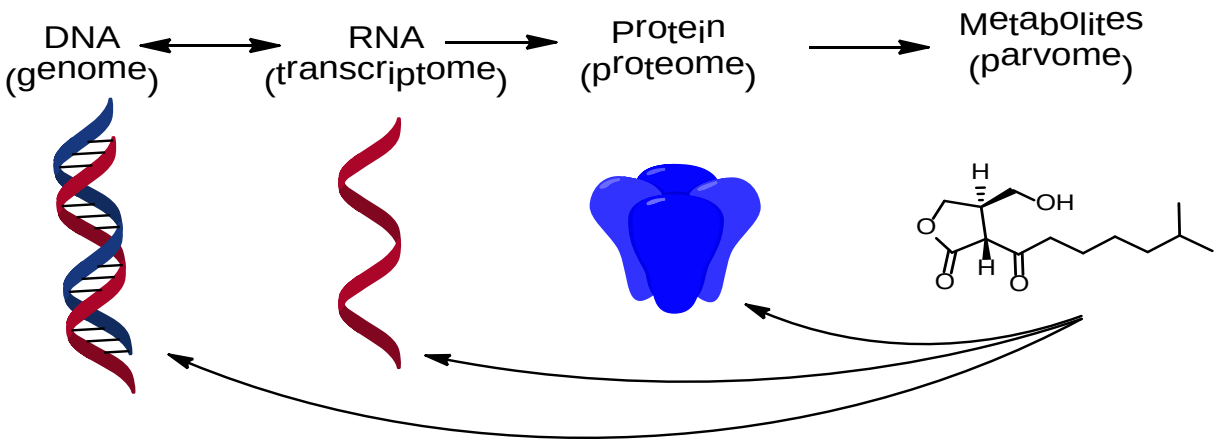


Figure 2-1. Central dogma of biology where metabolism is driven by protein expression which is determined by translation of RNA as a consequence of transcription of DNA with hypothetical points in the process which can be influenced by exposure to small molecules. Figure adapted from Davies, et. al.[13]

Media Composition as Chemical Perturbations

The process of natural product isolation begins with the generation of a complex extract that contains a multitude of chemical entities. This process can be done through a variety of methods depending on the nature of the compounds of interest. Extraction of the culture broth results in an extract containing primarily compounds excreted by the organism being investigated, in addition to media components that were not consumed during the process of cultivation. Whole cell extracts can be generated to target chemical entities that are retained within the cells by extracting just the cells. A combination of these extraction methods can be done to capture a complete chemical picture of the metabolic profile at the time of the extraction. Isolation of a single chemical entity within such a complex sample is an arduous and time consuming endeavor. Consequently, the process of isolating compounds is guided by some metric that can be applied throughout the isolation process to identify the compound's presence during the fractionation and purification process. Traditional metrics used for isolation of microbial secondary metabolites have focused on bioactivity against a specific target or the abundance of the compound relative to all others within the extract. Lead compound identification has been facilitated through a variety of methods, though the most common has been through the identification of bioactive compounds utilizing an assay organism.[15]

Initial studies on the metabolic influence caused by adjusting the media composition focused on the evaluation of crude extracts from hypogean actinomycetes. The extracts were generated through incorporation of solid phase extraction by using functionalized beads containing hydrophobic pores in which compounds excreted into the culture broth can be absorbed. Evaluation of the metabolic profiles for each extract was

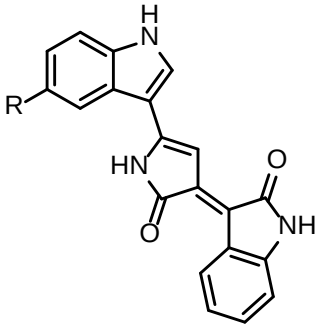
performed by combining mass spectrometry, UV absorption, and bioactivity against a panel of microbes. In initial studies, a variety of media components and media derived compounds were isolate due their abundance. As these compounds interfere with lead prioritization, a master list of these components has been generated to distinguish secondary metabolites produced by the organism being challenged from those that remain in the culture broth at the end of fermentation (Appendix A).

Compounds isolated from Differing Medium Composition

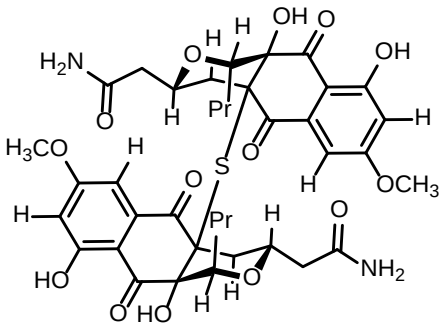
Proteobacteria, such as Lysobacter enzymogenes C3, are an attractive target for secondary metabolite production and isolation due to the rapid growth of the organisms.[16] As a consequence, Lysobacter enzymogenes C3 was selected to be cultured under a variety of medium composition conditions to evaluate the impact on the metabolic impact. Analysis of the prefractionated samples generated by employing gradient vacuum flash chromatography revealed the presence of an abundant compound that could not be readily dereplicated with the compiled analytical data, including proton NMR and UV spectra. COSY and HSQC fragments were assigned, and connected using HMBC data to incorporate the phenyl alanine fragment (Appendix B, Figures B1-B5). Through the use of HRMS and MS-MS analysis to confirm the presence of the phenyl alanine fragment facilitated complete structure elucidation. Searching the DNP database using the structure generated by NMR and HR-MS analysis led to the identification of the isolated compound as a diketopiperazine, cyclo(phenylalanine-4-hydroxy-proline).[17]

Compounds containing an extended chromophore are produced by secondary metabolic machinery and have been an attractive target for isolation due to the easy

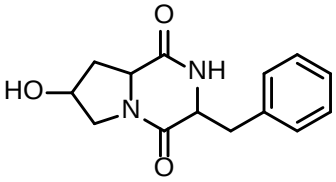
nature of tracking the compounds throughout the multiple rounds of purification required to achieve the level of purity necessary for structure elucidation and compound identification via NMR. Cultivation of an unknown actinomycete isolated from a hypogean environment resulted in the observation of a purple hue to the production media. Evaluation of this extract and purification of the compounds responsible for the pigmentation resulted in the conclusion that both of the pigmented compounds had been previously reported. Both fractions showed relatively pure compounds with λ_{max} around 580 nm and complementary ions in positive and negative ionization modes and were evaluated by ^{1}H NMR (Appendix B, Figures B6-B7). Compiling the acquired data for dereplication, including HRMS and MS-MS fragmentation, using the Dictionary of Natural Products Database (DNPD) facilitated the identification of the isolated compounds as violacein and deoxyviolacien.



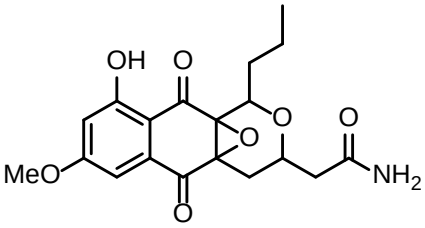
R=H deoxyviolacein
R=OH violacein
BBPARAUNK02BA01I1



BBHARD23OA01E1lh2c30



CRMLC3LY02I150MC14P17



BBHARD23OA04e1lh6c28

Figure 2-2. Compounds isolated through the application of differing medium compositions, the structures of which were determine though NMR and MS analysis.

Hypogeamycin A (BBHARD23OA01E1lh2c30) is an example of an unknown secondary metabolite isolated from an actinomycete isolated from a soil sample taken from Harding Cave and fermented under various media conditions. Crude extracts of the fermentation were generated and subjected to prefractionation utilizing size exclusion chromatography. The resulting fractions were evaluated for abundant isolatable compounds using RP-HPLC equipped with PDA-UV detection. Evaluation of the data and subsequent purification steps resulted in the isolation of hypogeamycin A and the epoxide precursor, hypogeamycin B. The structure of the hypogeamycins were

determined using various 2-D NMR techniques (Appendix B, Figures B8-B17), as well as, HR- MS, which revealed the dimeric nature of the compound. The relative stereochemistry between the propyl group and the ring was determined using 2-D NMR by through space magnetic interactions, or dipolar couplings, by utilizing the nuclear Overhouser effect (NOE). Unfortunately, absolute stereochemistry could not be determined without complementary synthetic studies where the absolute stereochemistry is known or extensive computational studies in conjunction with experimental data or through the use of complex chiroptical spectroscopy in combination of quantum chemical calculations.[18] Through the use of NMR analysis for structure elucidation of the ring system of hypogeomycin, MS data to reveal the dimeric nature of the compound, and the use of quantum chemical calculations of specific rotation and vibrational and electronic circular dichroism spectra the structural assignment and absolute stereochemistry were determined.

Principal Component Analysis of Crude Extracts

Identification of metabolic alterations within a crude extract can be a difficult process using the traditional “stare and compare” method in which individual datasets are manually compared for differences. This process is limited in its ability to identify compounds of interest, particularly those which co-elute. The application of an unbiased statistical analysis to determine lead compounds has the ability to rapidly identify metabolic differences between samples. The primary goal of multivariate statistical data analysis (MVDA) is to simplify a complex data set based on correlation within the samples while retaining as much variation as possible within the data set. The most commonly

employed MVDA tool is principal component analysis (PCA) which aims to establish relationships between biological samples and the metabolites they produce.[19]

The primary goals of PCA are to simplify and reduce the data to a manageable and easily interpretable dataset and detect outliers and variables which represent metabolic differences.[20] PCA is a statistical procedure that uses orthogonal transformation to identify the principal components within a data set. The first principal component accounts for the greatest variability in the data set, while each following component has the highest variance possible so long as it is orthogonal to all previously defined components.[21] The primary benefit of applying PCA to the comparison of metabolomes produced by altering a single component within the culture medium for a single organism is the ability to rapidly determine if there is any significant difference between the metabolomes. This is evaluated by visualization of the scores plot, in which samples with similar metabolomes will cluster within the same region while those with significant differences will fall within other quadrants. For samples that have significant differences, the metabolites responsible for the differences can be determined by evaluation of the loadings plot.[19] Analysis of *S. coelicolor* gene expression as a result of exposure to stress conditions including heat, cold, salt, and ethanol by PCA revealed potential interactions between developmental genes and stressors.[22]

Altering Culture Conditions to Induce Secondary Metabolite Production

While nutritional factors have been shown to have a significant impact on the metabolic profile of microorganisms, other methods of altering the culture medium have been proven effective for eliciting secondary metabolite production.[23] The addition of

organic compounds have been shown to influence antibiotic production by actinomycetes, increasing the production of some secondary metabolites, while decreasing the production of others.[24, 25] The addition of antibiotic compounds at subinhibitory levels has also seen shown to elicit secondary metabolism.[24] A range of these methods were applied to the model actinomycete, *S. coelicolor*, to determine alterations in metabolic output by altering only one component with the culture medium. Based on the data obtained followed by statistical analysis revealed this culture process as an effective method for altering gene expression, resulting in variable production of the many secondary metabolites known to be produced.[26]

Heat-shocking microorganisms during cultivation has resulted in the production of secondary metabolites and increased production of others.[7, 27] Heat-shock alters the metabolic profile by damaging the cell envelope and causing the accumulation of misfolded or unfolded proteins.[28] Metabolic responses of *S. coelicolor* to heat-shock revealed that 5-hydroxyectoine and its precursor, ectoine, were both observed for cultures which were maintained at 39 ˚C rather than 28 ˚C where ectoine, the precursor for 5-hydroxyectoine, were not observed.[29] Jadomycin B is produced in negligible quantities by *S. venezuelae* when cultured at 27 ˚C, though production can be increased to 25 µg/mL when cultured at 42 ˚C.[27] Another streptomycete microbe, *S. hygroscopicus*, is significantly influenced by increased temperatures.[30] The anti-fungal validamycin A, which is used as a control agent against sheath blight disease of rice and wheat plants, is produced at a concentration of 13 g/L when cultured at 42 ˚C while only 2 g/L were produced at 30 ˚C.[31]

While there are a multitude of parameters that can be adjusted to determine the metabolic impact of a single alteration to the culture medium during cultivation, a small selection is all that can feasibly be tested at the same time. With all of the options that have been tested on streptomycetes, the addition of low concentrations of DMSO and ethanol were tested to elicit production of secondary metabolites and identify lead compounds which were produced as a result of the chosen stressors *Astrosporangium hypotensionis*. Furthermore, the effect of temperature was tested by elevating the culture temperature to 38 ˚C and depressing it to 20 ˚C.

The Impact of Chemical Additives During Culture

A variety of chemical perturbations have been employed for activation of silent or cryptic gene clusters within streptomycetes to induce secondary metabolite production. The use of scandium, a rare earth element found sparsely dispersed throughout the earth's crust, was evaluated for its ability to induce production of secondary metabolites in a variety of model streptomycetes. At concentrations of 50-200 µM, scandium was capable of inducing actinorhodin production in *S. coelicolor.* Scandium chloride salt was also shown to induce actinorhodin production in *S. lividans* which contains the gene responsible for actinorhodin production, though the pigment is rarely observed. A fourfold increase in production of streptomycin by *S. griseus* was observed following cultivation in medium containing 20-100 µM scandium.[32] Lanthanum chloride salt was shown to increase antibiotic production if *S. coelicolor* at concentrations of 200-1900 µM.[33] The addition of DMSO to the culture medium at low concentrations increases the production of chloramphenicol, tetracenomycin, and thiostrepton two to three times when compared

to control cultures of *S. venezuelae*, *S. glaucescens*, and *S. azureus*, respectively. Ethanol also had similar impacts on the production of tetracenomycin C by *S. venezuelae* when added at low level concentrations.[34]

A. hypotensionis is an actinomycete that has been sequenced and shows a large number of secondary metabolic pathways for which no compound has been isolated. Furthermore, the single natural product isolated from this organism is produced by an unidentified gene cluster.[35] Genomic analysis of this microbe has revealed that, like all other actinomycetes, a variety of genes exist that are capable of producing secondary metabolites the structure of which can be putatively described prior to isolation and identification. Significant effort has been applied to this organism to expand on the understanding on the biological processes that influence the ability to induce proteins to synthesize metabolites associated with gene clusters.

The above mentioned chemical perturbations were applied to *A. hypotensionis* in an effort to induce secondary metabolite production. Biological reproducibility, which can vary between cultures, was also evaluated by cultivation of replicate control samples. Due to the complex nature of the extracts and to enhance detection of metabolic alterations even at low abundance relative to the extract, PCA was used to determine the miximal differences between the samples. Each extract was analyzed in duplicate and initial investigation of the data revealed that in PCA space, the replicates clustered well (Figure 2-4). Based on the clustering of the biological duplicates indicates that the metabolic variations observed are the result of the selected culture condition. Of all of the conditions that were chosen, the addition of ethanol resulted in the lowest metabolic impact as the data points which represent the entire metabolome occupy similar PCA

space as the control samples. The addition of lanthinum resulted in significanty altered metabolic output as the data points associated with the metabolomes lie along an opposing vector than that of the control samples, though not in a linear fashion relative to concentration. Further evaluation of the data revealed that while differences existed within the samples, there was a lack of good candidates for lead compounds to be isolated. As a result, alternative chemical additive methods were chosen to influence the metabolic output of this microorganism.

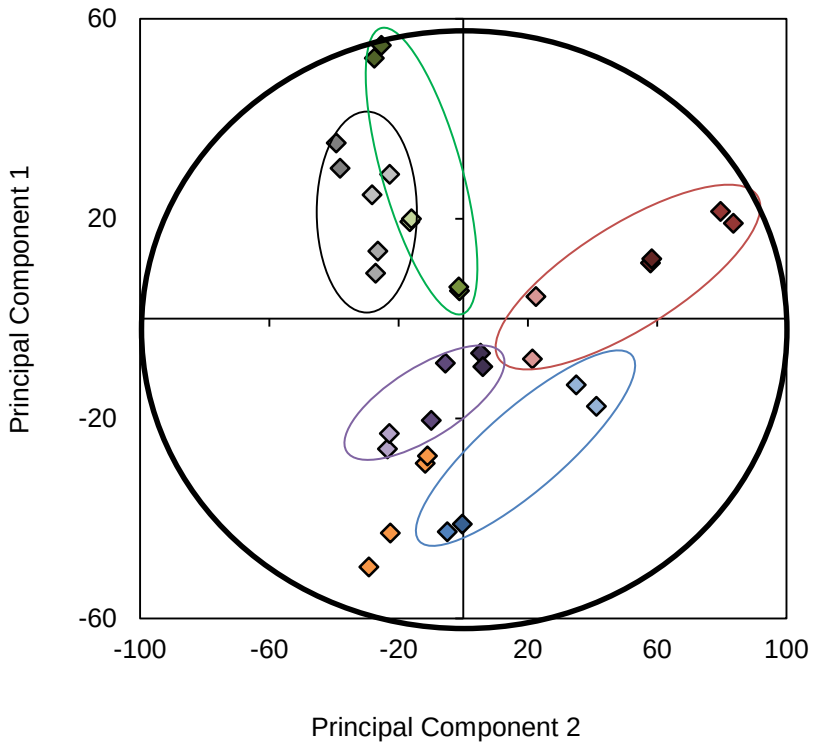


Figure 2-3. PCA analysis of K-26 following exposure to chemical additives or temperature changes to induce secondary metabolite production in a model actinomycete. Biological duplicates of control conditions (◆), 1% DMSO (◆), 3% DMSO (◆), 5% DMSO (◆), 1% Ethanol (◆), 3% Ethanol (◆), 5% Ethanol (◆), 50µM lanthanum (◆), 200µM lanthanum (◆), 500µM lanthanum (◆), 50µM scandium (◆), 200µM scandium (◆), 500µM scandium (◆), 23˚C (◆), and 37˚C (◆).

Impact of Chemical Additives Prior to Culture

An alternative to the use of chemical additives to elicit secondary metabolite production during culture is to use chemical additives to induce genetic mutations prior to cultivation of an organism. It has been shown that bioactive compounds that interact with the ribosome have the ability to alter the genome of the host microbe resulting in altered gene expression presumably as the result of unrestrictive transcription of genes resulting in the production of secondary metabolites.[36] To determine if this phenomena could occur with *A. hypotensionis*, wild type species were cultured on solid media containing 200 µg/mL of streptomycin. Following an extended culture period to allow the maximum number of mutants to develop, only six colonies were observed. These colonies were individually selected, homogenized, and cultured on solid media containing the same concentration of antibiotic used to induce the mutation to ensure that the mutation was

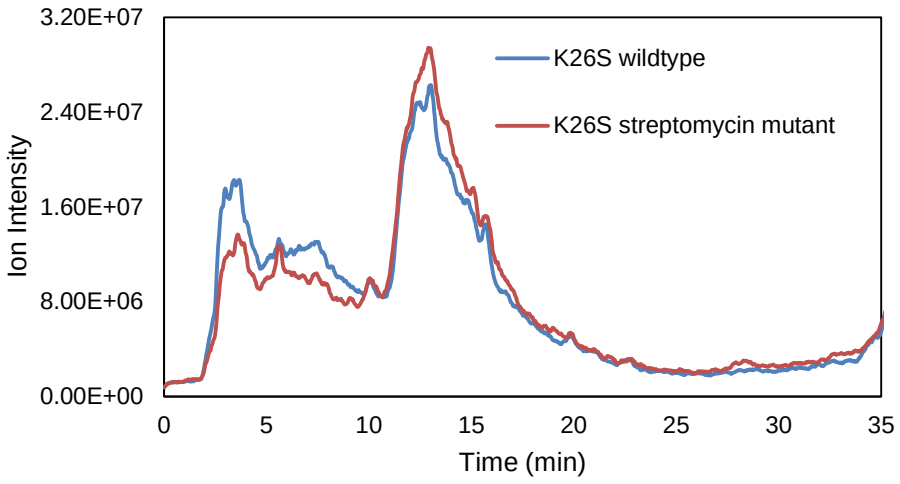


Figure 2-4. Overlay of UV absorbance following HPLC separation of the crude extract for K26 control and K26 streptomycin mutant to show significant overlap of the metabolomic profile of the generated extracts.

stable within the microbes and that the microbes had adapted to surviving the toxic environment. Interestingly, the colonies that expressed streptomycin resistance developed a hyper growth morphology on solid support and a more tubular rather than spherical phenotype when grown in liquid cultures.

The classic method of “stare and compare” of the metabolome by evaluating the chromatogram produced by UV absorbance was not a viable method for identifying lead compounds when comparing the metabolic profile of a single mutant against that of the wild type. (Figure 2-5). While there are visible differences, identification of ions associated with the observed alterations could not be definitively achieved. To tackle this analytical limitation, the application of PCA to the data set was employed. The resultant scores plot revealed that the streptmycin resistant mutants identifed as S2 and S3

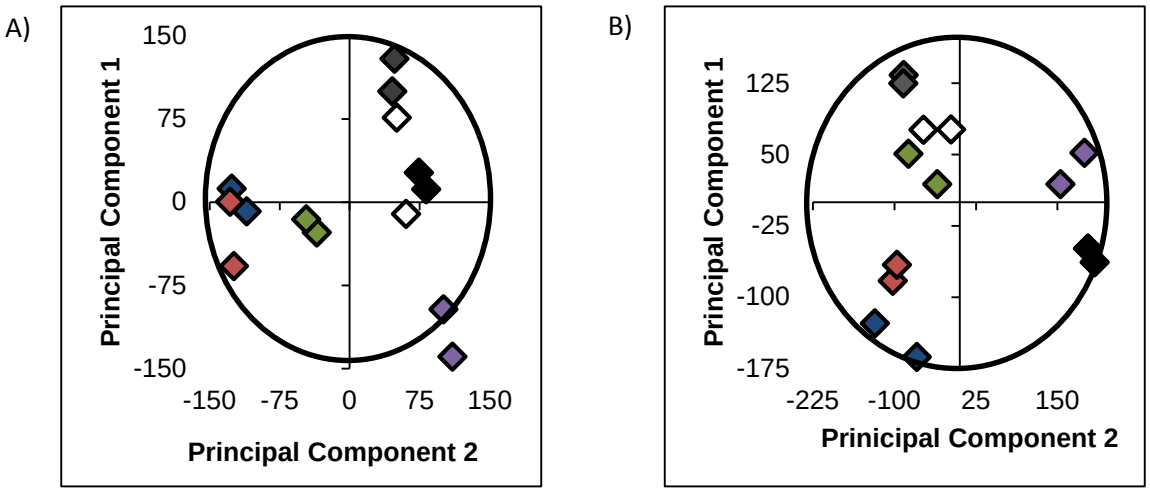


Figure 2-5. PCA analysis ions from *A. hypotensionis* following exposure to streptomycin prior to cultivation for metabolome evaluation. A) Loadings plot for the positive ions of technical duplicates. B) Loadings plot for the negative ions of technical duplicates. Streptomycin resistant mutants were identified as S1 (◇), S2 (◆), S3 (◆), S4 (◆), S5 (◆), S6 (◆) and evaluated against wild type sample (◆) for metabolic impact.

clustered in close proximity to each other and showed the greatest differences from all other cultures including the wild type organism for both positive and negative ions (Figure 2-6). As a result, these culture conditions were compared to the wild type to identify the metabolic features causing this differentiation through the use of OPLS-DA.

The use of OPLS-DA allows comparison of user defined samples to determine the ions responsible for the variations within metabolomes. Ion abundance is represented on the x-axis, while ion uniqueness is represented along the y-axis. For a variety of conditions, many ions were present in the control sample at low abundance relative to the extract, while a few conditions did result in the appearance of ions not present in the control condition. The resultant S-plot for comparison of the positive ions from the

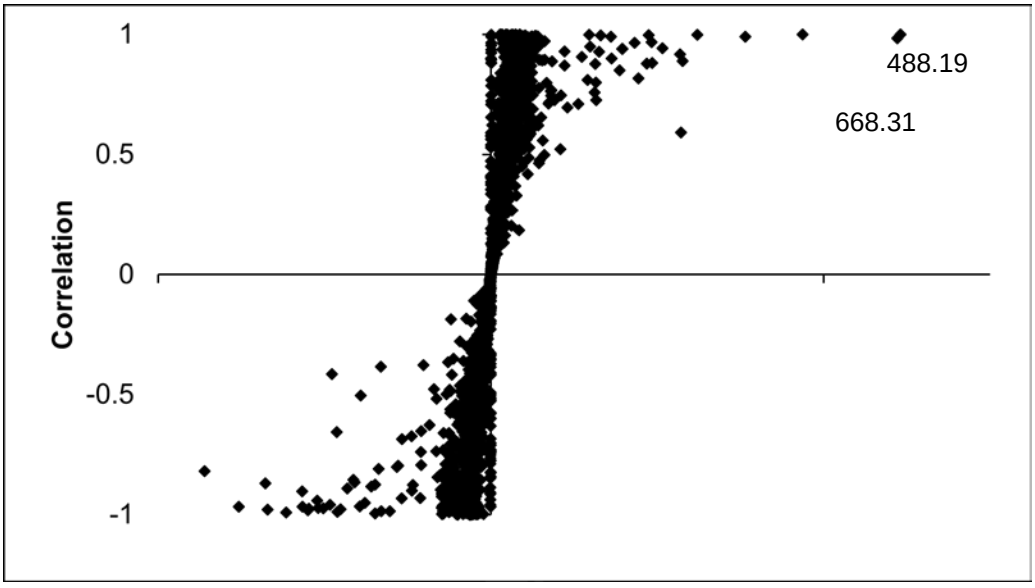


Figure 2-6. S-plot analysis of positive ions from S3 (quadrant I) vs wild type quadrant (III) to identify unique ions corresponding to secondary metabolites as a result of chemical exposure prior to cultivation. Ions with m/z 488.19 and 668.31 were identified as the compounds within the experimental culture extract as having the greatest influence for metabolic differences.

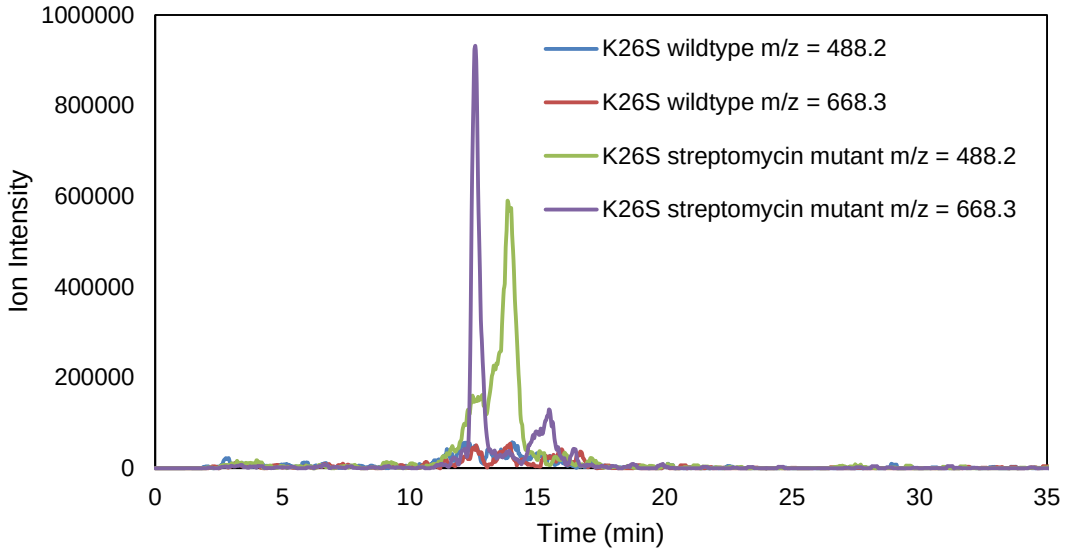


Figure 2-7. Overlay of extracted ions intensities for ions with *m/z* = 668.3 and 488.2 within a crude extract separated by HPLC to determine abundance differences between the wild type and streptomycin mutant S3 metabolomes.

metabolome of the streptomycin mutant S3 (quadrant I) mutant and wild type (quadrant III) can be evaluated to determine the presence of lead compounds (Figure 2-7). Identification of ions that are unique and abundant reveals *m/z* = 488.19 and 668.31 as being present in the culture of the mutant though not within the control sample. This data can be recapitulated by filtering the mass spectrum for the ions of interest. For ions that result in multiple peaks, as *m/z* = 668.31 does for the mutant extract, the peak of interest can be confirmed by comparison of the retention time. Applying this process to the ions identified in the S-plot confirmed the presence of ions in increased abundance within the mutant sample that would not have been detected without the application of PCA and OPLS-DA (figure 2-8). As the ions of interest are not present in the wild type sample, the presence of these compounds within the mutant extract are attributed to the genetic mutation induced by exposure to streptomycin prior to culturing the microbe.

Discussion

The search for secondary metabolites from microorganisms has beeen on ongoing process since the discovery of penicillin and the realization of its clinical significance. As a result of genomic identification of genes responsible for secondary metabolites, multiple approaches have been developed to maximize the number of compounds that a single microbe is capable of producing. Though the process of altering the medium composition to contain a variety of nutrients is random, it is an effective way of inducing secondary metabolism as shown for the isolation of diketopiperazine, violacien, and hypogeomycin.

Detection of the metabolic differences as a result of chemical additives would not have been possible without the use of PCA and OPLS-DA. This statistical anaylsis has the ability to identify significant differences between crude extracts, allowing a rapid method to determine lead compounds for isolation. The combination of genetic information with statistical analysis has the ability to greatly increase the likelihood of finding new chemcical entitites, and increase the potential of discovering new chemical scaffolds.

Significant efforts have been focused on selecting methods to induce secondary metabolism using the model streptomycete, *S. coelicolor*. While many of these methods are successful for this microorganism, they do not always work in the same manner when applied to other microorganisms though the genes that encode secondary metabolites are organized in the same fashion and produce the same chemical transformations. While the use of chemical addatives during cultivation of *S. coelicolor* were effective at increasing the production of secondary metabolites, the same application to *A.*

hypotensionis was ineffective at significantly increasing the production of secondary metabolites. The most promising approach to influencing the metabolic output of this microbe was the use of genetic mutations as a result of exposure to antibiotics prior to cultivation. Further analysis could connect the detected metabolic alterations with the corresponding gene cluster. Identifying the manner in which gene clusters from differing organisms are initiated could greatly increase the likelihood of rationally guided medium composition determination to improve the potential of secondary metabolite production.

Materials and Methods

Fermentation and Compound isolation

Cyclo(phenylalanine-4-hydroxy-proline) Isolation

A diketopiperazine compound identified as cyclo(phenylalanine-4-hydroxy-proline) was isolated from Lysobacter enzymogenes C3 by inoculating a loop full of cells into a 50 mL medium (5 g/L yeast extract, 3 g/L peptone, mannitol 5 g/L, at pH 7.2) in a 250 mL Erlenmeyer flask and cultivated for 3 days at 30 °C on rotary shaker at 180 rpm. A 10 mL aliquot was transferred to a 2.0 L fernbach flask and cultivated in the same medium for 24 hours at 30 °C on rotary shaker at 180 rpm. Extracts were generated by the addition of 200 mL of activated HP20 resin suspended in water on the seventh day of culturing. Following a 3 hour period of shaking under the culture conditions, the broth was separated from the resin and micellia and discarded. The resin and micellia were extracted with 100 mL of mass spectrometry grade methanol for 1 hour. The methanol extract was seprated from the resin and micellia and evaporated to dryness. The resin and micellia were subsequently extraced with acetone for 1 hour, which was then

separated and dried in vacuo. The methanol faction was separated by vacuum flash normal phase chromatography using a solvent stepwise gradient from 100% hexanes to 100% ethyl acetate to 100% methanol to produce 12 fractions. The 50:50 ethyl acetate: methanol fraction was subjected to reverse phase HPLC (RP-HPLC) using a C-18 column with a gradient from 95:5 water: acetonitrile to 5:95 water: acetonitrile over 30 minutes at a flow rate of 10mL/min. The compound of interest was collected along with unknown impurities which were removed by RP-HPLC using a phenyl-hexyl column with a gradient of 95:5 water: acetonitrile to 75:25 water: acetonitrile over 30 minutes at a flow rate of 5mL/min. Structure elucidation was completed using 2D NMR, MS–MS, and HR-MS. A search of the Dictionary of Natural Products database using the structure generated by NMR and HR-MS analysis led to the conclusion that this compound had already been reported as cyclo(phenylalanine-4-hydroxy-proline).

Violacien and deoxyviolation isolation

Isolation of violacien and deoxyviolation was achieved by metabolic analysis of an unknown actinomycete identified as BBPARAUNK02 purified from a soil sample from Parachute Cave utilizing the microbial trap method was grown employing varying media recipes. An aliquot of 50 µL of cryogenically stored spore suspension was applied to an agar plate containing 30 mL of ISP2 media. Following a period of time to facilitate growth and sporulation, a loop full of spores were inoculated into a 250mL Erlenmeyer flask containing 50 mL of ISP2 media (yeast extract 4 g/L, malt extract 10 g/L, and dextrose 4 g/L). Following a 7 day culture at 30 °C on rotary shaker at 180 rpm, a 20 mL aliquot was transferred to a 2.8 L Fernbach flask containing 500 mL of production media (Soy powder

15 g/L, glucose 10 g/L, soluble starch 10 g/L, NaCl 3 g/L, $MgSO_4$ x $7H_2O$ 1 g/L, 2 mL of trace elements solution ($FeSO_4$ x $7H_2O$ 0.01 g/L, $MnCl_2$ x H2O 0.08 g/L, $CuSO_4$ x 5 H_2O 0.07 g/L, $ZnSO_4$ x 7 H_2O, 1 drop of H_2SO_4) (pH 7.2)). The extract was generated by addition of 200 mL of activated HP 20 (Sephadex, GE Healthcare, Buckinghamshire, England) resin in water. Following a 3 hour period of shaking under the culture conditions, the broth was separated form the resin and micellia and discarded. The resin and micellia were extracted with 100 mL of mass spectrometry grade methanol for 1 hour. The methanol extract was seprated from the resin and micellia and evaporated to dryness. The resin and micellia were extraced with acetone, which was then separated and dried in vacuo. The crude extract was preliminarily fractionated using size exclusion chromatography employing LH20 resin. Two fractions contained a purple compound which were purified by RP-HPLC-MS in conjunction with UV detection using a C-18 column with a gradient from 95:5 water: acetonitrile to 5:95 water: acetonitrile over 30 minutes. Both fractions showed relatively pure compounds with λ_{max} around 580nm and m/z peaks that were complimentary in positive and negative ionization modes. Using the acquired data, the samples were easily identified as previously reported compounds using the DNPD. Proton NMRs for both compounds were obtained and compared to reported spectra to determine the identity of the compounds to be violacien and deoxyviolacien.

Hypogeomycin and epoxide isolation

Hypogeomycin was isolated from the actinomycete identified as BBHARD23. An aliquot of 50 µL of cryogenically stored spore suspension was applied to an agar plate

containing 30 mL of ISP2 media. Following a period of time to facilitate growth and sporulation, a loop full of spores were inoculated into a 250mL Erlenmeyer flask containing 50 mL of ISP2 media (4 g/L yeast extract, 10 g/L malt extract, and 4 g/L dextrose in distilled deionized water at a pH of 7.2 and autoclaved). Following a 7 day culture at 30 °C on rotary shaker at 180 rpm, a 20 mL aliquot was transferred to a 2.8 L Fernbach flask containing 500 mL of production media (glucose 1.0 %, glycerol 0.5 %, corn steep liquor 0.3 %, beef extract 0.3 %, malt extract 0.3 %, $CaCO_3$ 0.2 %, and 0.01 % thiamin, added after autoclave by sterile filtration (pH 7.2)). Fermentation was carried out for 7 days at. Ethyl acetate (250 mL) was added to the culture and the biphasic mixture was agitated to produce an emulsion via a rotary shaking at 180 RPM for 30 minutes. The phases were separated by centrifugation and the ethyl acetate layer was decanted, dried with anhydrous $MgSO_4$, filtered by vacuum filtration, and evaporated to dryness via rotary evaporation to produce a crude extract. Preliminary fractionation was performed by application of the crude extract in 3 mL methanol to a column filled with LH-20 (Sephadex, GE Healthcare, Buckinghamshire, England). Fractions containing abundant metabolites were combined and evaporated to dryness based on thin layer chromatographic analysis to generate six fractions which were evaluated by LCMS to determine prioritization of fractions for further purification allowed the purification of hypogeomycin and the epoxide precursor. Hypogeamycins were purified by reverse phase-HPLC (XBridge C18, 10 x 150mm, 5µm) at a flow rate of 5 mL/minute with water/acetonitrile/ammonium acetate gradient (5% acetonitrile in 10 mM ammonium acetate to 95% acetonitrile over 60 minutes and held at 95% acetonitrile for 10 minutes).

Culture conditions for chemical additives during culturing

A 50 µL aliquot of cryogenically stored glycerol stocks of *A. asperillus* were plated on solid K26S medium (glucose 10 g/L, soluble starch 10 g/L, beef extract 5 g/L, yeast extract 5 g/L, bacto tryptone 5 g/L, and $CaCO_3$ 2 g/L) containing bacto agar 20 g/L. Following a sifficient amount of time for robust growth to occur, a loop full of cells was inoculated into 10 mL of liquid K26S medium in a 50 mL falcon tube. Following 10 days of growth @ 30°C and 170 rpm in an environmentally controlled shaker, a 3 mL aliquot was transferred to a 250 mL erlenmeyer flask containing 30 mL of K26S medium. After 3 days, a 10 mL aliquit was transferred to a 2 L liter fernbauch flask containing 500 mL of K26S medium. To evaluate the effect of scandium and lanthinum chloride salts, an aliquot of concentrated salt solution that had been filtered through a 0.2 µm sterile membane and added to the culture media at a concentration of 50 µM, 100 µM, and 200 µM scandium, and 0.5 mM, 1.0 mM, and 2.0 mM lanthanum. Ethanol and DMSO were added in a sterile manner to a final concentration of 1 %, 3 %, and 5 %. All culture conditions containing additives were grown @ 30 °C and 170 rpm in an environmentally controlled shaker. Control samples were cultured in biological diplicates and non chemical addative samples were evluated at 23 ˚C and 37 ˚C for evaluation of tempature effects.

Culture conditions for chemical additives prior to culturing

A 50 µL aliquot of cryogenically stored glycerol stocks of *A. asperillus* were plated on solid media containing K26S medium containing bacto agar 20 g/L and 25 µg/mL, 50 µg/mL, or 100 µg/mL,of streptomycin antibiotic. Following a sifficient amount of time for robust growth to occur, identified colonies capable of surviving exposure to the

antibiotic were selected and inoculated into 1mL of sterile K26S media and homoginized. Antibiotic resistance was determined by application of a 200 µL aliquot to K26S medium with 20 g/L Bacgo agar and the antibiotic concentration identified as causing the mutation.

Generating the extract for K26 investigation

Complex extracts were generated by the addition of 200 mL of activated HP20 resin suspended in water on the seventh day of culturing. Following a 3 hour period of shaking under the culture conditions, the broth was separated form the resin and micellia and discarded. The resin and micellia were extracted with 100 mL of mass spectrometry grade methanol for 1 hour. The methanol extract was seprated from the resin and micellia and evaporated to dryness. The resin and micellia were extraced with acetone, which was then separated and dried in vacuo.

Evalution of metabolic differences by liquid chromatography mass spectrometry

LC-MS was performed on a Thermo TSQ triple-quad equipped with a Surveyor PC pump and Thermo Pal autosampler. Metabolites were separated on a 150 mm x 4.6 mm Waters C18 (5 µm Particle size) column. The auto-injector sample tray was held at 4 °C to minimize sample degradation and a sample size of 20 µL was injected. Chromatographic separation was achieved using a 30 minute gradient at a flow rate of 1mL/min using a gradient mixer of 10 mM ammonium acetate in 95 % water and 5 % acetonitrile (mobile phase A) and 10 mM ammonium acetate in 5 % water and 95 % acetonitrile (mobile phase B). The mobile phase was held at 100 % A for 1 minute, followed by a linear ramp to 100 % B over a period of 29 minutes and held at 100 % B for

20 minutes, followed by a 5 minute gradient to return the system to 100 % A and allowed to equilibrate for 10 minutes. Metabolites were analyzed using positive and negative mode electrospray ionization. Parameters for analysis include capillary voltage of 4.5 kV, source temperature of 275 °C. Sample sequence for PCA analysis followed the format of: methanol wash, 10 quality control injections (the first 5 of which will not be included in the data analysis), and 5 samples randomly selected followed by a quality control injection, with the entire sequence ending after duplicate quality control samples. The samples were analyzed at a concentration of 100 mg / mL. Quality control samples were generated by the addition of equal parts of each sample to evaluate within a single data set.

PCA to determine unique and abundant ions

Retention time and ion intesity data was extracted fro the .raw files produced by Xcalibur and converted to .mzxml files through the use of Proteowizard (xcms) using the msconver command using the filters of “polarity +” or “polarity –“ depending on the ionization to be investigated. The ions detected during the wash time imcorpated into the LCMS method were excluded from the extraction of the data using the filter “scanTime [start time in seconds,end time in seconds]. For example, a command line for data extraction in positive mode for 0-30 minutes would follow: msconver filelocation*.raw –mzXML –filter “polarity +” –filter “scanTime [0,1800]”. Peak alignment was perfomed using R software. The .tsv file generated was normalized to the total ion count using Microsaft Excel. Statistical analysis was performed using the extended statistics feature within MassLynx. Scores plots were generated using the Pareto PCA model, in which

the clustering of technical dublicates in based only on the variance and covariance with the m/z values associated with a particular retention time. Sample set was only considered acceptable if the quality control samples were clustered around the origin. Individual culture conditions were evaluated against the control culture through the use of OPLS-DA, a supervised method for utilizing PCA. The result of comparision resulted in a S-plot in which the abundance of an ion was represented by the incresed absolute value along the x-axis and the uniqueness of an ion was represented by the increased absolute value along the y-axis.

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CHAPTER 3

SOLID STATE CO-CULTURE TO PRODUCE LEAD COMPOUNDS

How to Investigate Chemical Communication Between Microbes

While traditional methods of cultivating bacteria have focused on mono-culture methods, a new wave of natural product discovery has focused on the evaluation of co-culture methods to induce secondary metabolite production. Since the discovery and understanding of the fact that bacteria utilize small molecules which are excreted by one organism and absorbed by a neighboring organism, a number of co-culture methods have been developed to determine if compounds excreted by an organism have the ability to impact the metabolic impact of a different species of microorganism. The bulk of the research into co-culture has focused on liquid culture conditions and searching for abundant metabolites. Additionally, the use of membranes to separate the co-culture organisms has been employed to determine if physical contact is needed to induce an altered metabolic profile. The development of a solid state co-culture method in conjunction with statistical analysis of the metabolic impact on a variety of actinomycetes could improve the likelihood of identifying a new chemical entity.

Chemical Communication Between Microbes

Chemical perturbations occur in nature through the process of intercellular communication and is fundamental to all microbiology.[1] The first confirmed chemical communication between bacteria was the identification of quorum sensing molecules to

communicate the overall population of bacteria in 1979.[2] Quorum sensing, the process by which bacteria send and receive chemical signals between microbes of the same species occupying the same space, can be understood as the process by which cell-cell signaling is used to determine the population density within a microenvironment[1]. Bacteria monitor their population by producing and excreting diffusible compounds where they accumulate in the surrounding environment and are acquired by bacteria within the proximity. At a minimal threshold, these molecules induce an alteration from primary to secondary metabolic processes in a process known as auto-induction.[3]

For quorum-sensing organisms, an increase in organisms leads to an increase in signaling molecules causing a population wide alteration in gene expression. This process allows the microorganisms to function more as a multicellular organism rather than individual cells, increasing the ability of the species to survive which could not be achieved by mono-cellular organisms. The full consequences of bioeffects induced by quorum sensing is not clearly understood, though it is known to have an impact on more than population control.[1] Chemical communication between differing species in the natural environment to facilitate mutualistic relationships also has the ability to increase the chance of survival for all microbes involved. Recent studies have shown that bacteria can form complex relationships with other organisms to produce new biologically active chemical entities the functions of which are currently unknown.[4]

The hypothesis that microbial competition is governed by a selective force that promotes survival of microbes if they are capable of biosynthesizing bioactive secondary metabolites is supported by several observations; the ability of antibiotic producing organisms to inhibit fungal pathogenesis on plants, the identification of soil microbes that

are resistant to naturally produced antibiotics, and various studies in soil and liquid culture to test the microbial competition have shown reduced cell density of sensitive species when grown in the presence of antibiotic producing actinomycetes.[4] While this hypothesis seems reasonable, extensive studies for antibiotic production in the natural environment have not been able to fully confirm that biosynthesis of these compounds is the result of environmental pressures, such as competition for nutrients by differing species of microbes.[5] Microbes are capable of producing compounds which result in cytotoxic effects for invading species though result in no phenotypic response on the producing organism, an evolutionary consequence of survival of the fittest. It is possible that antibiotics are produced in concentrations below current detectable methods, though still effective for survival of the producers in the natural environment.[5] In the competitive natural environment of microorganisms, it is possible to effectively sterilize a small area surrounding the producing organism with nanograms, or less, of bioactive compounds to allow the mycelium to produce extracellular enzymes for nutritional mobilization without competition.[6] Antibiotic production may not be exclusively used for chemical warfare between competing organisms. It has been shown that sub-inhibitory concentrations of antibiotics have induced an altered gene expressions in bacteria with the activation of genes with currently unknown functions indicating that these compounds may be part of a complex cell-cell signaling process.[1]

While it is known that microorganisms survive and sometimes thrive in biological communities where differing species of microbes are present, laboratory culturing procedures are dominated by single organism methodology utilizing nutrient defined conditions.[7] Given that the naturally complex environment from which microorganisms

are collected, the hypothesis that microbial communication between species can significantly influence growth and metabolic profiles has been tested using a variety of methods in recent decades.[1, 8-11] The impact of chemical exchange on a range of microorganisms has been evaluated for phenotypic changes (survivability, altered growth pattern, or pigmentation) and metabolic changes (gene expression, bioactivity, or detection of metabolites by MS or UV analysis).[4, 8, 10, 12, 13] The nature of chemical communication between microbial organisms and the consequences thereof are outlined in Figure 3-1. Quorum sensing between microbes of the same species allows population control to prevent over consumption of essential nutrients through use of signaling molecules which may be specific to each species. For *S. coelicolor*, the production of many secondary metabolites is controlled by the accumulation of a small diffusible molecule which accumulates in the surrounding environment.[14-16] Once the concentration of this compound reaches a certain concentration, secondary metabolism of prodigiosin s initiated and the concentration of the molecule declines as an increase in accumulation of the secondary metabolite increases in a process known as self-induced secondary.[14]

Chemical communication between microbes in a complex consortium of differing species can result in mutualistically beneficial exchange of compounds (Figure 3-1). Simple induced secondary metabolite production, the process by which a small diffusible molecule is excreted by one organism and incorporated into a neighboring organism, results in the activation of genes not expressed under mono-culture conditions and production of secondary metabolites, such as pestalone.[17] In some cases, the secondary metabolite has the ability to increase survivability, an excellent example of which is the incorporation of chelating compounds used to acquire iron from the environment.[16]

Siderophores produced by one organism can be used by another within the same environment to allow growth under laboratory controlled conditions resulting in the cultivability of organisms identified using environmental DNA (eDNA) which remained uncultivable in the absence of the siderophore producing organism.[16] Several studies have shown that interaction between differing species have resulted in the identification of novel chemical entities not produced utilizing conventional mono-culture conditions.[17]

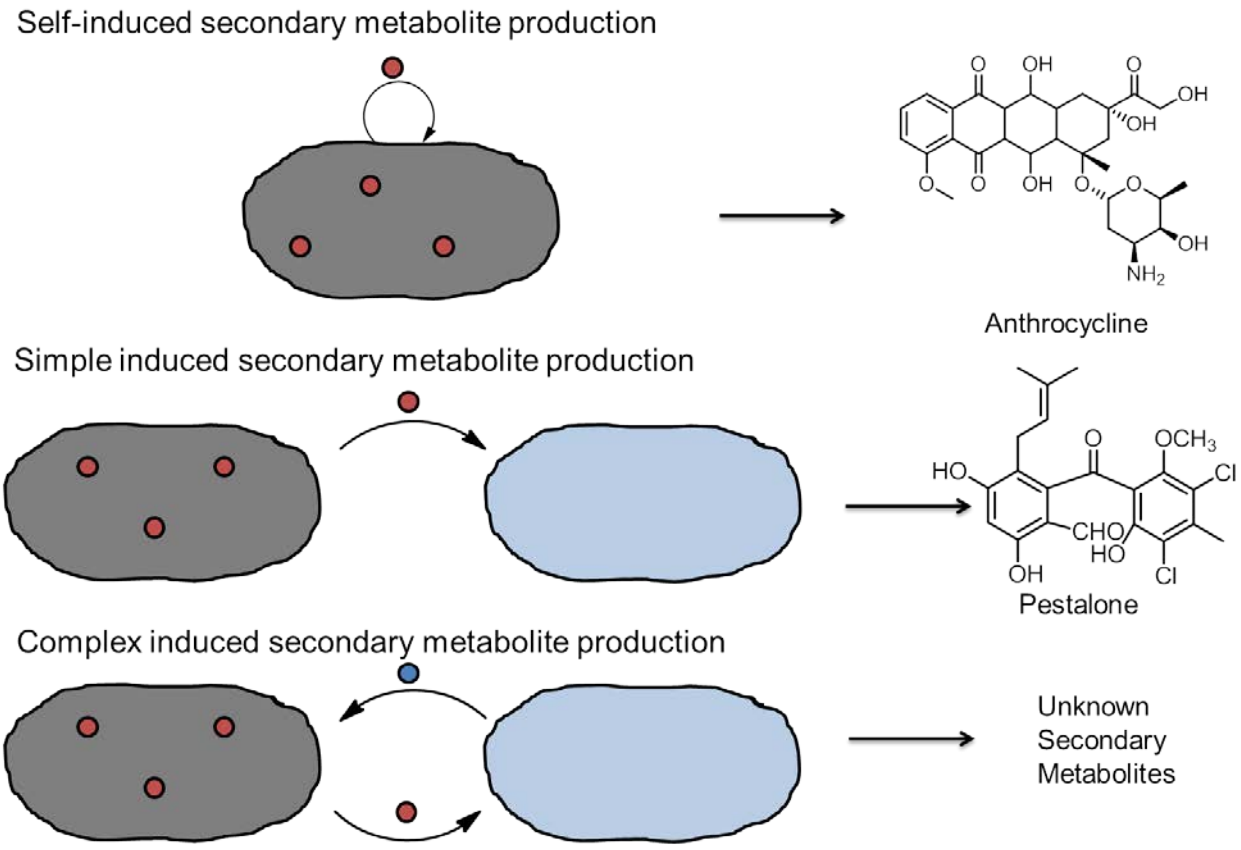


Figure 3-1. Signaling molecule activation of secondary metabolite production. Self- induced production as a result of incorporation of a small, diffusible molecule produced. Simple induced secondary metabolite production as a result of incorporation of a small, diffusible molecule produced by a different microbe. Complex induced secondary metabolite production as a result of incorporation of a small, diffusible molecule produced by a differing organism which is incorporated into the production of another small, diffusible molecule which is incorporated into the alternate microbe resulting in the production of secondary metabolites.

A more complicated scenario for chemical communication between differing microorganisms is complex induced secondary metabolic production. This process requires that a microbe excrete a signaling molecule which is recognized by a neighboring organism and results in the production and excretion of a signaling molecule. This second signaling molecule is then incorporated by the organism which produced the original signaling molecule, which results in the production of a secondary metabolite. While there is no published confirmation of the complex induced secondary metabolic profile, this phenomenon is hypothetically possible.

Self-Induced Metabolic Impacts on Actinomycetes

Signaling molecules have been identified in a variety of actinomycetes and shown to regulate the growth cycle and induce production of bioactive compounds. The first actinomycete signaling molecule was identified as A-factor [2-(6'-methylheptanoyl)-3R-hydroxymethyl-4-butanolide] in 1967.[14] The growth dependent accumulation of A-factor, which only has a half-life of several hours in liquid cultures, results in phenotypic and metabolic changes as part of a complex programmed development.[18] During the A-factor sensitive period of the streptomycete lifecycle a series of metabolic events for healthy development are initiated. This results in the expression of specific genes which alter the metabolic processes from primary metabolism to secondary metabolism.[18]

A-factor was identified as a key component in secondary metabolite production and sporulation, not only of *Streptomyces griseus,* but for a variety of actinomycetes which are not producers of A-factor.[15] Through the application of microbial genetics, in which A-factor producing genes were disrupted, phenotypic and genetic alterations occurred in which a lack of spore formation, a process known as balding, and secondary metabolite production was diminished.[18] Addition of purified A-factor to the culture medium for genetically modified *S. griseus* negated these effects resulting in the recovery of sporulation and streptomycin production.[14] Just as the discovery of penicillin led to significant efforts to isolate bioactive compounds from microbes, the discovery and

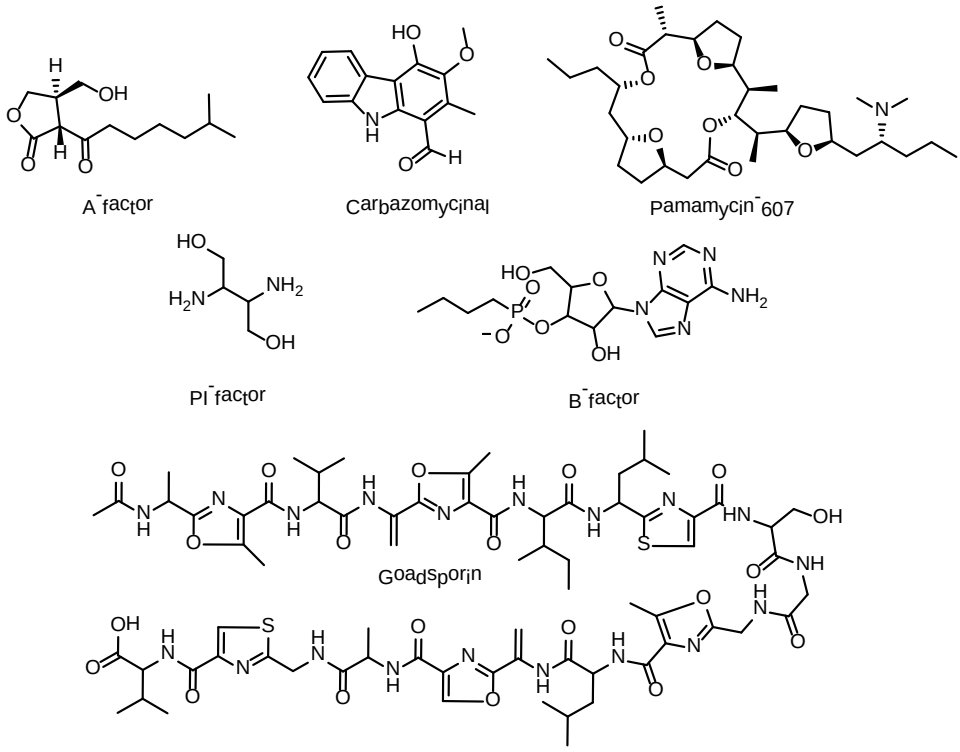


Figure 3-2. Selected Signaling molecules isolated from actinomycetes capable of altering the secondary metabolic profile of a variety of actinomycetes not capable of producing these compounds. A-factor from S. griseus , Carbazomycinal from *Streptoverticilliuin*, Pamamycin-607 from S. alboniger, PI-factor from S. natalensis, B-factor from commercial yeast, and Goadsporin from S. lividans TK23.

understanding of how A-factor influenced the phenotypic and metabolic changes in a variety of streptomycete organisms resulted in significant efforts to identify other small molecules capable of acting as signaling molecules which can influence metabolic and phenotypic responses (Figure 3-2). A variety of signaling compounds with similar chemical structure to A-factor have been identified as virginiae butanolides.[19] Signaling molecules from differing species have also been identified, such as, B-factor, which was isolated from yeast extract and homologues have been identified in both prokaryotes and eukaryotes.[20] Carbazomycinal has been identified as a key component capable of inhibiting the differentiation of substrate mycelia into aerial mycelia, a phenotypic alteration associated with the production of secondary metabolites.[21] Alternatively, the discovery of pamamycin and evaluation of its ability to influence phenotypic responses resulted in the development of aerial mycelia a production of secondary metabolites with antibiotic properties.[22] The production of pimaricin, a glycosylated polyene with potent antifungal activity, is controlled by a unique hydrophilic auto inducer identified as pimaricin inducing (PI) factor which induces standard quorum-sensing kinetics as a response to population density.[23] The identification and structure elucidation of goadsporin in 2001 was the result of the phenotypic response of *S. lividans* TK23 production of actinorhodin, the red pigment produced by *S. coelicolor*, can be initiated under specific laboratory conditions.[24] While many signaling compounds are capable of influencing the metabolic profiles of numerous species of microbes, there is still some specificity which is not fully understood.

Co-Culture for Induced Metabolites from Actinomycetes

Based on the observed metabolic impact of small diffusible molecules within a micro-environment, a multitude of methods have been developed to determine to the impact of co-culture in liquid medium conditions.[6, 9, 13] The addition of heat-killed, nonviable challenge organism, such as *E. coli*, as a media additive to *S. coelicolor* resulted in increased production of the secondary metabolite, undecylprodigiosin.[8] To determine if a co-culture dependent metabolite is produced as a response of physical contact or diffusion of a small molecules, the utilization of membranes which permit chemical communication while maintaining monoculture conditions for each organism

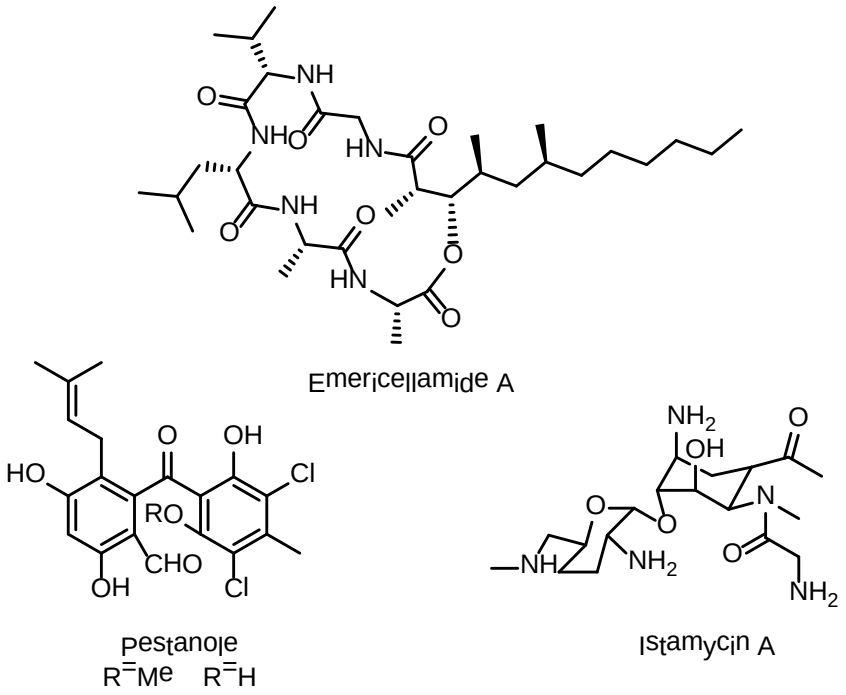


Figure 3-3. Selected new chemical entities produced as a consequence of co-culture in liquid medium. Emericellamide from marine-derived fungus *Emericella* sp. and actinomycete *Salinispora arenicola*, Pestalone from deuteromycete and an unidentified marine bacterium, and Istamycin from *S.tenjimariensis.*

have been employed.[12] Specific cases have revealed that physical contact between different species of micro-organisms is required to elicit an alteration of the metabolic profile resulting in the production of new chemical entities.[8, 9] The various methods employed for co-culture in liquid medium have resulted in the expression, isolation, and identification of multiple compounds representing a variety of biomolecular classes were produced as a response to poly-culture (Figure 3-3).[9] The discovery of a new lipopeptide secondary metabolite, Emericellamide A, from the marine-derived fungus *Emericella* sp. was facilitated by the application of co-culture with a marine actinomycete *Salinispora arenicola* which resulted in 100 times the production of the compound compared to mono-culture conditions.[25] Pestalone is also produced as a result of co-culture between two marine microbes, a deuteromycete and an unidentified marine bacterium, and has shown potent bioactivity against pathogens that are resistant to current clinically relecent antibiotics. The identification of the producing organism was determine as a result of minimal production of pestalone when 1 % ethanol was added to the mono-culture broth in which the fungus was cultured.[17] The production of istamycin by *Streptomyces tenjimariensis* was evaluated as a response to co-culture conditions employing a variety of competing microorganisms. The results revealed that 53 bacterial species (22 % of those tested) were capable of causing a significant increase in the production of istamycin.[4]

A large portion of the research focusing on co-culture of actinomycetes has been developed utilizing liquid culture conditions.[7] One consequence of liquid culture when culturing streptomycetes is the formation of cell pellets as a consequence of allowing the microbes to free-float within a homogenous aqueous semi-aerobic environment.[26] As a

result of the pellet formation, cells within the sphere become nutrient deprived, while those on the surface are less likely to feel this effect, the result of which is differing metabolic conditions.[26] On solid media, streptomycetes grow by extending mycelium into the solid substrate and aerial mycelium above the substrate.[27] Streptomycetes undergo a series of morphological and biochemical changes throughout their lifecycle, which begins with germination of spores in a nutrient compatible environment to initiate vegetative growth and results in the development of aerial hyphae capable of generating spores.[28]

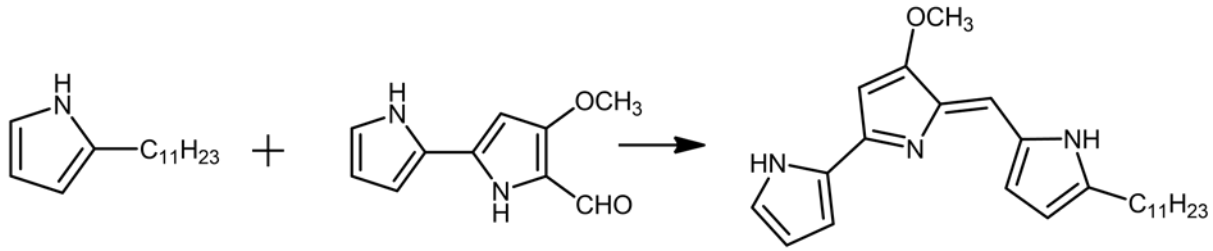
Developing a Method for Solid State Co-culture

To avoid the complications associated with liquid cultures, the development of a method in which the microbes would be cultured on solid media was developed to evaluate the influence of competing microbes on the metabolic output of the actinomycetes to be tested. Analysis of the environment surrounding aquatic and terrestrial microbes geometrically fixed to solid support for cultivation has revealed a variety of excreted molecular plumes. Furthermore, these chemical plumes correspond to phenotypic responses observed for differing microbes cultured in close proximity to each other.[29] This analysis has led to the hypothesis that many secondary metabolites act as chemical signaling molecules capable of influencing microbes within the same species (population density), encroaching species (chemical warfare), and companion species (commensal relationships).[30]

A variety of co-culture studies have been carried out using the model streptomycete, *S. coelicolor,* due to the extensive knowledge of this microbe.[8, 13, 28, 31, 32] Phenotypic responses in the production of actinorhodin and prodigiosin, the red and blue

pigments, respectively, have been used to evaluate the effectiveness of multiple experimental designs including genetic mutation, chemical probing, and co-culture.[32] To evaluate the effectiveness of the developed solid state co-culture process, *S. coelicolor* was used as the proof of principle organism due to the visual appearance of the red pigment, prodigiosin. The biosynthetic process of producing prodigiosin has been well characterized facilitating the assemblage, annotation and genetic modifications of the gene cluster responsible for production.[31] Using genetically modified organisms, provided by the laboratory of Dr. Greg Challis, in which the prodigiosin gene cluster was disrupted such that prodigiosin production was dependent on the diffusion of substrates into the surrounding environment followed by the incorporation by a neighboring organism. As shown in Figure 3-4 A, the enzymatic combination of 2-undecylpyrrole (UP) and 4-methoxy-2, 2'-bipyrrole-5-carboaldehyde (MBC) are required to produce prodigiosin. *S. coelicolor* mutants capable of producing and excreting only one of the necessary compounds were applied to the solid media by employing a 96 well plate replicator with a guide for reproducible geometric fixation of microbes (Figure 3-4 B). Evaluation of the mono- and co-culture conditions of the selected mutants showed that no pigmentation developed for the mono-culture conditions (Figure 3-4 C left and center panel) while production of prodigiosin was clearly visible for the co-culture condition

A)

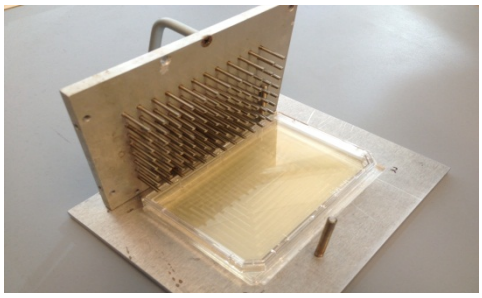


2-Undecylpyrrole (UP)

4-methoxy-2, 2'-bipyrrole-5-carboaldehyde (MBC)

Undecylprodigiosin

B)



C)

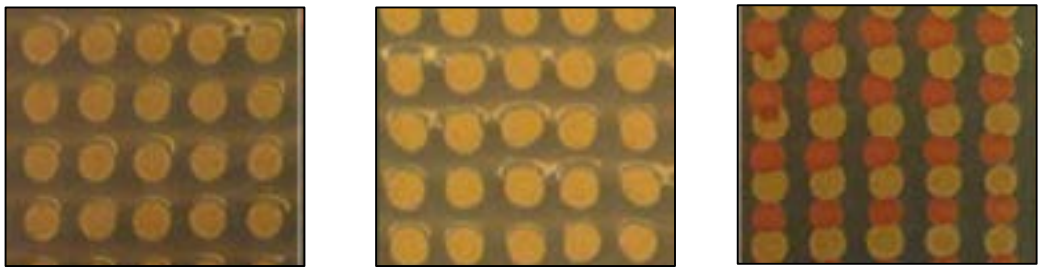


Figure 3-4. A) Chemical structures of UP and MBC which are enzymatically joined to produce undecylprodigiosin, the characteristic red pigment produced by *S. coelicolor*. B) Replicator and guide used for application of the spore suspension to the solid medium for co-culture. C) S. coelicolor mutants grown in mono-cultures where undecylprodigiosin is not produced and co-culture where appearance of undecylprodigiosin was visually monitored as a consequence of small diffusible molecules produced by neighboring organisms.

(Figure 3-4 C right panel). Additionally, the nearly uniform appearance of the red pigment implies that the entire colony exhibits the same metabolic and phenotypic alterations in

response to the diffusible compounds produced by the neighboring organism, an ideal cultivation method that cannot be achieved in liquid medium.

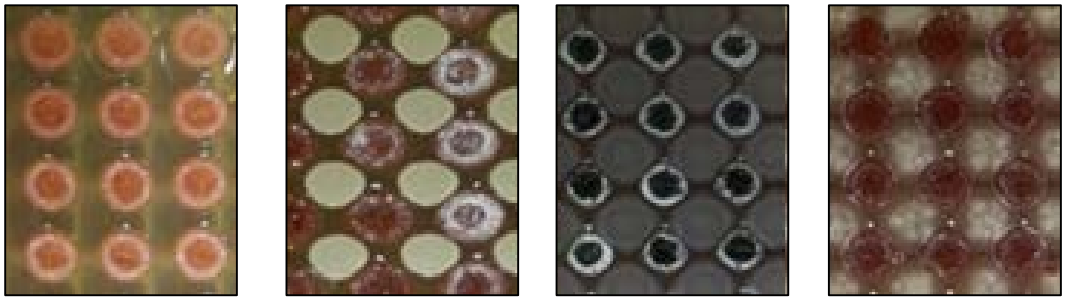
Following the successful development of a co-culture method utilizing solid medium, an appropriate extraction method had to be developed to provide the best representation of metabolic profiles while providing enough material for analysis and determination of lead compounds produced as a result of the co-culture environment. Initial extraction methods relied on the diffusion of molecules contained within the solid substrate used for culture into the designated extraction solvent. This method resulted in generation of an extract which captured the metabolic profile for each of the culture conditions employed, though only negligible amounts of crude extract produced in this manner. As diffusion relies on the movement of molecules from areas of high concentration to low concentration over a period of time, the extraction method was adapted to eliminate the need for compounds to diffuse into the extraction solvent by altering the solid support. Incorporation of a solid support with a low melting point, which would liquefy after a period of gentle heating and facilitate the release of all compounds diffused into the solid support by the microbe resulting in a significant increase in crude extract. Further optimization of the extraction method incorporated solid phase extraction (SPE) utilizing HP-20 resin covered with pores which allow a broad range of compounds to be extracted from the liquefied culture medium. The compounds were then extracted off the resin, allowing an acceptable amount of crude extract for analysis and determination of lead compounds.

Identifying competing organisms

Once appropriate culture and extraction methods were developed, competing organisms were identified based on their ability to influence metabolic profiles of actinomycete when cultured in liquid media. The evaluation of prodigiosin production by *S. coelicolor* was been shown to increase when exposed to *B. subtilis*, either heat killed or biologically viable.[32] *E. coli* has also been shown to increase the production of pigmented compounds during co-culture experiments with *S. coelicolor*.[13] A recent study has shown that organisms within the Tsukamurella and Rhodococcus families have the capacity to elicit an altered metabolic pathway of several actinomycetes resulting in the identification of previously unreported compounds that are not observed in the mono-culture conditions for these microbes. This phenomenon has been putatively identified as a metabolic response to bacteria containing mycophenolic acid within their cell walls, which cannot be recapitulated by the addition of purified mycophenolic acid. Interestingly, the metabolic impact is observed only when liquid culture conditions allow physical contact between the organism being investigated and the microbe containing mycophenolic acid[33]. Evaluation of the genetic comparison utilizing 16 sDNA revealed an organism (BBSNAI13) isolated from Snail Shell Cave in Rockville, TN, as a rhodococcus species, a genus known to contain mycophenolic acid within the cell wall. Combining the above information guided the selection of *E.coli, B. subtilis*, Rhodococcus, the reported *T. pulmonis* strain available from ATTC, and *M. luteus* as the challenge organisms for co-culture.

The appropriate culture conditions and metabolic impact of the chosen challenge organisms were evaluated utilizing the model actinomycete *S. coelicolor* A3 (2). While

actinomycetes are not typically capable of invading territory already colonized by an established microorganism, there is evidence that they are capable of repelling invaders if colonies are already established.[34] As the chosen challenge microorganisms grow at a faster rate than most actinomycetes, the actinomycetes to be investigated were applied to the cultivation substrate prior to application of the challenge organisms and incubated to allow establishment of actinomycete colonies. Due to the propensity of actinomycetes to produce antibiotic compounds, the challenge organisms were applied to the solid media before the established actinomycete colonies entered stationary phase, the most common period in their lifecycle for production of bioactive secondary metabolites. Phenotypic responses for the developed co-culture method of *S. coelicolor* A3(2) reveal that rhodococcus increases sporulation, while *M. luteus* increases actinorhodin (blue pigment) production and *T. pulmonis* increases undecylprodigiosin (red pigment) production based on the visualization of the pigmented culture media (Figure 3-5).



S. coelicolor Mono-culture | Rhodococcus Co-culture | *M. luteus* Co-culture | *T. pulmonis* Co-culture

Figure 3-5. *S. Coelicolor A3 (2)* grown in mono- and co-culture conditions with the selected challenge organisms after four days of cultivation. Phenotypic responses observed include increased sporulation in co-culture with BBSNAI13, actinorhodin production as a response to co-culture with *M. luteus* and increased production of prodigiosin in co-culture with *T. pulmonis*.

Impact of Solid Medium Culture Compared to Liquid

Many of the actinomycetes that have been evaluated for their ability to produce secondary metabolites have been isolated from terrestrial samples while the cultivation methods to evaluate secondary metabolite production have been evaluated utilizing liquid medium conditions.[35] Comparison of the metabolome of *S. coelicolor* grown in liquid media and solid media resulted in the observation of increased production of several metabolites including desferrioxamine, calcium-dependent antibiotic (CDA), and undecylprodigiosin. The increased production of desferrioxamine is not surprising given the fixation of the microbes resulting in diminished availability of iron as colony formation and development depletes this essential element from the localized area surrounding the colonies. The production of this compound in co-culture condition was not significantly impacted by the challenge organisms with the exception of *M. luteus*, which caused a significant decrease in the observed abundance when compared to the mono-culture condition. Production of CDA was significantly increased when compared to the liquid condition in which the detection of this molecule was negligible, though *co*-culture with *M. luteus* showed the greatest decrease for the challenge organisms. While undecylprodigiosin was increased to detectable levels on solid support media when compared to the liquid conditions, co-culture with *T. pulmonis* diminished production to basal level limits observed in liquid media. Co-culture with *M. luteus* significantly increased production of this molecule while the greatest increase was detected when *S. coelicolor* was cultured with the rhodococcus cave derived competing organism consistent with the phenotypic observations for the co-culture conditions (Figure 3-6).[36]

Based on the analysis and determination of metabolic differences using *S. coelicolor* A3 (2), the solid-state co-culture method was expanded to evaluate a variety of actinomycetes.

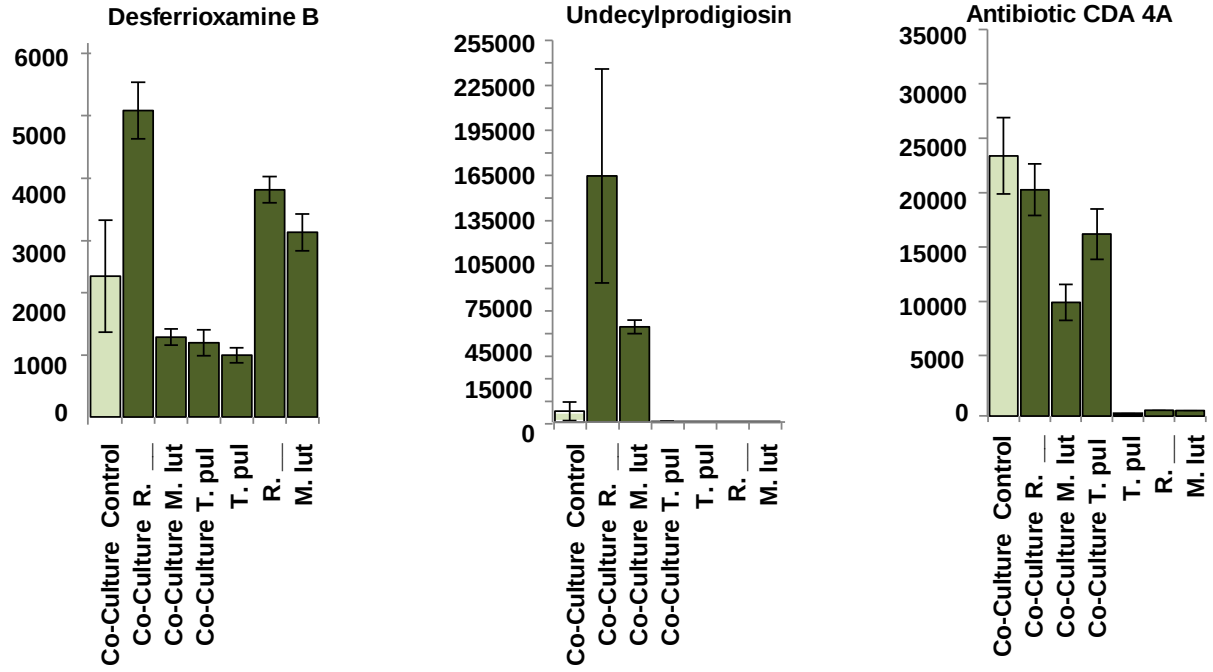


Figure 3-6. Comparison of Desferrioxamine B, undecylprodigiosin, and Antibiotic CDA produced by *S. Coelicolor A3 (2)* grown in mono- and co-culture conditions with the selected challenge organisms after four days of cultivation. Figure adapted from Goodwin, et. al.[36]

Co-Culture to induce secondary metabolite production

The developed co-culture technique was applied to a variety of genomically sequenced microorganisms known to produce secondary metabolites. Among these was the actinomycete *Nocardiopsis* (FERM BP-5871) known to produce apoptolidin.[37] A variety of apoptolidin molecules, the core structure of which is a 20-membered macrolide, have been reported under specific mono-culture conditions (Figure 3-7).[38] Interest in the isolation, synthesis, and study of apoptolidin macrolides is associated with their observed selective cytotoxicity against various cancer cell lines providing an opportunity for the development of a unique class of anticancer therapeutic.[39] The biosynthetic production of the apoptolidin compounds has been elucidated and the gene cluster responsible fully annotated, including post translational modifications. This knowledge facilitated the development of a genetically modified species which lacked the ability to produce the apoptolidin molecules.[40]

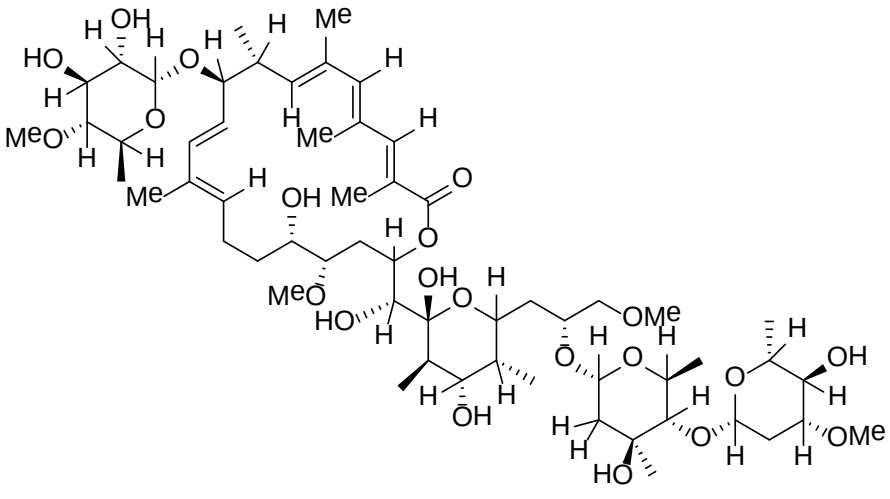


Figure 3-7. Structure of apoptolidin A with the 20-membered macrocycle core produced by *Nocardiopsis* (FERM BP-5871).

Co-culture conditions were employed to determine the metabolic responses, specifically the production of apoptolidin, as a consequence of exposure to the selected challenge organisms. Manual examination of the data was performed to identify any chemical entities which were noticeable via UV detection or ionization when employing HPLC-MS. Of the challenge organisms cultured in the presence of the apoptolidin producing actinomycete, only *E. coli* was capable of inducing production of a variety of compounds putatively identified as apoptolidins. To validate this conclusion, the genetically modified Nocardiopsis ΔApoS8, which is incapable of producing apoptolidins, was cultured in mono-culture and co-culture with *E. coli* as the challenge organism. As expected, the previously observed compounds were not produced, supporting the

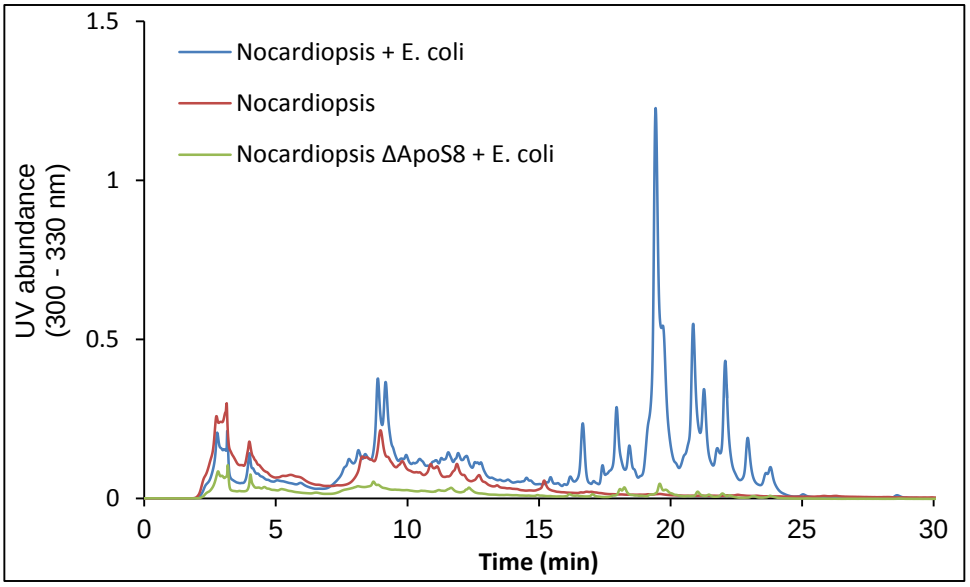


Figure 3-8. UV analysis of apoptolidin producer in mono- and co-culture of Nocardiopsis with *E. coli* and co-culture of *E. coli* and Nocardiopsis ΔApoS8.

Compound	exact mass	Observed m/z	ions	retention time
Apoptolidin A	1128.64	1146.7	$[M+NH_4]^+$	19.35
Apoptolidin B	1112.65	1130.72	$[M+NH_4]^+$	22.06
Apoptolidin F	1128.64	1146.8	$[M+NH_4]^+$	23.58
Isoapoptolidin A	1128.64	1146.8	$[M+NH_4]^+$	20.83
Isoapoptolidin B	1112.65	1130.9	$[M+NH_4]^+$	22.89

Table 3-1. Putative identification of apoptolidins produced by Nocardiopsis as a result of co-culture with *E. coli*.

conclusion that co-culture on solid medium has the ability to elicit production of apoptolidin when *Nocardiopsis* is challenged by *E. coli* (Figure 3-8). Comparison of the extract mass spectrum to the extracted metabolic profile of Nocardiopsis cultured in liquid medium optimized for apoptolidin production facilitated the putative identification of the compounds within the co-culture metabolome based on retention time and ion profile (Table 3-1).

Co-Culture of Hypogean Actinomycetes

Cave, or hypogean, environments are home to a variety of microorganisms, including visible colonies of actinomycetes.[41] Unlike other environments from which microorganisms have been isolated, hypogean environments maintain static conditions year round and have limited nutrients available for the microorganisms, leading to a greater competition for survival.[41] Microorganisms within nutrient limited environment develop an oligotrophic lifestyle and must adapt to chemoautotrophy or chemolithoautotrophy survival mechanism. [42,43] Due to the nutrient poor environment within cave systems, production of antibiotics by one organism may assist in the competition for nutrients.[44] Actinomycetes are known to survive on minimal nutrients and

play an active ecological role by selectively degrading lignocelluloses and humic materials, the most abundant dissolved organic material within caves.[41] Another food source for hypogean actinomycetes may be the decomposition of other organisms, a possible adaptation to nutrient deprived environments. Bacteria within hypogean environments are classified into two different categories depending the duration of their lifecycle within the caves: those that are accidentally introduced to the cave environment and those that spend their entire life cycle within this environment.[44] To avoid microbes that were introduced accidentally or those that are present at the entrance of the cave due to environmental conditions, isolation of microorganisms for investigation were taken from soil samples collected from remote locations within the various cave environments.

Microbes were isolated from the soil samples through the use of the microbial trap method which selectively promotes the growth and isolation of actinomycetes from complex microbial environmental samples.[45] Once the organisms were purified to a single species, analysis of the 16s DNA was performed to reveal the closest genetic relative through the use of phylogenetic comparison to known microorganisms. Selection criteria for candidates to be exposed to co-culture cultivation was based on genetic similarity to organisms known to produce secondary metabolites (streptomycetes), while those that were potentially pathogenic (nocardia) were avoided.

Determination of Co-Culture Dependent Lead Compounds

While phenotypic responses are indicative of altered metabolic profiles, as observed for *S. coelicolor*, the results can be misleading. One example of this is the evaluation of the metabolic output for a cave derived actinomycete, BBSNAI11 (Figure 3-8). Through visual inspection of the co-culture conditions of BBSNAI11, the greatest phenotypic response results from the co-culture condition with *T. pulmonis*, though no significant alterations of the metabolic profile were detected for this condition when compared to the mono-culture of the actinomycete. Visual inspection of the metabolic profile for each of the experimental culture conditions by UV absorption and ionization in positive and negative mode revealed significant differences between the samples.

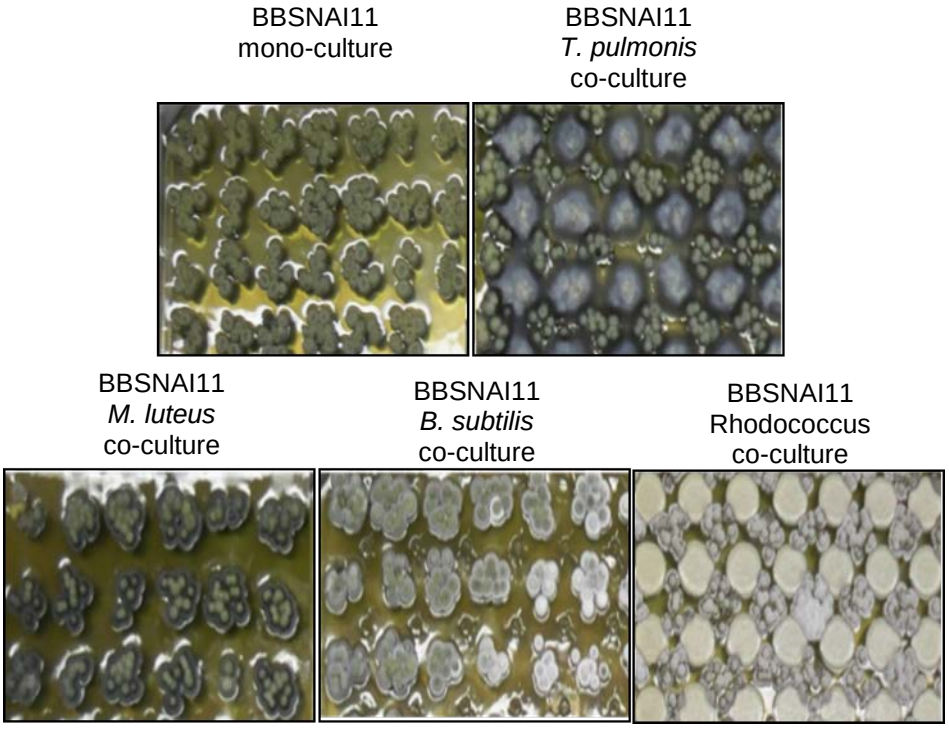


Figure 3-8. Visual inspection for phenotypic alterations during culturing of BBSNAI11 when exposed to co-culture with selected challenge organisms.

Appearance of new metabolic features as a result of co-culture were difficult to detect, though manual inspection of the metabolic profiles for the cave actinomycete BBSNA11 revealed the appearance of a new metabolite when challenged with rhodococcus in co-culture. Manual inspection of a microbial metabolic profile has the ability to determine lead compounds within the extract based on abundance determined by UV absorption or ionization. In many cases, an abundant peak will overshadow some of the less abundant molecules which co-elute based on the chromatographic separation employed. An additional complication to evaluate to metabolic profiles by manual inspection of the metabolic impacts as a result of co-culture is the complex nature of the extracts themselves. Significant similarities within the extracts generated from mono- and co-culture conditions as a consequence of the presence of the actinomycete being investigated make manual inspection a difficult task.

PCA to identify unique ions

Incorporation of statistical analysis, such as principal component analysis (PCA), to evaluate metabolic differences between samples has the ability to discriminate co-eluting or non-abundant components within a metabolic profile, allowing rapid identification of novel metabolites expressed as a result of co-culture. PCA converts multidimensional mass spectrometry data for each sample into lower-dimensional plots to identify maximally distinguishing metabolic features, each of which is then used as a separate dimension within PCA space.[46] This provides sample grouping and separation based on patterns in the most significant detected features where each data point represents multiple features or intensities, generated by extraction of mass spectrometry

(MS) data.[47] Evaluation of the metabolic profiles of all samples within a data set without defining the culture conditions is achieved by unsupervised PCA. This statistical approach is used to determine similarity and divergence of each of the culture conditions when compared across the entire data set for all culture conditions. Application of this type of analysis was applied to the extracts generated from cave derived actinomycete co-culture extracts utilizing the data generated from the positive ionization for samples examined by HPLC-MS. Initial investigation of metabolic differences is based on evaluation of the scores plot to determine the spatial distribution of each metabolome represented as a single data point in PCA space. Interrogation of the PCA results for all

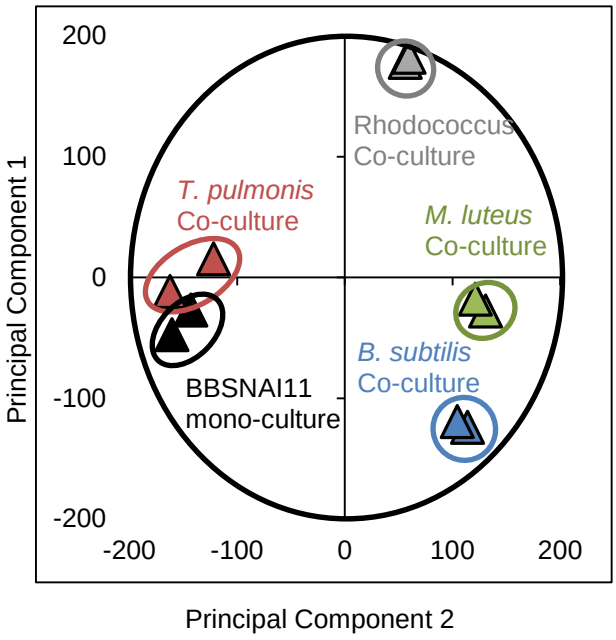


Figure 3-9. PCA scores plot of BBSNAI11 mono- and co-culture extracts. The greatest metabolic similarity between the samples can be seen by the close proximity of the mono-culture of *S. coelicolor* (◆) and co-culture with *T. pulmonis* (◆), while the greatest differences between the mono and co-culture samples can be seen for *rhodococcus* (◆) and M. luteus (◆) both of which occupy different quadrants.

culture conditions employed for BBSNAI11 revealed that the co-culture combination of the actinomycete with *T. pulmonis* had the greatest similarity to the mono-culture, while *M. luteus, B. subtilis,* and rhodococcus had the greatest influence the metabolic profile (Figure 3-9).

The significant separation of the data points representative of the metabolic profiles for the co-culture conditions with BBSNAI13 and *M. luteus* reveals that these challenge organisms had the greatest impact on *S. coelicolor* as each condition occupies a different quadrant than the mono-culture metabolome. The significant impact on the metabolome as a result of co-culture with *M. luteus* correlates with the observed phenotypic results, while phenotypic responses observed for co-culture with *T. pulmonis* did not result in significant metabolic profile alterations.

Given the successful separation of each metabolome within PCA space, analysis of the loadings plot provided information about how each component within the metabolic profile influenced the separation of the samples within PCA space (Figure 3-10). To interpret these plots, the features aligned along the vector of the metabolome's location in PCA space represent those ions that contribute most to the separation in PCA analysis. Features can be evaluated based on uniqueness and intensity, based on their alignment with the vector identified in PCA space and distance from the origin, respectively. Examination of the presence of the ions detected through the application of PCA revealed that the co-culture conditions had significant and differing impacts on the secondary metabolites produced by the actinomycete being challenged. The component with the greatest abundance and contribution to the separation of the mono-culture condition from the Rhodococcus and *M. luteus* co-culture conditions corresponds to an ion with *m/z* =

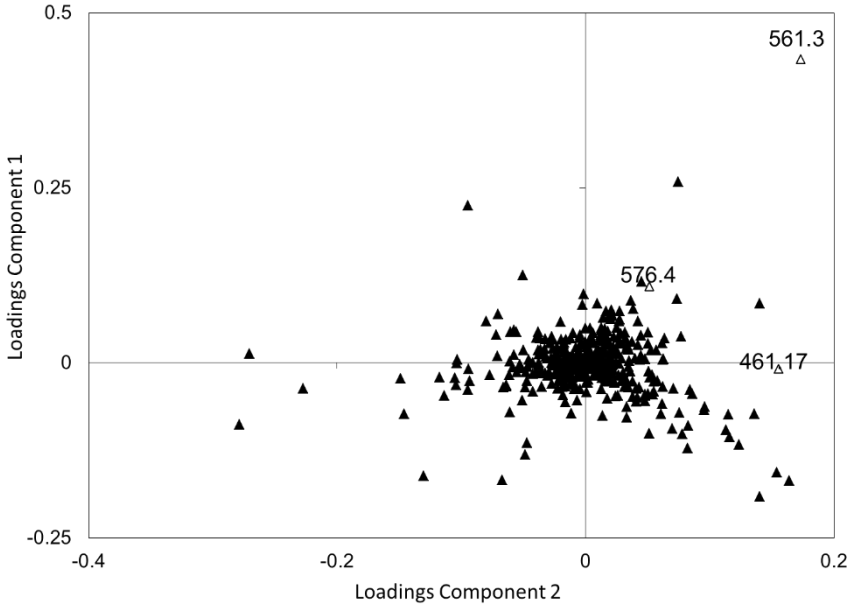


Figure 3-10. PCA generated loadings plot for BBSNAI11 mono- and co-culture conditions with selected ions associated with lead compounds present in the metabolic profiles as a result of co-culture.

561.3 in quadrant II, the 2-D location which lies between the vectors identified in PCA space for these co-culture metabolomes. Examination of the location of ion with *m/z* = 461.17 is indicative of significant contribution for the separation of all co-culture conditions, except *T. pulmonis,* as it lies along a vector opposite of the mono-culture and co-culture conditions with *T. pulmonis*.

Comparison of the abundance of the selected ions, *m/z* = 561.3 and 461.2, revealed that the compounds are up-regulated when compared to the mono-culture condition of the actinomycete and are not present in the mono-culture of the challenge organisms (Figure 3-11). It has been speculated that many of the genes that are responsible for production of secondary metabolites which have not been linked to an isolated compound are produced, though the limits are such that the compound is not identified as a lead compound for isolation[48]. Through the process of co-culture, the production of these compounds could be significantly increased, which would facilitate

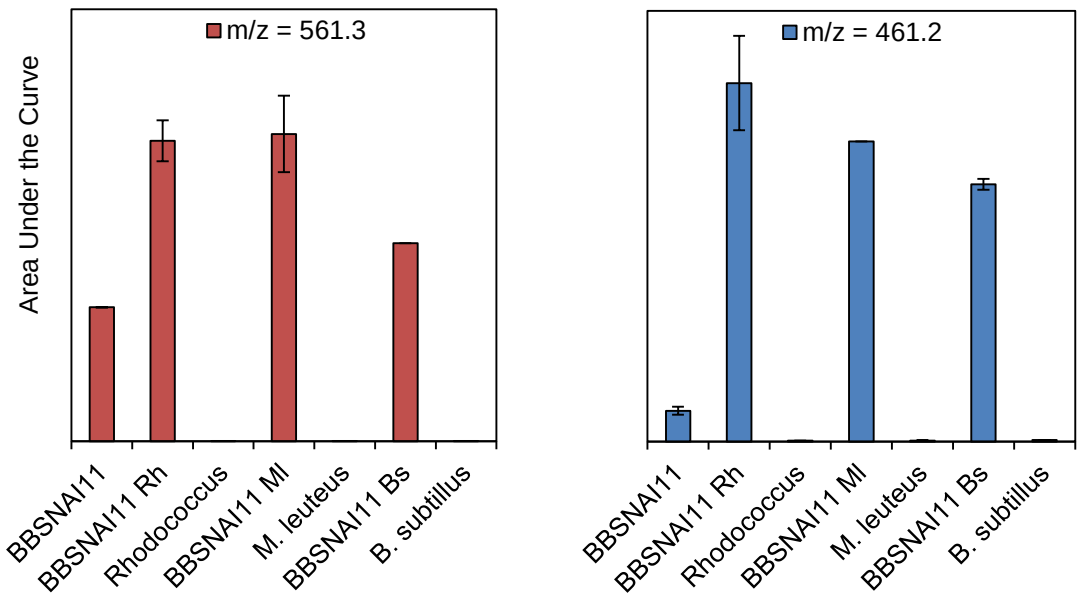


Figure 3-11. Comparison of ion intensity for each of the culture conditions employed to evaluate the metabolic impact of increased production of secondary metabolites produced by BBSNAI11 for ions associated with lead compounds identified through loadings plot analysis.

the ability of isolate and identify previously unreported secondary metabolites. Co-culture conditions resulted in approximately doubling of the production of the compound associated with *m/z* = 561.3 and a significantly more increased level of up-regulation observed for *m/z* = 461.2. The detection of compound production in the mono-culture condition can definitively determine that the actinomycete is the producing organism, which is further confirmed by the lack of production in the mono-culture conditions of the challenge organisms.

The application of co-culture not only has the ability to increase production of secondary metabolites, but can also result in the production and accumulation of components not seen in the mono-culture condition. Examination of the less abundant, closer to the origin, components resulted in the detection of an ion with *m/z* = 576.4 as an influencing component for the separation of the metabolic profiles in PCA space, specifically Rhodococcus. Unlike the up-regulated components previously described, detection of this component was negligible in all of the mono-culture conditions. However, it can still be hypothesized that this component is produced by the actinomycete for several reasons. Actinomycetes have large genomes relative to other microbial species, with 50-70% of the genome dedicated to the production of secondary metabolites.[49] The competing organisms, with the exception of the Rhodococcus microbe (BBSNAI13), have small genomes when compared to other microorganisms. Furthermore, the presence of

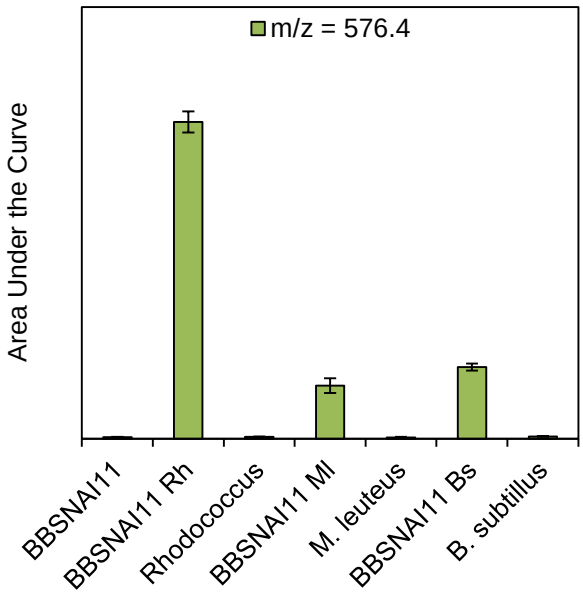


Figure 3-12. Comparison of area under the curve for *m/z* = 576.4 which appears only as a result of the co-culture conditions employing each of the challenge organisms.

this component in all of the co-culture conditions, in which the only similarity between the samples is the presence of the actinomycete, supports the hypothesis that this component is produced by the actinomycete (Figure 3-12). As a consequence of the uniqueness of this component to the production only in co-culture conditions, especially that employing Rhodococcus as the challenge organism, this method has the ability to induce production of secondary metabolites previously unobserved, presumably through the activation of gene clusters not expressed in mono-culture conditions.

Discussion

The phenomenon of bacteria utilizing small molecules to communicate critical information between individual organisms is not fully understood.[18] There has been significant effort to understand how chemical communication impacts the population of microbes within a specified environment, particularly with regards to pathogenic organisms such as *Pseudomonas aeruginosa*.[30] Recent studies have focused on communication between disparate microbes to determine the metabolic consequences in a manner more similar to that of the natural environment. While several studies have focused on the evaluation of the consequences of co-culture in liquid medium, the development of solid state co-culture method more similar to the natural environment has not been investigated until now. Development of a solid state co-culture system was evaluated utilizing the model actinomycete *S. coelicolor*. Significant differences were observed for this microbe when compared to culture conditions employing liquid medium, with some metabolites accumulating at greater abundance while others were diminished. Application of this method to an actinomycete known to produce a secondary metabolite,

for which the biosynthetic process has been determined, revealed that the compound apoptolidin was only produced when the application of co-culture with *E. coli* was employed. Based on the metabolic responses of known actinomycetes, this method was evaluated for metabolic alterations when applied to unknown cave derived actinomycetes.

Evaluation of the metabolome via LC-MS in conjunction with PCA was used to determine lead compounds produced by cave derived actinomycetes only during co-culture with the selected challenge organisms. In most experiments, evaluation of the various metabolomes revealed a significant decrease in the most abundant metabolites produced in mono-culture when compared to co-culture samples. The most promising cave derived actinomycete evaluated was an organism identified as BBSNAI11, whose closest phylogenetic relative determined by 16 sDNA was *S. coelicolor*. While differences between the metabolic profiles of mono- and co-culture were observed for the multiple challenge organisms, the most significant was determined to be the result of co-culture with the cave derived *rhodococcus* species identified as BBSNAI13. Both organisms were isolated from the same environment and may have developed a mutualistic relationship in the natural environment. The increased production of metabolites produced by the actinomycete BBSNA11 and the observation of a metabolite not produced in mono-culture show the capacity of this method to identify lead compounds for isolation which would not be observed using conventional mono-culture methods.

The developed method has been shown to elicit the production of metabolites which are produced only in the presence of a challenge organism utilizing the selected culture conditions. While this extraction method employed has been improved to facilitate an increase in the amount of extract produced by each experiment to evaluate the

influence of co-culture on cave derived actinomycetes, the limited quantity of extract recovered limited the ability to isolate and identify the chemical structure through the use 2-D NMR techniques and HR-MS. Further improvements to the extraction and cultivation method would facilitate the isolation and identification of new chemical entities at quantities sufficient for biological activity testing against a variety of pathogens and cell lines leading to the development of new therapeutics.

Materials and Methods

Co-culture

Co-culture plates were prepared with 40 mL of sterile ISP2 media (4 g/L yeast extract, 10 g/L malt extract, 4 g/L dextrose, 15 grams of agar in distilled deionized water at a pH of 7.2 and autoclaved) to a one well Omni Tray plate. Cryogenic spore suspensions of *S. coelicolor* were cultivated on agar plates (100 x 15 mm) containing 30 mL of ISP2 media and incubated at 30 °C until the production of spores occurred. The spores were removed from the surface of the plate using a sterile loop and suspended in 25 mL of ISP2 liquid media at a concentration of approximately 10^8 spores/mL of media. This solution was homogenized and decanted into a one well plate. The pins of a 96 well replicator were submerged into the spore solution and applied to the surface of the solid support within the one well plate without puncturing the surface. The plates were incubated for 24 hours at 30 °C. Cryogenically stored *E. coli* stock was inoculated into 5mL of sterile LB media 6 hours prior to application to the co-culture plate. Cryogenically stored *B. subtilis* stock was inoculated into 5 mL of sterile nutrient media 8 hours prior to application to the co-culture plate. Cryogenically stored *M. luteus* was inoculated into

5 mL of sterile ISP2 8 hours prior to application to the co-culture plate. BBSNAI13 (cave rhodococcus) stock were inoculated into 5 mL of sterile ISP2 24 hours prior to application to the co-culture plate. Cryogenically stored *T. pulmonis* stock was inoculated into 5 mL of sterile heart infusion media 24 hours prior to application to the co-culture plate. The O.D. 600 value was measured in a 10 mm quartz cuvette on a Spetracmax Plus 3. For all competing organisms, once an O.D. 600 of ≈ 1 was achieved, the sample was diluted to 30mL in a separate one well plate. The pins of a 96 well replicator were submerged into the solution and applied to the surface of the solid support within the one well plate without puncturing the surface in an offset manner relative to the actinomycete. The cultures were incubated for 7 days and extracted. The appearance of phenotypic differences was noted for co-culture conditions when appropriate.

Extraction of co-culture samples

Extraction of co-cultures using standard agar was achieved by mincing the solid substrate in 1 cm X 1 cm segments and extracted with equal volumes of the solvent of choice for 3 hours by shaking at 170 rpm and 30 °C. Samples were centrifuged and the extraction solvent was decanted off and evaporated to dryness. Extraction of co-cultures incorporating low melting agar was achieved by cutting the solid substrate into 1” X 1” segments and placed into a 50 mL falcon tube. A volume equal to the volume of agar of saturated NaCl solution was added to each tube and incubated in a 60 °C water batch for 3 hours or until liquefied. The suspension was transferred to a 250 mL Erlenmeyer flask and equal volume of activated HP20 resin was added. The samples were extracted for 3 hours by shaking at 170 rpm and 30 °C. Samples were centrifuged and the HP20 resin

for each culture condition was transferred to a clean 250 mL Erlenmeyer flask. High purity methanol (1 mL/ 1 mL HP20 resin) was added to each flask to extract the compounds from the resin for 1 hour with shaking at 170 rpm and 30 °C. The methanol was decanted off and evaporated to dryness.

Liquid Chromatography Mass Spectrometry

Dried extract samples were suspended in high quality methanol at a concentration of 100 mg/mL. LC-MS was performed on a Thermo TSQ triple-quad equipped with a Surveyor PC pump and Thermo Pal autosampler. Metabolites were separated on a 100 mm x 3 mm Waters C18 column with guard column. The auto-injector sample tray was held at 4 °C to minimize sample degradation and a sample size of 20 µL was injected. Chromatographic separation was achieved using a 30 minute gradient at a flow rate of 1 mL/min using a gradient mixer of 10 mM ammonium acetate in 95 % water and 5 % acetonitrile (mobile phase A) and 10 mM ammonium acetate in 5 % water and 95 % acetonitrile (mobile phase B). The mobile phase was held at 100% A for 1 minute, followed by a linear ramp to 100 % B over a period of 29 minutes and held at 100 % B for 20 minutes, followed by a 5 minute gradient to return the system to 100 % A and allowed to equilibrate for 10 minutes. Metabolites were analyzed using positive and negative mode electrospray ionization and UV absorption by flowing 80 % of the eluent into the PDA detector and 20 % into the mass analyzer. Parameters for MS analysis include capillary voltage of 4.5 kV, source temperature of 275 °C. Detection of UV active compounds was achieved through PDA analysis with a wavelength range of 200-800 nm.

Principal Component analysis

Sample sequence for PCA analysis followed the format of: methanol wash, 10 quality control injections (the first 5 of which will not be included in the data analysis), 5 samples randomly selected followed by a quality control injection, and duplicate quality control samples at the end of the sequence. Technical duplicates were run for each sample to be investigated. The samples were analyzed at a concentration of 100 mg / mL. Quality control samples were generated by the addition of equal volume of each of the samples to be evaluated within a single data set. Visual inspection was performed to evaluate the reproducibility of retention time and ionization efficiency for duplicate injects and quality control samples throughout the entire sequence. Positive and negative ionization spectra were converted to mzML files through the use of Proteowizard employing filters for ionization mode and retention time. Peak picking and retention correction were performed in R.

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CHAPTER 4

BIOAUTOGRAPHY IMAGING MASS SPECTROMETRY (BIMS)

Deconvolution of Metabolic Impact of Compounds within a Complex Mixture

Through the use of co-culture, it has been determined that microorganisms secrete a variety of compounds in a chemical plume which have the ability to impact the metabolic output of neighboring organisms. This process has been visualized through the use of imaging mass spectrometry which has the ability to identify ions associated with a specific 2-D location within a sample. While this method is effective at identifying the metabolic impact as a result of exposure to a chemical plume, it is limited in the ability to identify which chemical entity within the plume is responsible for the metabolic response. Expanding on the current imaging mass spectrometry methods has the ability to determine the individual compounds within an extract from a producing organism and the metabolic impact of each on an assay organism.

Mass Spectrometry Methods of Identifying Localized Molecules

Traditional methods of imaging biological samples rely on stains, tags, or radioactive labeling to identify localization of compounds within a specimen. While these techniques are useful to qualitatively determine if a compound is localized, no further information to characterize the compound can be obtained. In a complex mixture such as tissue samples, obtaining the molecular weight of a compound localized to a certain region has been difficult to achieve until recent analytical techniques were developed.[1]

Imaging mass spectrometry (IMS) allows the detection and determination of ions for molecules localized within a complex sample by detection of analytes across the entire sample.[2] The sample is analyzed over a specified mass range in a predefined *x* and *y* coordinate system and the ion intensity for each pixel is plotted to determine compound localization and abundance. Combining the multichannel (*m/z*) measurement capabilities of mass spectrometers with imaging allows for each pixel to stand as a single experiment containing a complete mass spectrum over the specified range.[3] The application of MSI to biological samples has facilitated the detection of localized compounds within organs, organelles, and cells in a complex biological system.[4-8]

The first use of MSI was performed in the 1960's using secondary ion mass spectrometry (SIMS)[9], which relies on ionization to occur as a result of striking the sample surface with primary ions, such as Ar^+, Ga^+, and In^+,[10] and penetrates a sample to a depth of a few nanometers.[11] The principle advantage of using SIMS over other methods is increased resolution, which is the greatest for imaging MS, on a scale of approximately 100nm.[12] Unfortunately, the ion beam frequently results in fragmentation of molecules limiting the m/z range to <300 Daltons.[9] Desorption electrospray ionization (DESI) imaging was first introduced in 2004, as a method of performing mass spectrometry at ambient pressure with minimal sample prep for detection of natural products on plant surfaces.[13] The primary advantages of DESI are the range of samples that can be analyzed (solids, liquids, frozen liquids, and adsorbed gases), the lack of sample preparation, atmospheric pressure analysis of samples, and the variety of compounds that can be detected (protein, carbohydrates, oligonucleotides, industrial polymers, and small organic molecules).[14-16] Application of DESI to IMS analysis revealed unique

phospholipid composition of healthy liver tissue versus cancerous tissue in 2005.[17] In 2006, analysis of rat brain showed localized lipids consistent with reported biology at a resolution of 500 µm, a spatial resolution significantly poorer than SIMS and slightly less than MALDI.[18] Improvements to resolution were achieved in 2006 with a reported resolution of 400 µm[14], 250 µm[12], and 200 µm which facilitated detection of ecological interactions along the surface of algae.[6] In 2009, DESI-IMS led to the identification of the secondary metabolites identified as bromophycolides,[6] the algal diterpene-benzoate macrolides that were isolated from *C. serratus* with micromolar concentration IC_{50} values when tested against a variety of cancer cell lines.[19]

The most commonly used ionization method for IMS is Matrix Assisted Laser Ablation/Desorption Ionization (MALDI). Since its introduction in 1986 by Karas[20], MALDI has provided rapid detection of singly charged, intact molecules at concentrations in the femptomole to attamole range for peptides and proteins.[2] Insulin in rat pancreas tissue sections at an estimated concentration of 38 amol was detected by MALDI-IMS in 1997.[2] MALDI-IMS presents the first label free method for imaging of biological tissues with high sensitivity and potential molecular identification based on *m/z* values from ions generated by a soft ionization technique.[21] Adaptation of this method to incorporate a blotting technique resulted in the detection of localized proteins within mammalian tissue samples.[4]

To overcome the disadvantages of the use of matrix during imaging analysis, which can interfere with the detection of small molecules, new methods have been developed where no matrix is required for ionization of molecules of interest.[22] Desorption ionization on silicon (DIOS) traps analytes using porous silicon. The use of porous silicon

acts as an organic matrix by absorbing UV energy and transferring the energy to compounds trapped within the pores which are desorbed from the surface using a pulsed laser.[15] Ionization results in little or no fragmentation of compounds at concentrations as low as attomole without the use of matrix.[23] The primary benefit of DIOS is that no matrix needs to be applied to the sample, allowing unambiguous detection of small molecules in the mass range of current organic matrices used for ionization in MALDI.[15] DIOS has been shown to ionize a range of molecules, from small drug molecules to peptides, even in the presence of salt which significantly hinders ionization in MALDI.[23]

Similar to DIOS, nanostructure initiated mass spectrometry (NIMS) is a matrix free method of ionizing analytes by using initiator molecules trapped in pores.[22] The first NIMS imaging experiment was reported in 2007 of mouse embryo tissue sections to identify ion profile differences within developing tissues.[24] A variety of irradiation sources, surfaces, and liquid fluorinated initiators have been used to facilitate ionization in NIMS.[24] A variety of initiators have been developed and utilized to tailor the ionization efficiency of the analyte of interest.[25] The primary advantages of using the initiators in NIMS is the fact that they do not ionize when irradiated, leading to significantly diminished chemical noise.[25] Additionally, the lack of analyte fragments due to the 'soft' nature of this desorption/ionization technique facilitates the determination of parent ions.[22]

Applying IMS to TLC Bioautography

Evidence derived from metabolomic inventories reveals important roles that microbial secondary metabolites play in interspecies and interkingdom relationships.[5, 7, 26, 27] Secondary metabolites are traffickers of microbial information related to a wide

variety of processes such as biofilm formation [28], mutualism [29], commensalism [30] and antagonism (antibiosis).[31] Additionally, a large fraction of antimicrobial secondary metabolites used in the clinic, including those in clinical applications beyond their initially observed antibiotic activity, are produced by soil-dwelling actinomycetes.[32] Recently, sequencing of resistance genes from soil microbiomes has revealed that most clinically presented antibiotic resistance elements can also trace their origins to soil microbiotica.[33] The propinquity of secondary metabolic biosynthesis and resistance in the soil suggests a dynamic co-evolutionary chemical ecology and it may be argued that basic insight into these systems, in addition to providing profound insight into microbial ecology, may provide tools and strategies for treating diseases with similar molecular bases.

To study these interactions, a wave of new methods has recently been developed to inventory molecular microbial interactions in co-cultures *in situ*.[6, 7, 26] Notably, the technique of tissue imaging mass spectrometry has been adapted with spectacular results in the field of microbial metabolomics.[34-38] Two-dimensional mass images of microbial co-cultures have revealed potential agents of microbial ecological communication, offense, and defense. A unique benefit of the image is that these molecular entities are revealed by the patterns of their topology of their planar distribution. Some potential limitations include sensitivity for low concentration bioeffectors, analytically difficult to detect molecular entities, and coincidental (non-causal) apparent phenotypic molecular diffusion plumes.

A method addressing these limitations, capable of imaging secondary metabolites in concert with affected global metabolomic inventories, can potentially accelerate discovery of compounds and their effects on targeted organism. Correspondingly, we

propose here the combination of *in situ* microbial imaging mass spectrometry with thin layer chromatography (TLC) bioautography. In this method a complex concentrated microbial extract is arrayed chromatographically via 2-dimensional TLC and an indicator organism, embedded in a medium containing a solidifying matrix, is overlaid onto the TLC plate. Incubation results in phenotypic changes in the indicator organism in proximity to the chromatographically arrayed bioactive extract compounds. Phenotypic changes can then be imaged at the mass-feature level using MALDI imaging mass spectrometry. This new approach adds a chromatographic dimension to bioeffector discovery in enriched microbial extracts, amplifying bioeffectors and deconvoluting coincidental molecular plumes. Additionally, simultaneous indicator microbe mass imaging permits the discovery of metabolomic consequences on the indicator organism providing a basis for mode of action and target determination. We herein develop and implement Bioautographic Imaging Mass Spectrometry (BIMS) using a mixture of known antibiotics against *Bacillus subtilis* and demonstrate sensitive compound detection and compound-specific metabolomic responses. Application of BIMS to the analysis of a crude extract from a cave-derived actinomycete using a microbe which presented with less sensitivity to the compounds within the crude extract. Using *Escherichia coli* as the assay organism resulted in the discovery of unreported bioactive and non-bioactive compounds.

Development of BIMS methodology

The application of MALDI imaging MS towards the chemical analysis of aluminum backed TLC plates has been previously described showing greater sensitivity versus chemical staining or UV detection.[39] Antibiotics chosen for the development of BIMS

were initially subjected to MALDI when the samples were applied directly to the TLC substrate intended to be used for separation of the components. Compounds that were readily ionizable and detectable were evaluated by bioautography to determine potency against the designated assay organism. Ideal regions of null growth, or zones of inhibition, as a result of exposure to the antibiotics should be approximately the same size as the spot containing the compounds, excess bioactivity would prevent determination of

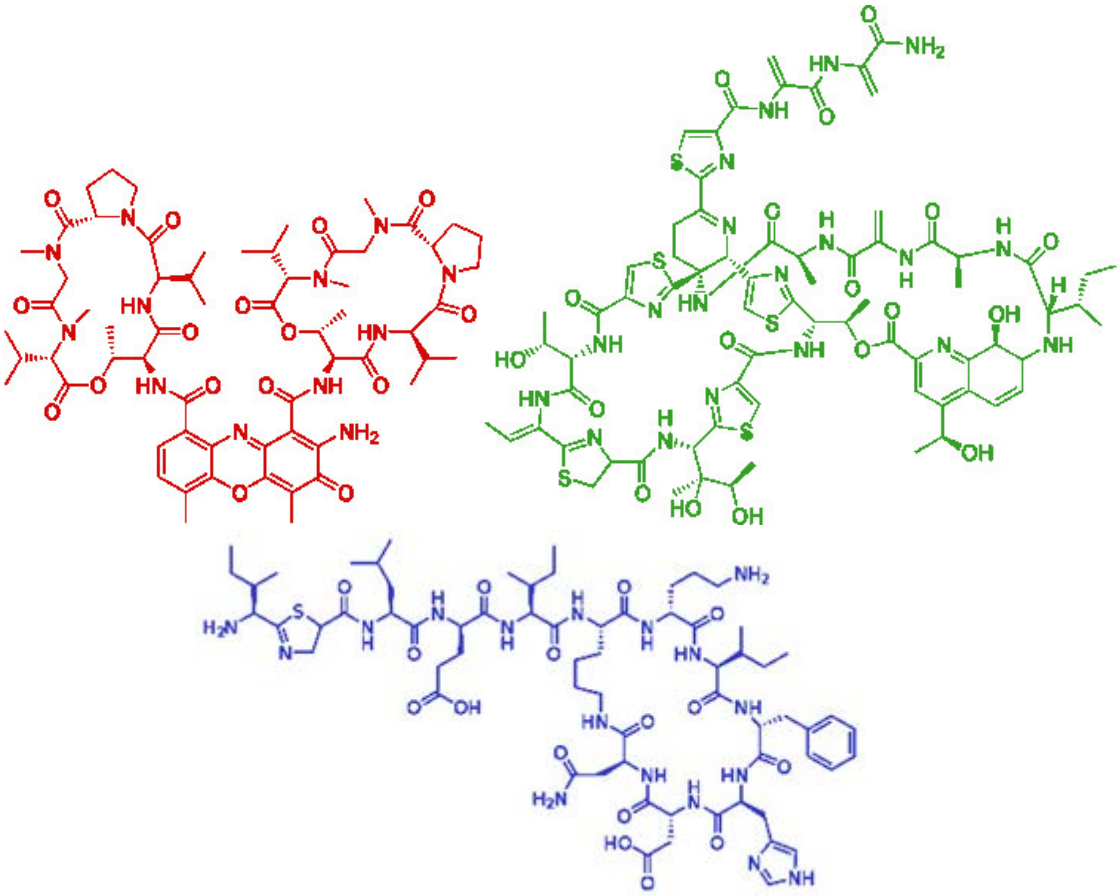


Figure 4-1. Antibiotics selected for development of BIMS methodology. Each sample is color coded to coordinate with all subsequent data analysis with actinomycin (red), thiostrepton (green), and bacitracin (blue).

metabolic impact on the assay organism in regions were overlap between different compounds resulted in null growth. In an effort to determine the metabolic impact of compounds with differing modes of action (MOA), the final mixture for BIMS method development consisted of actinomycin (1 µg, MOA - RNA transcription)[40], thiostrepton (5 µg, MOA - protein synthesis)[41], and bacitracin (10 µg, MOA - cell wall biosynthesis)[42], each of which resulted in an appropriate null growth region (Figure 4-1).

Following the selection of antibiotics with appropriate concentrations for assay organism growth inhibition, detection through traditional methods (UV detection or chemical staining), and detection via MALDI-MS, chromatographic conditions had to be determined for optimum separation of the chosen antibiotics and cultivation of the assay organism. For maximum separation of the compounds, 2-D TLC was selected in which the mixture was applied to the lower left corner of the TLC plate and eluted with a high polarity mobile phase. Following a drying period to remove any residual solvent, the plate was rotated 270° and eluted with a second, less polar mobile phase (Figure 4-2A). Evaporation of the second mobile phase was followed by application of molten agar and the assay organism and incubated overnight to allow planktonic (bacteria embedded in the agar layer) or biofilm growth (bacteria applied to the solidified agar surface) and null growth, or zones of inhibition, to develop as a result of microbial exposure to the various antibiotics (Figure 4-2B).

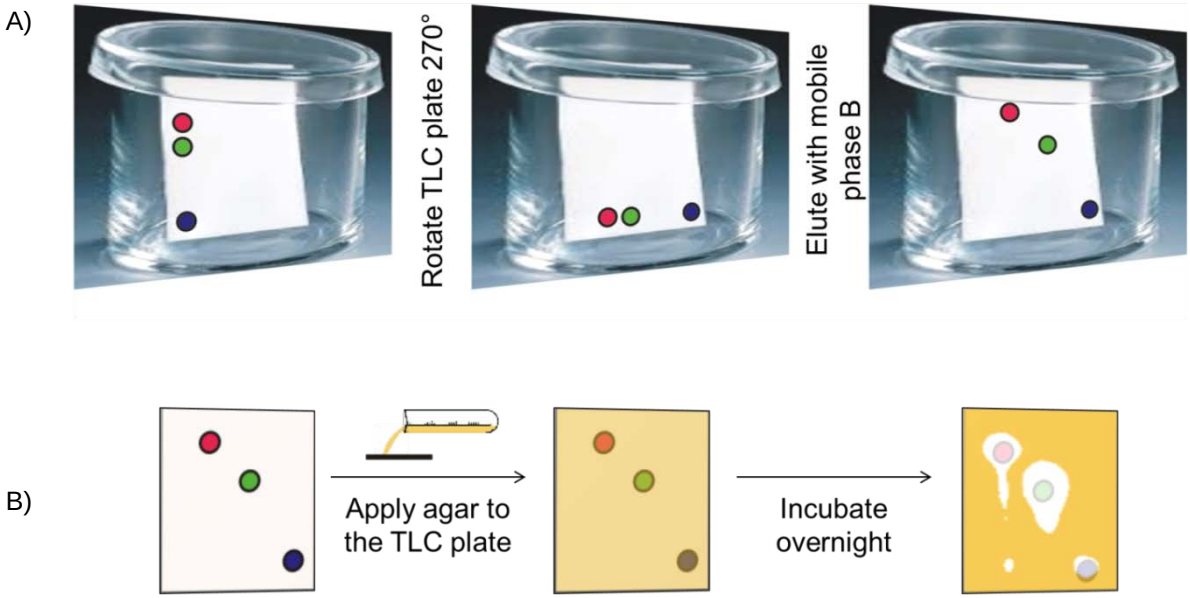


Figure 4-2. Preparation of 2-D separated antibiotics for analysis by bioautography. A) Separation of antibiotic mixture by 2-D TLC. Antibiotic mixture is applied to lower left corner of the TLC plate and separated using mobile phase 1. The plate is removed, allowed to dry, rotated 270°, and separated using mobile phase 2. B) Bioautography of separated antibiotics. Following separation of the compounds, the TLC plate is thouroughly dried to remove any residual solvent. Agar is applied to the TLC surface and incubated to facilitate growth of the assay organism.

The analysis of microorganisms growing on and in agar growth medium has been previously reduced to practice utilizing MALDI-IMS.[43] Additionally, the investigation of compound identification within a complex mixture separated by thin layer chromatography via MALDI-IMS has also been performed.[39] However, significant technical challenges were encountered in combining the two applications. Sheer forces developed in the agar films during the drying process that cause rapid delamination and disintegration of the thin fragile agar film (Figure 4-3 A). To address this issue, the medium composition was adjusted to a decreased content the solidifying agent agar from 3 % to 1 % while the addition of 0.5 % glycerol allowed greater flexibility of the agar layer (Figure 4-3 B).

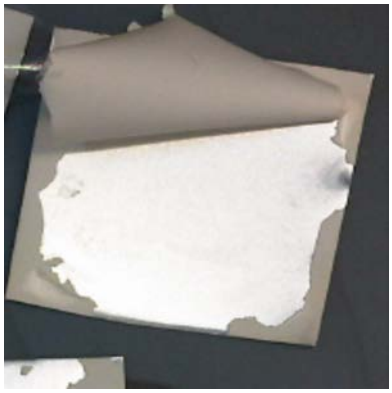
A)  B) 

Figure 4-3. Delamination of agar layer following bioautography. A) Delamination of agar layer applied directly to TLC plate following chromatographic separation and overnight incubation of the antibiotics sample using a medium compositions of 3% agar. B) Intact agar layer after layer applied directly to TLC plate following chromatographic separation and overnight incubation of the antibiotics sample using a medium compositions of 1% agar and 0.5% glycerol.

Unfortunately, signal intensity was greatly attenuated or absent for MALDI-IMS imaged TLC plates surmounted by a thin agar film regardless of laser source/ intensity, matrix chemistry, or agar media formulation. This may be the result of losses in conductivity due to the insulating nature of the agar layer. This speculation is supported by conductivity measurements, in which the insulation capacity of the agar layer eliminated the surface conductivity of the sample. Addition of traditional matrix molecules to the agar used for incubation was tested to determine if this would correct the conductivity issue. One consequence of these compounds was the lack of growth as a result of toxicity, as with 2, 6-dihydroxyacetphenone, which eliminated all growth of the chosen assay organism *B. subtilis*. Gold nanoparticle solutions were also tested as a medium additive, resulting in non-uniform growth of the assay organism. The addition of dithranol had negligible effects on the growth of the assay organism, but it made evaluation by UV or chemical staining difficult and resulted in delamination of the agar layer as the sample dried. This

result led to the hypothesis that removal of the intact agar layer would result in a sample capable of withstanding the high vacuum and laser intensities required for MALDI-IMS. By incorporating a hydrophobic release agent into the TLC mobile phase (5 % naphthalene), dried agar films were able to effectively be separated from bioautographically developed plates.

Attempts to relieve these sheer forces by removal of the agar layer and application directly to a MALDI target were unsuccessful at preventing delamination of the sample to be imaged upon exposure to the high vacuum conditions required for MALDI analysis (Figure 4-4). To address these problems, the concentration of agar was adjusted to determine the minimal amount required for the layer to solidify and support the growth of the assay organism being used. Samples containing less than 1 % agar were not capable of solidifying sufficiently, while those with higher agar concentrations resulted in excessive delamination and curling of the thin film after the drying process. Removal of the agar

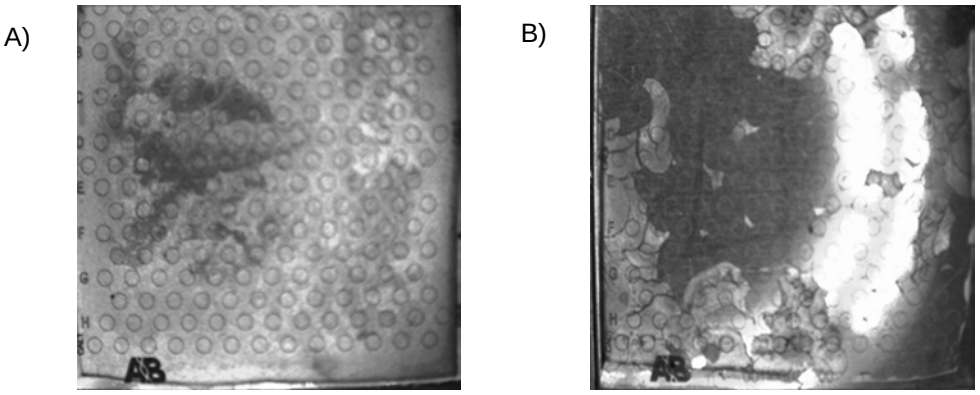


Figure 4-4. Delamination of agar layer following exposure to high vacuum. A) Intact agar layer following transfer of agar layer to MALDI target for drying and imaging. B) Delamination of agar transferred from 2-D TLC developed plate containing the various antibiotics to a MALDI target for drying.

layer from the TLC plate followed by application directly to the MALDI target prior to drying was successful, unfortunately, the agar layer would delaminate in the high vacuum during the acquisition of the image. As a result, the thin film was then joined to a layer of conductive carbon filled tape, a process that has been shown to sufficiently conductive to permit ionization of compounds[44], and surmounted to a stainless steel target prior to imaging via MALDI MS.

To maximize the information gained from the developed BIMS method required six replicate samples: UV and chemical stained, control, biofilm, and three planktonic (INT stained bioautography and inverted and normal for imaging) (Figure 4-5). Initial evaluation of the separation was performed by UV absorption followed by chemical staining which rendered the plate incapable of being used for further analysis. Bioactive regions within the sample were determined by applying 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) which reacts with healthy cells resulting in a very distinctive purple color and can only be used on planktonic samples as staining of the biofilm sample washed the assay organism off the agar surface regardless of stain application methods.[45] Control samples were analyzed by application of agar without an assay organism, followed by incubation, agar delamination and mounting to

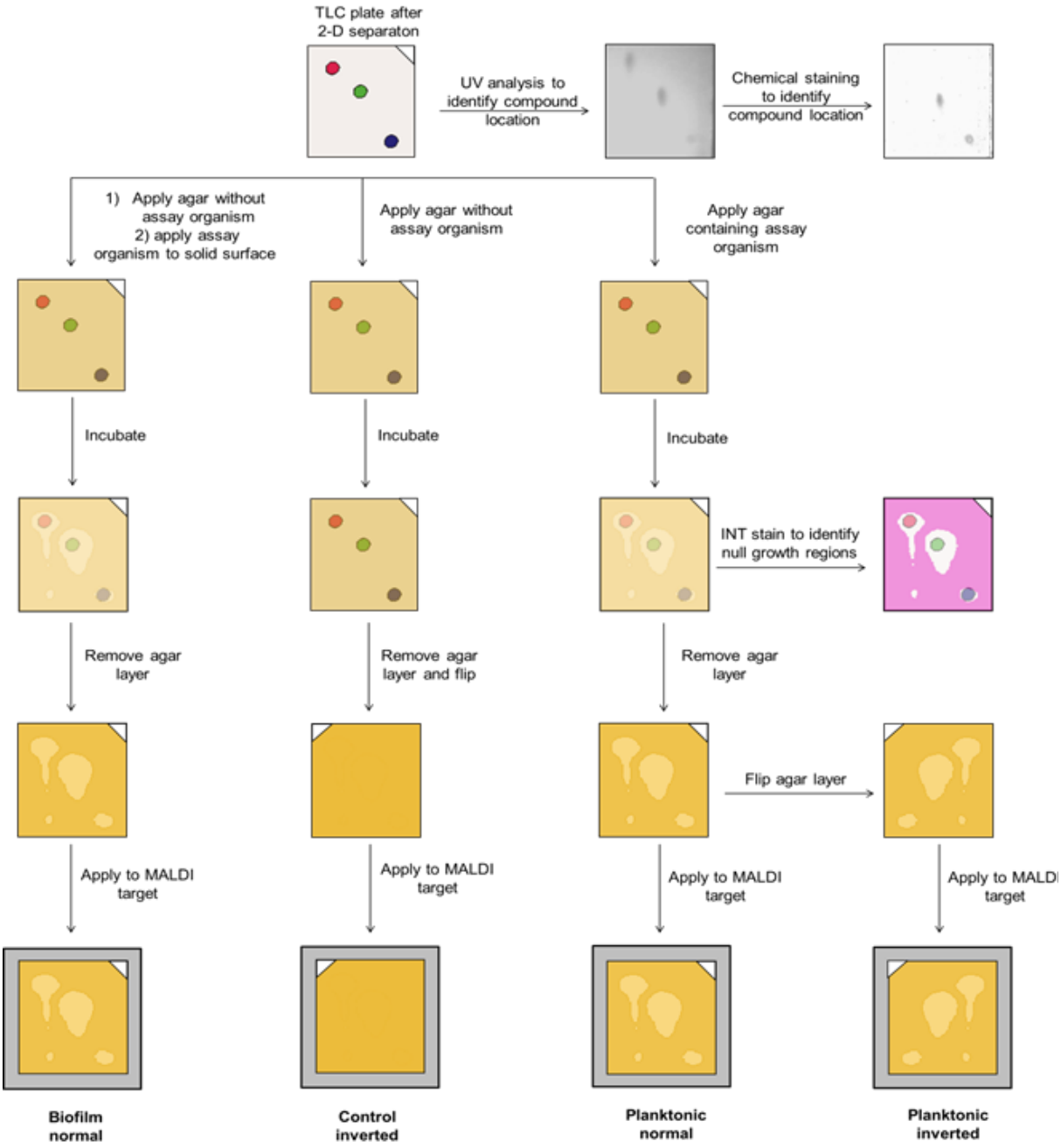


Figure 4-5. Schematic of sample preparation for BIMS identifying the procedure for each of the samples analyzed where actinomycin (●), thiostrepton (●) and bacitracin (●) have been separated via 2-D TLC.

the MALDI target in an inverted manner so that the TLC contact surface of the agar was being imaged. Analysis of this sample via imaging facilitated determination of compound location and degradation of compounds as a result of the incubation process. Images obtained from this sample were compared to those acquired from the planktonic inverted sample. This sample was prepared by addition of agar containing assay organism and incubated and mounted to the MALDI target in a manner identical to the control sample. This analysis facilitated the detection of compounds responsible for bioactivity, though the intensity for each compound detected was significantly diminished when compared to the control sample. Imaging of planktonic normal samples facilitated detection of bacterial responses which overlaid with the observed healthy and null growth regions revealed by INT stain. Additional information was derived from the biofilm normal sample in which agar without an assay organism was applied to the chromatographically separated compounds on a TLC plate. Once the agar solidified, a cell suspension containing the assay organism was applied to the surface and incubated. This method facilitated the formation of biofilms in null growth regions identified using INT stain and confirmed through the application of BIMS.

Development of appropriate imaging conditions were evaluated on small bioactive regions due to the significant amount of data generated for each sample and the time involved collecting the data from the image. Evaluation of the actinomycin region from a TLC plate which utilized ditranol as the delamination agent was performed using multiple *m/z* ranges of the same sample. Analysis of the low mass range (75 - 500 amu) image revealed significant low mass ions associated with the edge of the zone of inhibition (Figure 4-6). Subsequent imaging of the same sample was inconclusive, leading to the

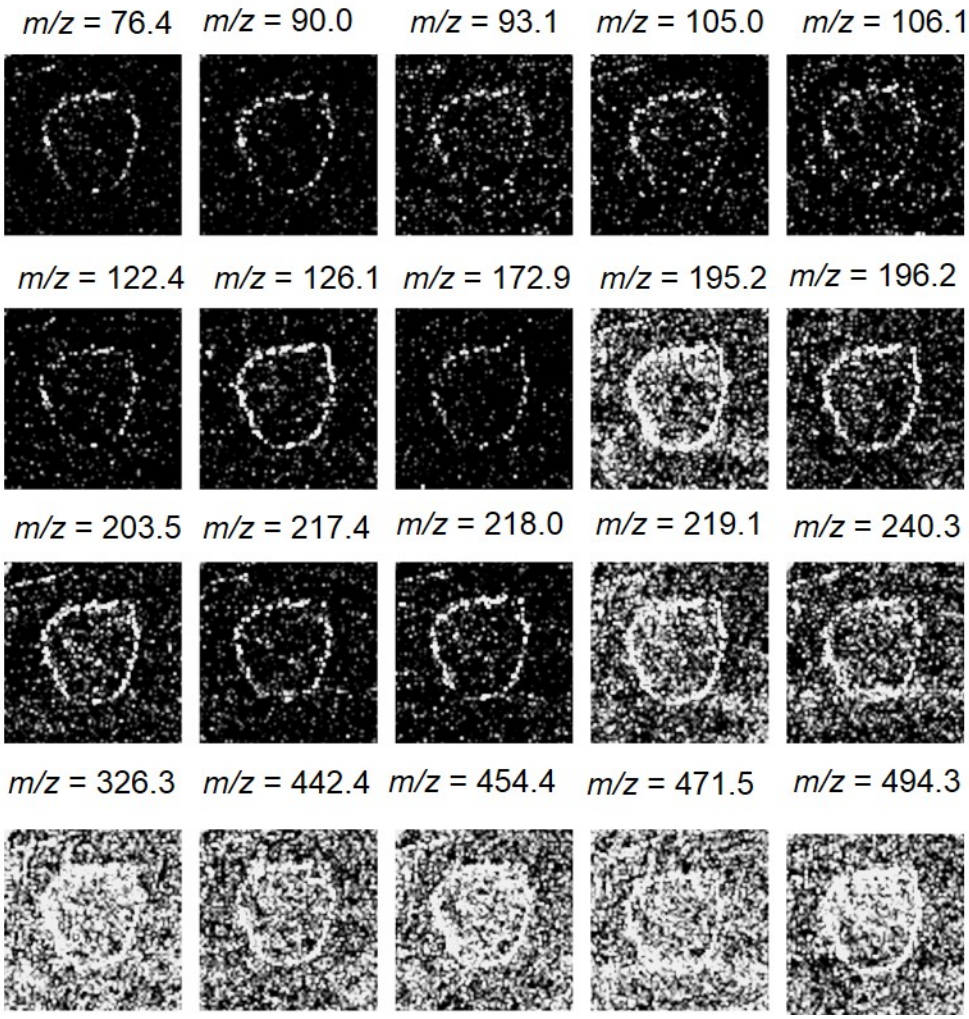


Figure 4-6. Low mass ions associated with the edge of the zone on null growth for *B. subtilis* as a result of exposure to actinomycin.

hypothesis that the sample is not robust enough for multiple rounds of imaging. As a consequence, the spatial resolution and mass range to be investigated were adjusted to facilitate acquisition of the greatest amount of information from a single sample to be imaged.

Laser source was compared for N_2 internal-laser and Nd:YAG external laser. Images were generated utilizing BIOMAP and revealed successful detection of the antibiotics, with spatial separation which correlates to the observed bioactivity, UV analysis, and chemical staining of ninhydrin (Figure 4-7). Optimization of compound detection for each laser source was determined by adjustment of parameters, such as, laser energy, shots / pixel, and image resolution. A significant consequence of employing an external laser for ionization was the number of shots / pixel which posed a significant

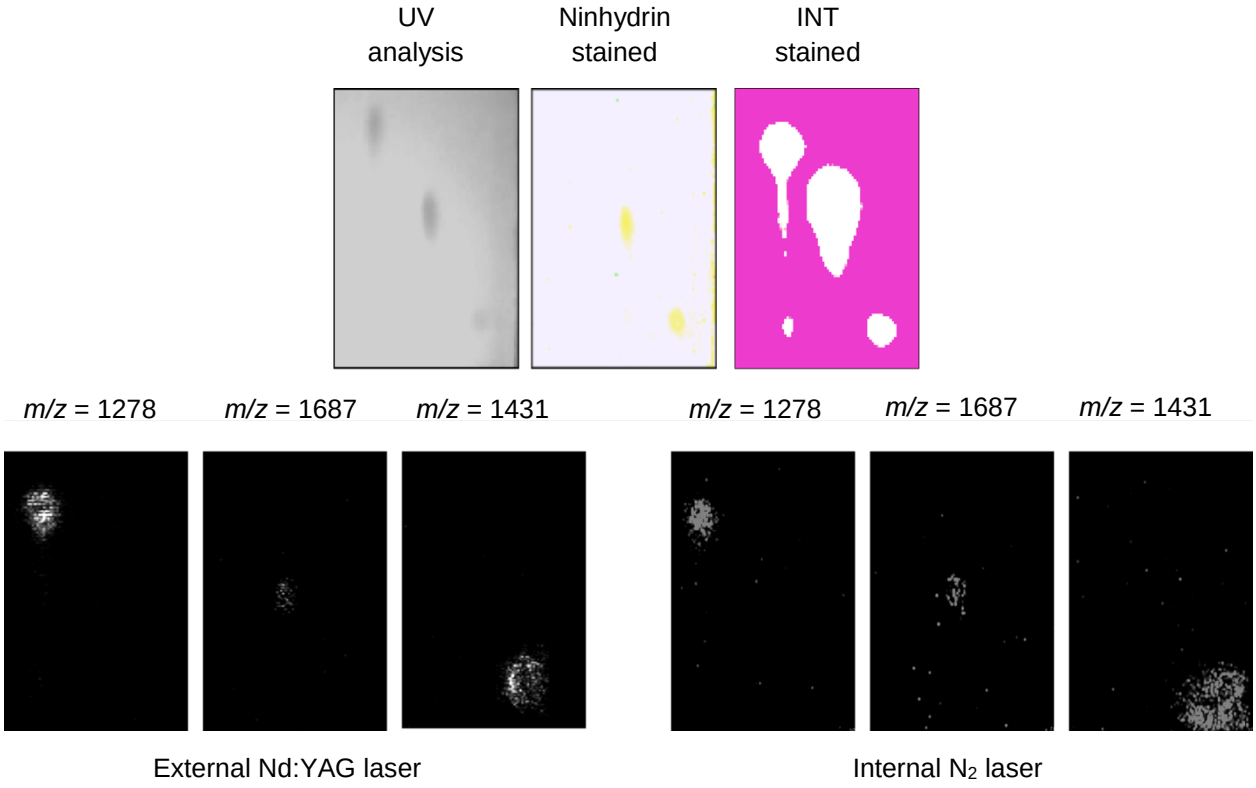


Figure 4-7. Comparison of laser type for ionization efficiency of known antibiotics separated by 2-D TLC. A) Visualization of antibiotic location via UV analysis and ninhydrin stain and zones of null growth as a result of the antibiotics via INT stain following incubation of planktonic indicator organism in agar. B and C) Detection of actinomycin (*m/z* = 1278), thiostrepton (*m/z* = 1687), and bacitracin (*m/z* = 1431) detected by external Nd:YAG and internal nitrogen laser, respectively.

destructive capacity for the focusing mirrors used to direct the laser beam to the sample stage. The same information could be acquired by employing an internal N_2 laser with half the shots / pixel, significantly reducing the amount of time required for imaging of a sample with approximately 4 cm x 4 cm dimensions. Given the large surface area to be imaged, significant consideration had to be given the file size of the data generated. While many of the developed IMS methods use a greater spatial resolution, the BIMS method was developed to investigate relatively large areas requiring less spatial resolution, a parameter that was adjusted to decrease imaging time and file size. As a consequence, images were obtained as a m/z range of 350 - 2000 Daltons with a spatial resolution of 200 x 250 µm resulting in data sets approximately 1GB is size / image.

The summary of BIMS methodology is shown in Figure 4-8 for the analysis of a mixture of three antibiotics with *B. subtilis* as an indicator stain. The mixture of compounds is pipetted onto the corner of a square SiO_2 TLC plate, run using a low polarity mobile phase, rotated 270 degrees and run in a relatively high polarity mobile phase containing naphthalene as a releasing agent. This separates and arrays the mixture according to two chromatographic dimensions. After evaporation of chromatographic solvent, agar containing medium at 60 °C is decanted onto the TLC plate. Incubation at 30 °C for 16 hours results in bacterial growth in the agar layer agar layer containing the assay organism, revealing patterns of antibiotic activity. Agar surmounted TLC plates are dried, the agar film is removed by carefully scrolling it off of the TLC plate and manual removal of residual SiO_2 prior to unscrolling the film onto double sided carbon tape affixed to a MALDI target. After mounting the sample, submicron particles of dihydroxybenzoic acid (DHB) were applied to the entire surface of the sample. Images were obtained using a

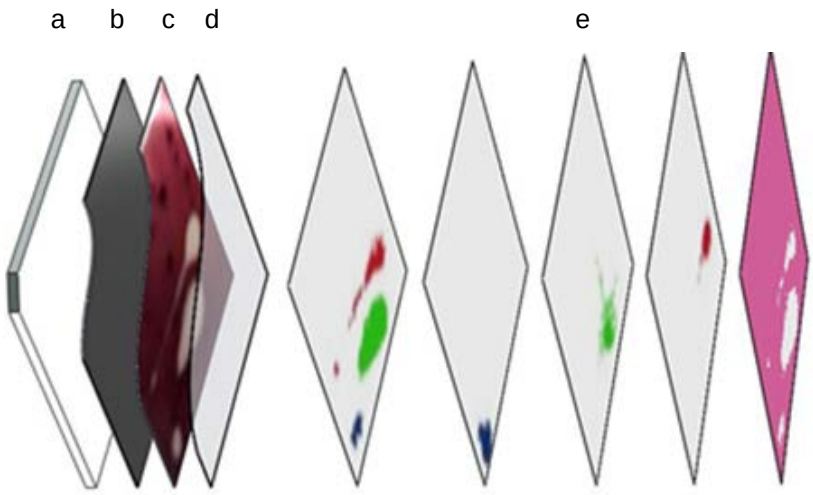


Figure 4-8. Data and physical layers of BIMS with examples of m/z images associated with, from left to right: e) unperturbed bacteria (m/z = 407.0); actinomycin peptide (m/z = 1278.8); thiostrepton inhibition zone feature(m/z = 766.8); bacitracin peptide (m/z = 1444.6), microbial mass ion common to all inhibition zones (m/z = 1061.9); d) MALDI matrix; c) dried agar layer with diffused compounds and target bacteria; b) conductive carbon tape, and **a)** stainless steel MALDI target.

resolution of 200 µm x 250 µm to generate a file size approximately 1GB and analyzed using BIOMAP software. The physical and data layers for BIMS analysis of the planktonic inverted sample are shown in Figure 4-8.

Analysis of BIMS data

The BIMS method can potentially reveal several classes of mass features of interest: a) signals associated with antibiotics in regions of diminished or null cell growth, and features associated with microbial metabolomic inventories including b) features associated with unperturbed bacteria, c) features associated with altered phenotypes (e.g. antibiosis) and d) features reflecting metabolomic perturbation of growing bacteria with no observable changes in growth phenotype (i.e., phenotypically cryptic). Manual investigation of the images generated over the mass range selected revealed ions

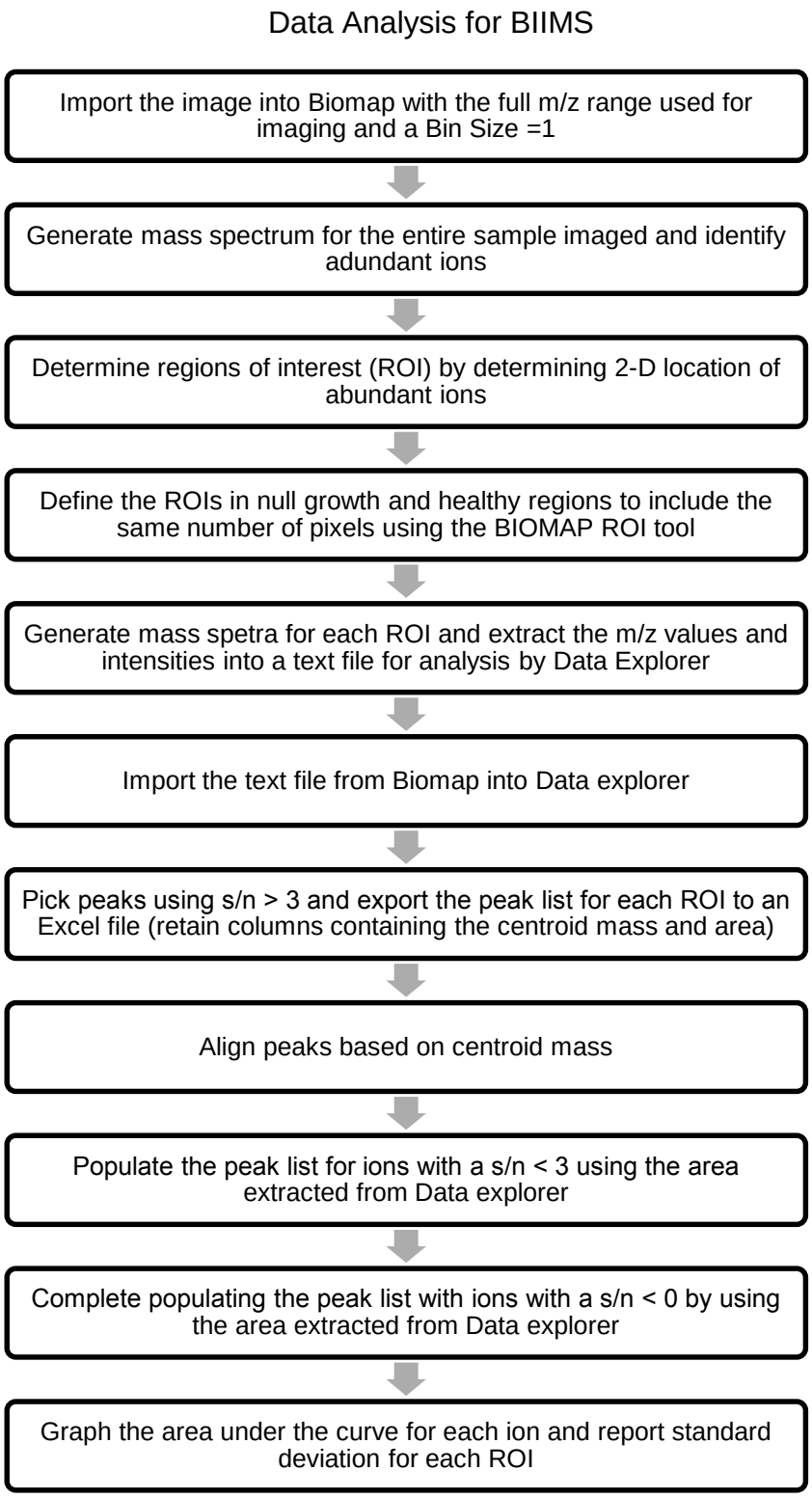


Figure 4.9. Workflow of data analysis for samples subjected to BIMS.

associated with each class. The identification of regions of interest, null growth, stressed growth, or unperturbed bacteria through the use of BIMS which correlated with the observed phenotypic responses facilitated the application of statistical analysis.

Comparison of the regions was performed by analysis of replicates from a user defined region of interest (ROI) utilizing Biomap ROI feature. The full workflow form compiling the data matrix for statistical analysis is outlined in Figure 4-9 and screen captions associated with the process are presented in Appendix B. Briefly, the images acquired were evaluated using BIOMAP software where ROIs were determined based on images that correlated to the observed phenotypic image provided by INT staining of a replicate sample. Once the ROIs were identified, the m/z spectrum were extracted for

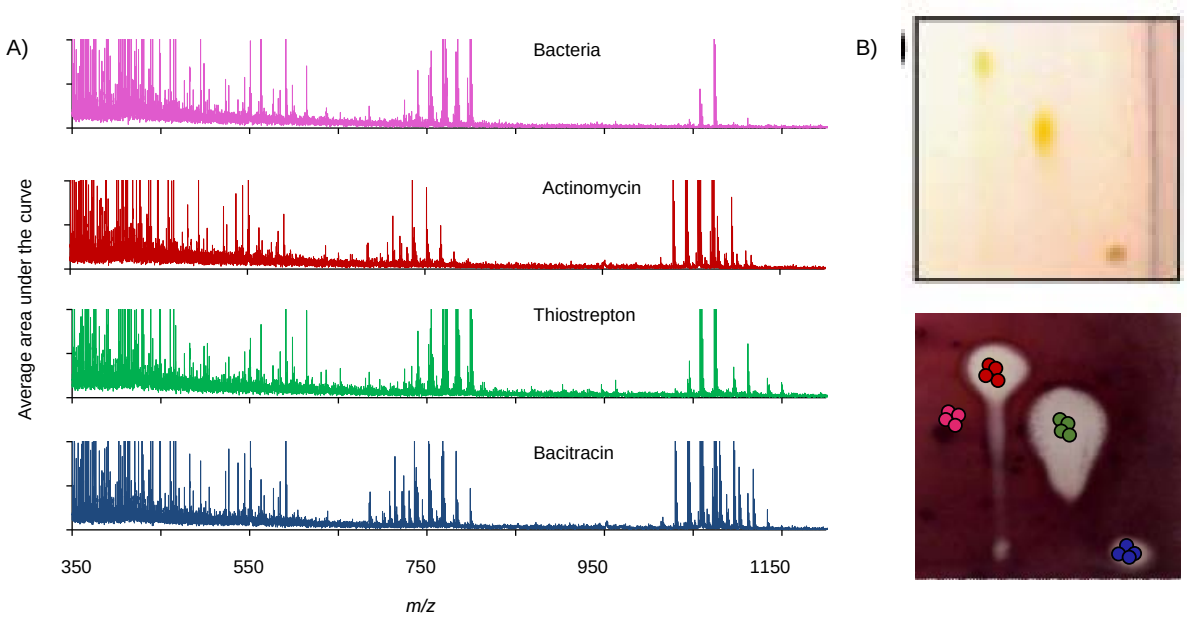


Figure 4-10. Evaluation of the metabolic profiles for each ROI within the biofilm antibiotics sample. A) Extracted mass spectra for each of the identified ROIs for the biofilm antibiotics sample. B) Separated antibiotics visualized by anisaldehyde staining (above) and INT staining to identify regions of unperturbed bacteria and null growth regions.

each ROI containing the same number of pixels. The spectrum were normalized to the TIC and imported into Data Explorer where the m/z values and area under the curve were extracted. Peak alignment was performed in Excel and the resulting data matrix was used for statistical analysis using EZinfo software.

The first sample evaluated was the biofilm sample in which the assay organism was applied directly to the surface of the agar layer once it had solidified. Initial investigation of the m/z spectrum associated with the identified ROIs revealed multiple metabolic alterations between the selected regions. The most obvious differences occur in the mass ranges 700-800 Daltons and 1000-1100 Daltons (Figure 4-10). A more comprehensive method of determining the metabolic alterations as a result of exposure to the bioactive compounds is the use of principal component analysis (PCA).

Evaluation of ion uniqueness was performed by comparison of the averaged area under the curve for each user defined region. Statistical analysis was used to identify the uniqueness of ions associated with the various ROIs which corresponded to antibiotics with differing modes of action. This type of analysis to simplify and rapidly identify ions of interest within the various ROIs can be achieved through the use of PCA. Investigation of the PCA separation (Figures 4-11, insert) reveals that replicate ROIs correspond to a specific quadrant associated with a specific phenotypic region. Further analysis of the data utilizing the loadings plot (Figure 4-11) which identifies ions associated with the separation in PCA space based on alignment with the vector of the samples in PCA space. Based on this analysis, of the loadings plot reveals ions with m/z = 799 associated with healthy bacteria, m/z = 1058 with shared distribution within the thiostrepton and actinomycin regions.

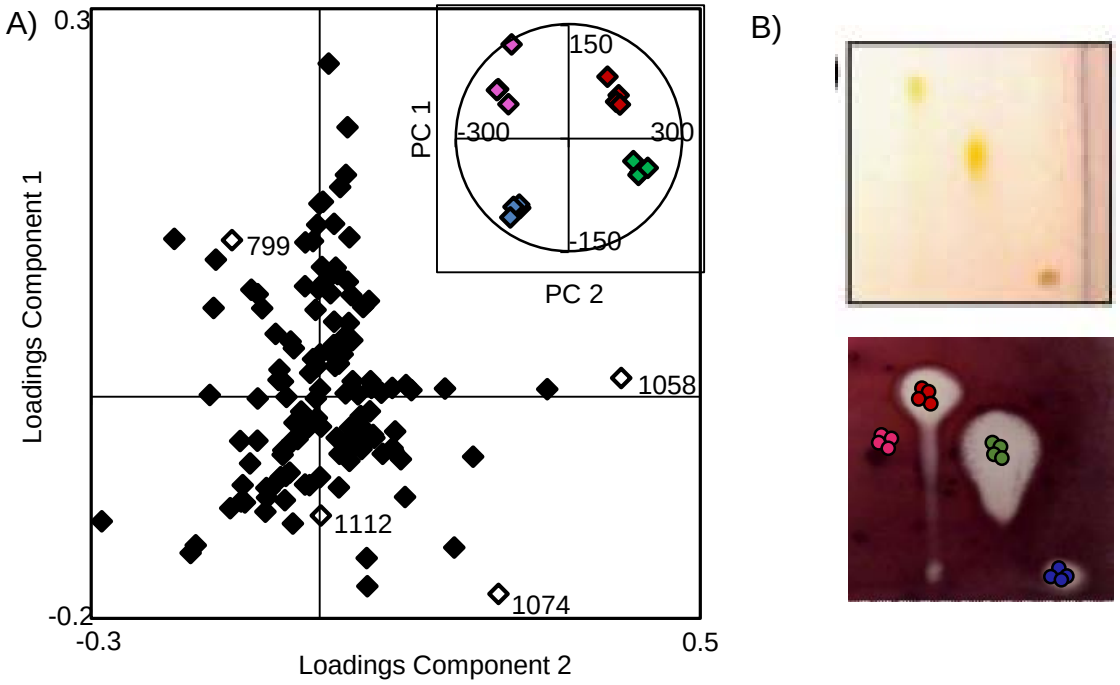


Figure 4-11. Statistical analysis of the biofilm antibiotics sample. A) Loadings plot for ions with s/n ration greater than 3 with selected ions associated with the various identified regions of interest. Principle components plot to identify clustering and location of regions of interest within principle component space (insert) for healthy bacteria (◆), actinomycin (◆), thiostrepton (◆), and bacitracin (◆). B) Separated antibiotics visualized by anisaldehyde staining (above) and INT staining to identify regions of unperturbed bacteria and null growth regions.

The identified uniqueness is supported by the corresponding extracted comparison of area under the curve for the selected ions identified in the loadings plot (Figure 4-12). This data supports the conclusion that *m/z* = 799 is a unique feature associated with healthy bacteria, while *m/z* = 1058, 1074 and 1112 are associated with regions in which the chosen antibiotics are present. Image analysis can potentially also reveal features that are associated with only one antibiotic or antibiotic class and these features have potential use as markers of mode of action or starting points for target identification via reverse genetics.

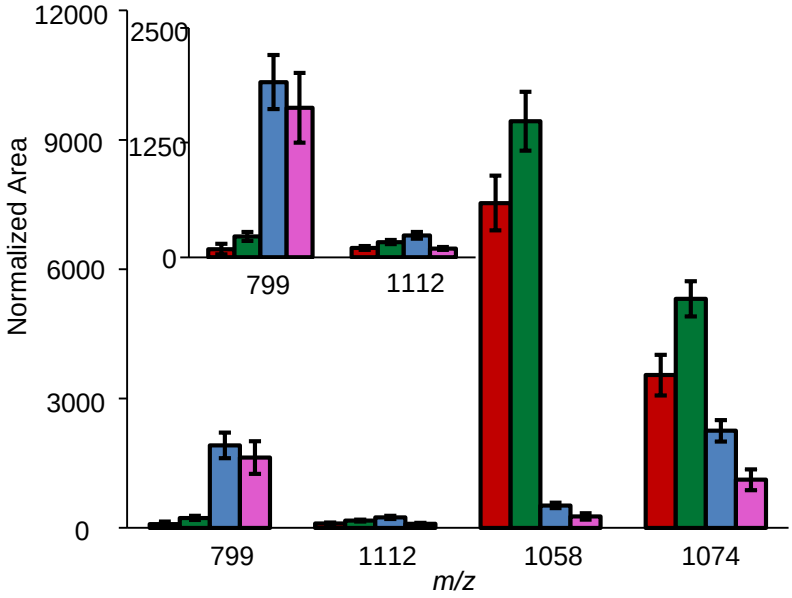


Figure 4-12. Graphical comparison of ions identified via PCA. The average area under the curve for replicate samples within each identified ROI for each ion with error bars to represent the standard deviation among the samples for healthy bacteria (◆), actinomycin (◆), thiostrepton (◆), and bacitracin (◆), with enlarged view of *m/z* 799 and 1112.

A more effective statistical approach to identify the metabolic impact on the assay organism is the comparison of the ROIs associated with the unperturbed bacteria to those associated with the antibiotics chosen for this study using orthogonal partial least squares discriminant analysis (OPLS-DA). This statistical approach facilitates the identification of ions in a supervised method which orients principal component one to describe the greatest differences between two defined experimental groups (*i.e.,* ROIs), and principal component two to be orthogonal to principal component one and describe the greatest intragroup variation. These resulting plots represent features as a function of both ROI correlation (ordinate) and intensity, or loadings contribution (abscissa). Outliers in quadrants I and III correspond, in this instance, to features associated with unperturbed bacteria vs. null-growth regions as a result of antibiotic exposure, respectively. Application of OPLS-DA to the biofilm dataset revealed the ions most responsible for the

metabolic differences between the null growth regions and unperturbed bacteria had a *m/z* = 1057.8 and 1073.7 (Figure 4-13). These values correspond to those reported as surfactin, a lipopeptide produced by B*acillus subtillus*[46], the assay organism used in this analysis. Surfactin (Figure 4-14) has been implicated in motility of the bacterium by lowering the surface tension of substrates allowing for rapid cell growth and colony expansion.[47] Recent IMS experiments have shown that surfactin is excreted by bacterial colonies and diffused into the medium. Consistent with the observation that surfactin is excreted by the bacteria to facilitate expansion of bacterial colonies; a uniform surfactin response to the zones of inhibition for the 2 largest regions, while no signal was observed for the smallest null growth region, though it could be observed through the use of OPLS-DA. The origin of these *m/z* features may be a result of bacterial attempts to overcome toxicity, increased bacterial growth as a result of diffusion of nutrients not consumed in the null growth regions, or cellular debris or metabolism from antibiosis as a result of

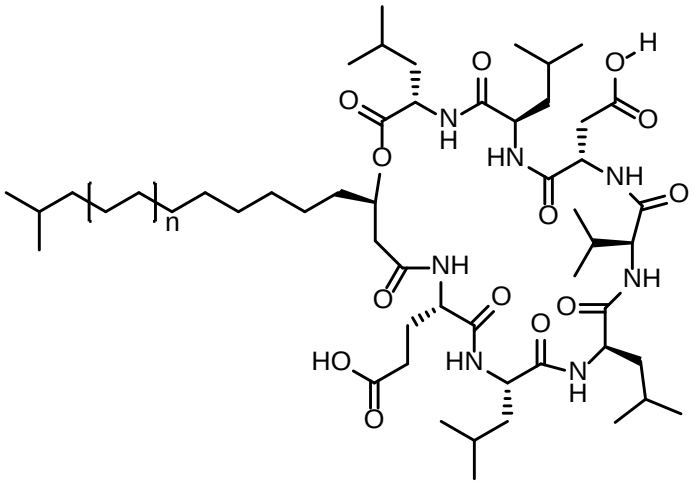


Figure 4-13. Chemical structure for surfactin where the acyl chain has a variable length.

diffusion of bioactive compounds into areas where healthy bacteria were established.

The full utility of BIMS lies in the detection of microbial phenotypes based on metabolomic inventories. Mass features that recapitulate a typical bioautography image with ions associated with bacteria growing in unperturbed regions are shown for *m/z* = 366.6, 388.7, and 406.5 and are the analytical equivalent of cell staining reagents, for example, iodonitrotetrazolium (INT) violet for bacteria (Figure 4-15). However mass imaging offers a range of masses to use as indicators to visualize cell metabolism beyond live/dead phenotypes. Mass feature maps associated with bacterial null growth

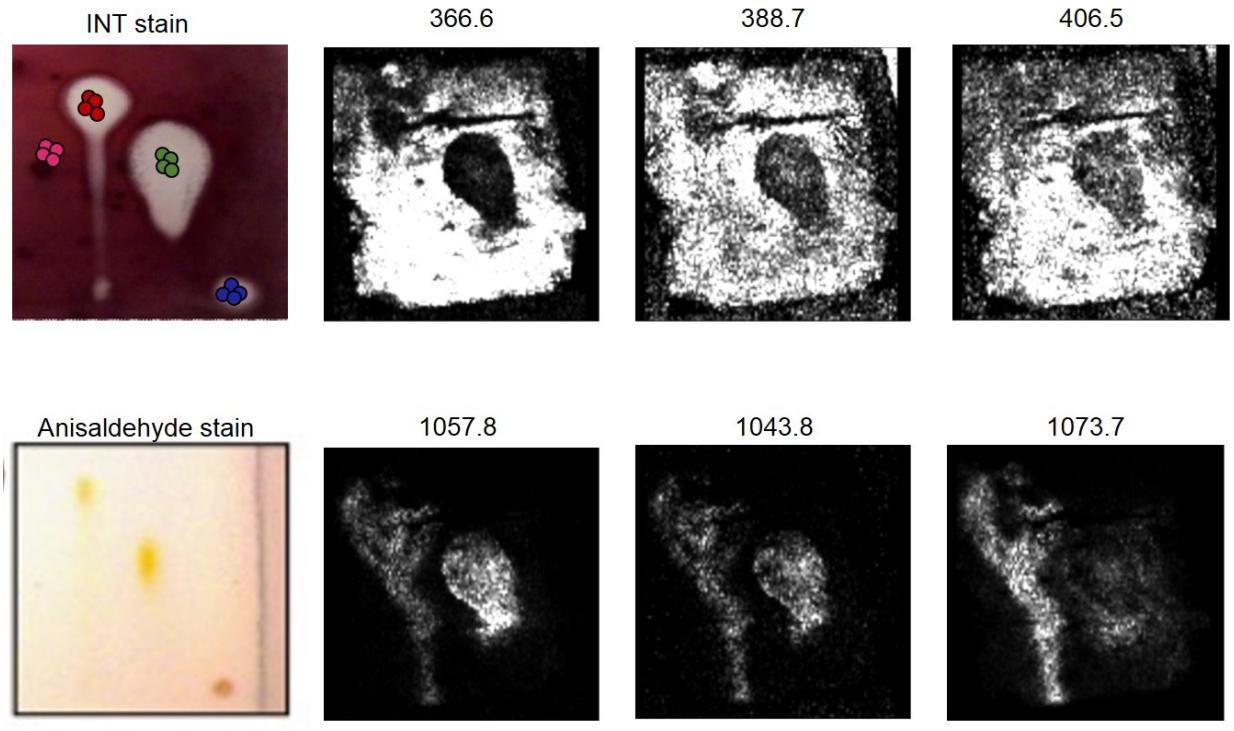


Figure 4-14. Images for selected ions from the biofilm normal antibiotics sample identified as unique to each of the ROIs which correspond to the separated antibiotics visualized via bioautography (top left) and anisaldehyde staining (bottom left).

phenotypes that are not antibiotics, could be the result of unconsumed media components. This is unlikely as the identified ions are not present at significant quantities in the control sample. These features are identifiable by their relatively uniform distributions in the null growth region (*m/z* = 1057.8, 1043.8, and 1073.3, Figure 4-15) and ostensibly represent features associated with bacterial cell death, (i.e. bacterial "death screams"), or otherwise represent an aged snapshot of the mass feature inventory specific to the indicator bacteria that were unable to grow. The images associated with null growth are candidates for either antibiotics or metabolomic perturbations that are a function of antibiosis. For example, potential origins of signal may be the result of cell depolarization and lysis, proteolysis, undigested media components, cell wall components of stationary phase indicator bacterium prior to inoculation, metabolic responses to overcome toxicity, or modification of the bioactive compound to decrease toxicity. Feature origins can be partially inferred by comparison to features from other null growth regions. Features that are present in all null growth regions are likely derived from these general m/z features of antibiosis. A final class of features identified via m/z image our initial data set comprises those associated with metabolic perturbations of bacteria not otherwise demonstrating an altered growth phenotype. A compiled set of images for the biofilm antibiotic sample can be seen in Figure 4-16.

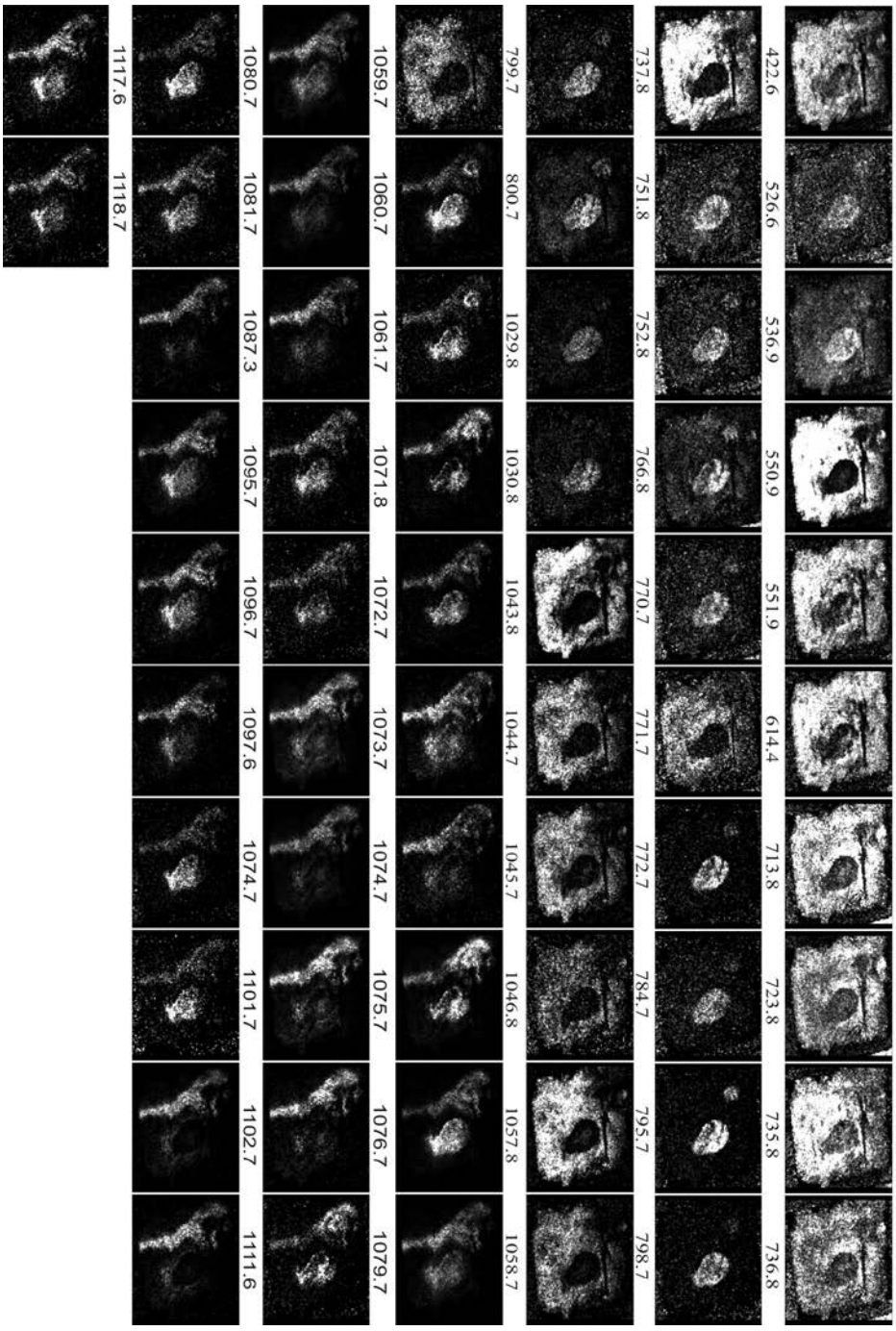


Figure 4-15. Images for ions from the biofilm normal antibiotics sample.

Applying the same statistical analysis to the normal planktonic antibiotic sample revealed ion associated with unperturbed bacteria, null growth region, and a new phenotype in which compounds were detected at the edge of the null growth region with m/z values observed in the biofilm sample and presumed to be surfactin compounds. Additionally, the application of OPLS-DA to the sample revealed a number of low molecular weight ions that would have been difficult to observe through manual comparison of the extract mass spectra for each ROI (Figure 4-17). Unlike the biofilm

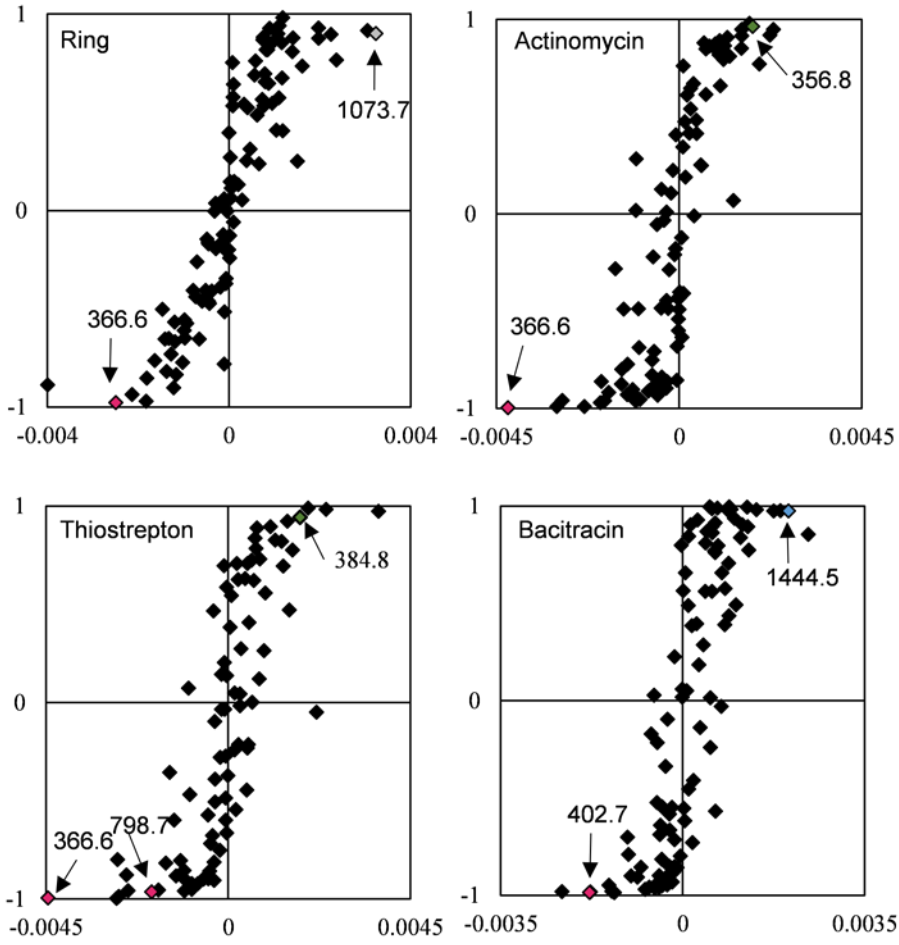


Figure 4-16. S-plot generated as a result of OPLS-DA comparison of unperturbed bacteria and null growth regions within the planktonic normal sample.

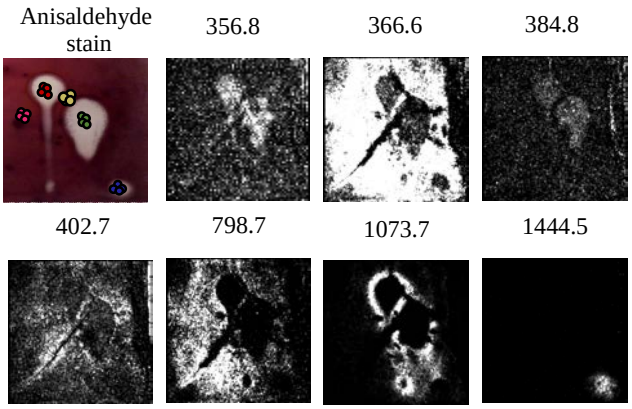


Figure 4-17. INT stained sample with ROIS identified for statistical analysis and selected images for ions identified by OPLS-DA analysis of the planktonic normal antibiotic sample.

sample, in addition to a null growth region observed for bacitracin, a number of ions observed during analysis of the control sample associated with the parent compound were observed, most notably the ion with m/z = 1444.5 (Figure 4-18). Extraction of the corresponding images from Biomap are compiled in Figure 4-19, while the selected ions associated with the metabolic alterations identified through the use OPLS-DA can be seen Figure 4-18. Further investigation of the data revealed a number of ions in both the biofilm and planktonic normal samples, many of which were putatively identified as surfactins. Investigation of the planktonic inverted sample revealed an entirely new set of images (Figure 4-20), some of which are not recapitulated in either the biofilm or normal planktonic samples. Furthermore, the high intensity ions associated with surfactins were not observed. With the combined data sets and images for each of the possible sample preparations, is may be possible to build a library of ions associated with bioactive compounds and identify potential differences as a result of varying modes of action.

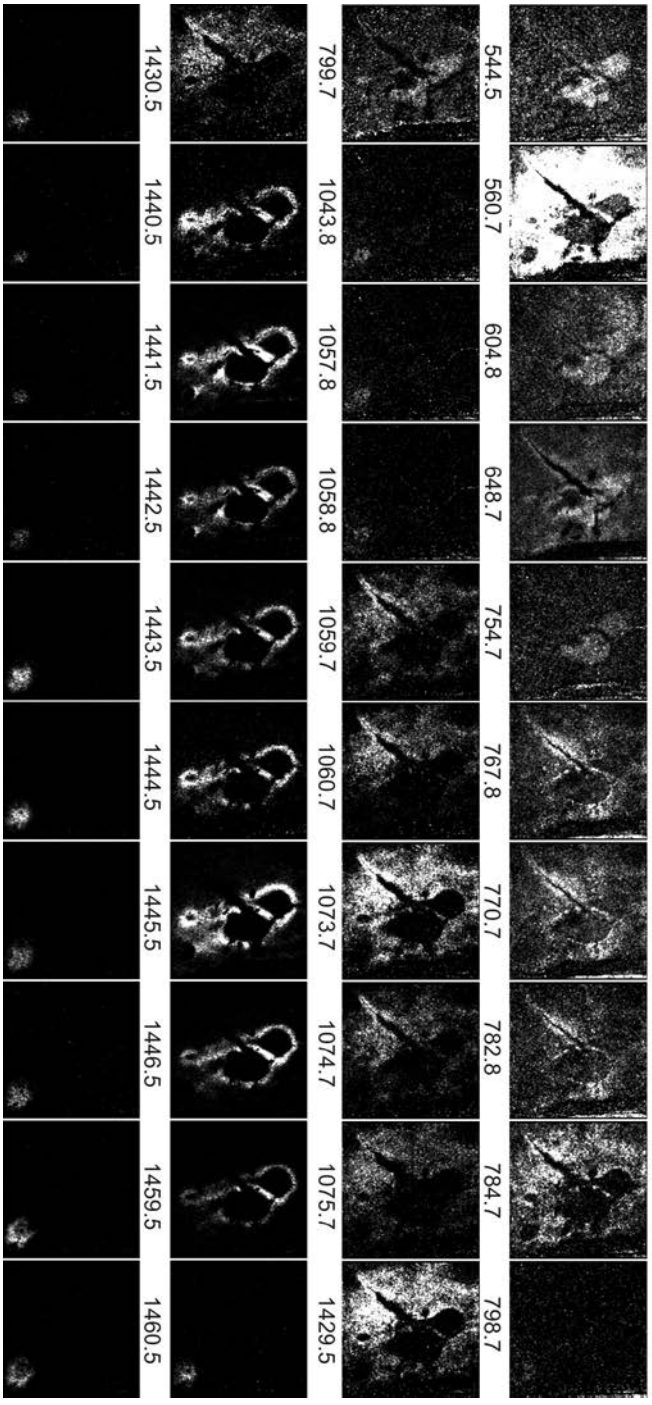


Figure 4-18. Images for selected ions from the planktonic normal antibiotics sample.

Figure 4-19. Images for selected ions from the planktonic inverted antibiotics sample.

Applying BIMS to the Analysis of a Complex Extract

Identification of compounds of interest for priority isolation within a complex extract remains a difficult task for discovery of new chemical entities. Extracts from biological sources, the primary sources for discovery of biologically active compounds, may contain several thousand compounds, both primary and secondary metabolites. Adapting BIMS methodology for determination of lead compounds for isolation required the use of a more robust microbial indicator. The use of *E.coli* as the assay organism allowed the application of higher concentrations of a crude extract containing biologically active secondary metabolites. The extract chosen for method development of analysis of a microbial extract was known to contain a variety of baumycin-like compounds many of which are active against *E. coli*. Due to the complex nature of crude extracts, multiple ROIs were identified and evaluated for ion uniqueness of both bioactive and non-bioactive regions within the sample as determined by the INT stained sample (Figure 4-21).

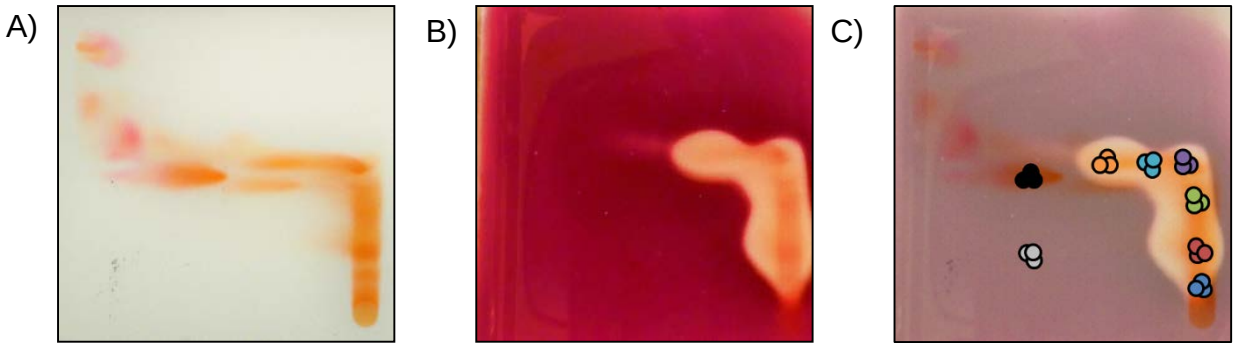


Figure 4-20. BIMS analysis of a crude extract. A) Visualization of chromatographically separated crude extract via 2-D TLC. B) Bioautography analysis with INT stain to identify regions of null growth. C) Overlay of INT stained image and visual image with identified regions of interest which contain bioactive and non-bioactive compounds.

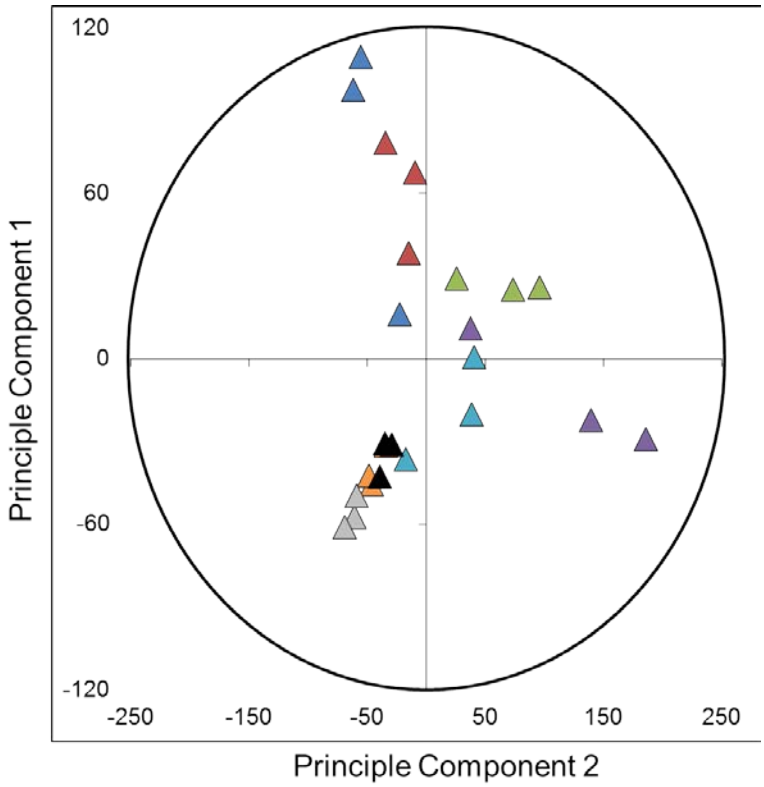


Figure 4-21. Principle component analysis in triplicate of the various ROIs identified in the planktonic inverted sample after exposure of E. coli to the chromatographically separated actinomycete crude extract.

Application of PCA to the analysis of the selected ROIs resulted in overlap of several selected regions to be interrogated (Figure 4-22), making evaluation by loadings component a difficult task. Due to the complex nature of crude extracts, PCA does not adequately differentiate metabolic differences contained within the ROIs in two-dimensional space. To address this limitation of PCA, ROIs were evaluated by orthogonal partial least squares-discriminant analysis (OPLS-DA). The application of this approach is illustrated in Figure 4-23, where user identified ROIs were compared to healthy bacteria molecular profiles to determine the ions corresponding to the unperturbed and null growth regions observed in bioautography.

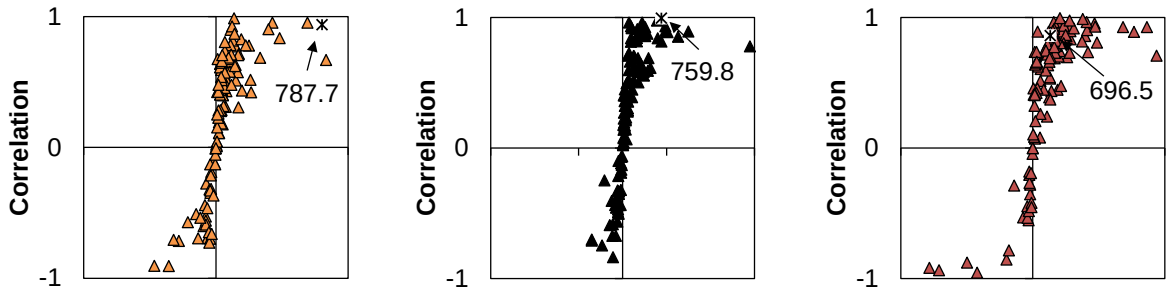


Figure 4-22. S-plots of various ROIs when compared to unperturbed bacteria for the crude extract planktonic inverted sample. (787.7, 759.8, and 696.5)

Abundant and non-abundant ions were identified for each of the selected ROIs when compared to the unperturbed bacteria within the crude extract planktonic inverted sample which can be compared to the bioautography analysis of the crude extract (Figure 4-2). This analysis facilitated the detection of ions specific to a region which corresponds to observed chromatographically separated chemical entities. Comparison of the non-bioactive region of interest revealed an ion with *m/z* = 787.75 as having the greatest intensity and uniqueness versus the region analyzed as representative of healthy bacteria. The same type of comparison for the bioactive region revealed a relatively abundant and unique ion with *m/z* = 815.88. The application of OPLS-DA can also be instrumental in determining ion uniqueness for a region of the image populated by non-abundant compounds, as seen for evaluation of a bio-active region known to contain baumycin, with an observed *m/z* = 696.472, which corresponds to the sodiated adduct. Analysis of this crude extract through the use of BIMS shows the versatility of the method to not only identify microbial responses to compounds with differing mechanisms of

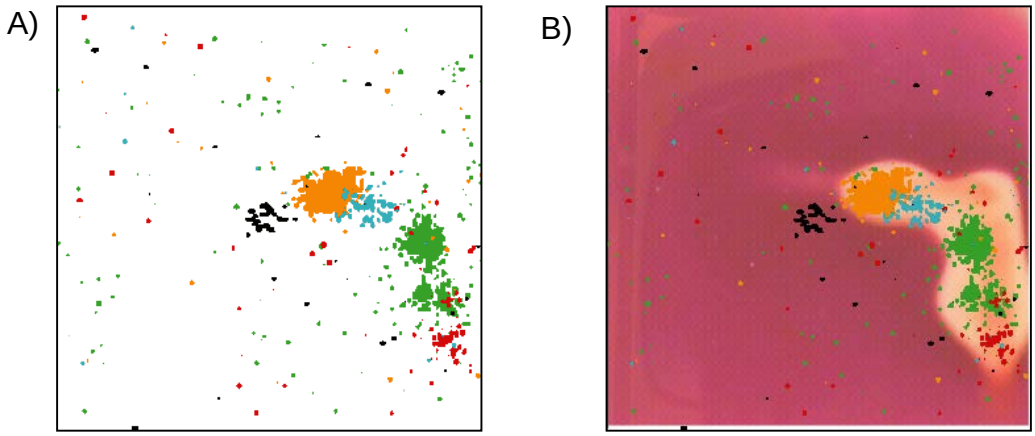


Figure 4-23. A) Selected images for ions identified as being unique to the ROIs investigated when compared to unperturbed bacteria in the crude extract planktonic inverted sample. B) Overlay of the INT stained sample and an ion for each ROI identified within the crude extract planktonic inverted sample.

action, but also to guide the isolation process for bioactive and non-bioactive compounds.

Isolation attempts for each of the ROIs was performed resulting in the isolation and structure elucidation of baumycin and a less abundant compound identified as an unreported analogue (Figure 4-24). The structure of each of the compounds was determined through the use of various NMR techniques (Appendix E, Figure E1-E6). With the assembled contiguous fragments generated from the COSY analysis and the determination of hydrogen – carbon connectivity identified by the HSQC facilitated the assemblage of potential chemical structures. Combining the NMR data with the MS and UV data revealed the baumycin compound as a previously reported compound. However, the less abundant compound was determine to be an unreported chemical entity. Following the identification of the compounds, the purified compounds were used

to confirm 2-D TLC compound locations and bioactivity as observed during the sample preparation for BIMS analysis. A compilation of the images associated with the crude extract planktonic normal sample can be seen in Figure 4-25.

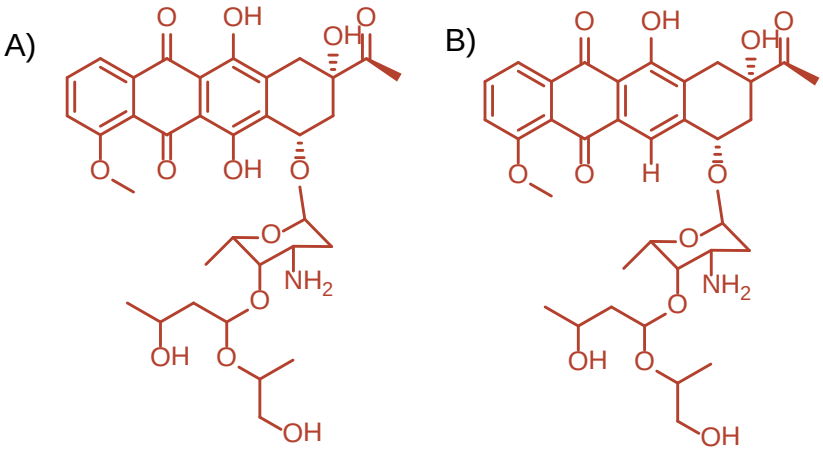


Figure 4-24. Chemical structure of baumycin (A) and previously unreported analogue (B) identified and isolated as a result of BIMS analysis.

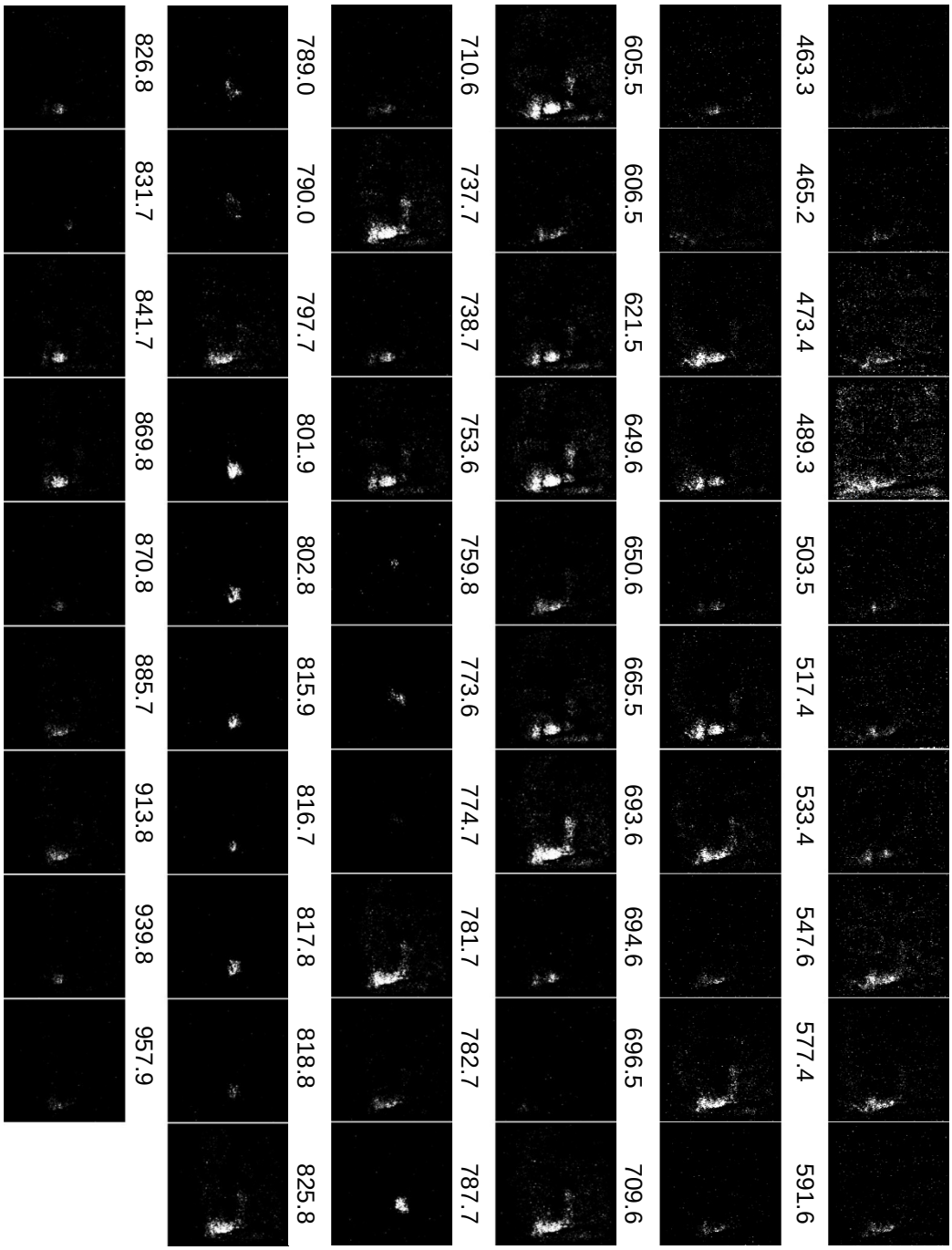


Figure 4-25. Images for selected ions from the planktonic inverted crude extract sample.

Discussion

The versatility of the current method proposed in this work is clearly demonstrated by both the detection of bacterial metabolic responses and the isolation and identification of compounds within a complex mixture. The detection of compounds corresponding to bacterial metabolic responses that are elicited *in situ* when *B. subtillis* is used as the indicator organism has potential applications well beyond secondary metabolite discovery, particularly in the areas of chemical mediators of metabolism, mode of action determination, and target identification within all kingdoms of life. BIMS analysis of a crude extract generated from a cave actinomycete revealed the presence of several bioactive and non-bioactive compounds, culminating in the isolation and identification of several compounds within complex mixtures when the more robust indicator organism *E. coli* is used as the indicator organism. There are currently no reports of imaging MS for *E. coli*, in which analysis of intact cells results in the visualization of healthy bacteria. An increase in sensitivity could result in the detection of ions associated with healthy *E. coli* and reveal any unique ions associated with exposure to anti-infectives in a manner similar to the surfactin ions observed for *B. subtilis* via BIMS analysis.

Expanding on the current study could potentially lead to identification of ion profiles which would have the potential to rapidly determine a new chemical entities mode of action against pathogens. Identification of microbes based on their chemical profile has already been achieved via MALDI analysis, with particular interest in the identification of wild-type organisms when compared to those that have become resistant to current antibiotic therapies.[48] Expanding the BIMS method to analysis of the metabolic profile for microbes which have developed resistance could lead to the

understanding of how resistance arises and potentially lead to new methods to combat the pathogenic microbes.

Materials and Method

TLC analysis

Antibiotics were mixed at a concentration of 1 mg / mL actinomycin, 5 mg / mL thiostrepton, and 10 mg / mL bacitractin in a mixture of 50:50 methanol:dichloromethane. For all samples, the specified amount of antibiotic mixture was spotted in the lower left corner of a TLC plate cut to 8cmX8cm. The length of the run was determined by marking the TLC plate at a distance equal to the length of the MALDI target from solvent front to 50mm from the center to the spot. The sample was then eluted with the first mobile phase 90:10 dichloromethane:methanol. Samples were removed and allowed to thoroughly dry then rotated 270° and run with the second mobile phase consisting of 90:10 ethyl acetate:methanol with 50 mg/mL naphthalene. The TLC plates were cut the 4.4cm x 4.4cm, the size of the MALDI target used in this experiment, and stained or used for bioactivity after evaluation using UV detection. The ninhydrin stained sample was run with 2uL for better staining, 1uL was used for bioautography.

Bioautography imaging assay

Bacillus subtilis was grown from a frozen stock in 5 mL nutrient broth to an OD greater the 0.7 at 30 °C with 170rpm. Samples were run in duplicate with 5 % inoculum with 0.5 % nutrient agar and 0.5 % glycerol. Samples were incubated for 15hours at 30 °C. Samples were chose based on the observable zones of inhibition and taken for further

testing. INT stain was used at a concentration of 3mg/mL in a mixture of 80:20 water:ethanol and pipetting on to the agar until a uniform pick color was observed. Samples used for imaging were aired dried in the hood for 6 hours. A razor was used to trim 2 mm from the edge of the plate and the agar and silica gently peeled off the aluminum backing. The agar layer was gently removed from the silica layer to leave a tissue thin sample for mounting. The sample was mounted on double sided conductive tape that covers the entire target for imaging. Excess tape was trimmed away from the sample and crushed DHB was painted on the entire surface.

Imaging

MALDI-IMS was done using an Applied Biosystems Voyager DE-STR using a 337 nm nitrogen laser operated at 20 Hz and controlled by MMSIT software. 2,5-Dihydroxybenzoic acid (DHB) was crushed through ball milling using ceramic ball bearings to submicron particles and applied using a dry paint brush to the entire surface. The TOF was calibrated using angiotensin II, DHB matrix cluster, and bradykinin applied to a control agar sample after drying and mounting to the MALDI target using double sided carbon tape. Images were collected with 7 shots per pixel at a resolution of 200 x 250µm to generate a complete imaging file approximately 1GB in size within 4 hours.

BIMS data analysis

Following the identification of regions corresponding to the various null growth regions, data were extracted for further processing in Data Explorer. The numbers of pixels contained within the user defined circle for ROI evaluation were 25 pixels for the known

antibiotic analysis and 15 pixels for the unknown bioactive compounds within the crude extract. The extracted mass spectra was normalized to the TIC in Excel and imported into data explorer. Ions of interest were identified as having a s/n > 3, and the complete data set was fully populated by extraction of all ions with a s/n > 0 for each region of interest. The ion intensity, area under the curve for each ion, and *m/z* value associated with the ions were compiled followed by peak alignment (less than 5 % standard deviation was used as the cutoff for peak alignment). This data was then evaluated to determine ion uniqueness based on the area under the curve for each ion through the use of graphical comparison and principle component analysis (PCA).

Multivariate Statistical Analysis

Data analysis was performed by importing the peak-picked, aligned, and normalized data generated using Excel into EZinfo (Umetrics, Umeå, Sweden). Principal component analysis of the known antibiotic samples resulted in clustering of the replicate ROIs and identification of ions associated with each known compound. Supervised MVSA methods were applied to the analysis the crude extract. Each ROI was compared to a region of equal size associated with unperturbed bacteria to determine the greatest difference between the two groups.S-plots were utilized for OPLS-DA loadings interpretation, leading to the identification of both abundant and unique of ions of interest.

Validation of the ions observed for antibiotics

Validation of the ions detected for the known compounds was performed by completing the 2-D TLC separation and bioautography. Regions of bioactivity corresponding to each

antibiotic used were excised for the TLC plate and extracted with 100 uL of methanol. The supernatant was removed and dried to vacuum. The samples were suspended in H_2O with 0.1 % formic acid and analyzed Acquired on Synapt G2 HDMS (Water, Milford, MA) with nanoAcquity UPLC system with autosampler, with instrument parameters of resolution mode, capillary voltage 3.5 kV, source temp 120 °C, sampling cone 5.0, source gas flow 400mL/min, desolvation temp 400 °C, helium cell flow 180 mL / min, IM gas flow 90 mL / min, TWIMS wave height 40 V, TWIMS wave velocity 650 m / s, mass range 50-1700 Da, continuum mode, acquired with MSE with an energy ramp from 10-45 eV, acquired with leucine enkephalin lock spray two point correction on the fly, calibrated to less than 0.1 mDa using sodium formate salt clusters. Chromatographic separation was achieved by gradient flow from H2O with 0.1 % formic acid (A) to ACN with 0.1 % formic acid (B) with a flow rate of 85uL/min. The gradient ran 100 % A for the first minute, ramped to 100 % B over the next 11 min, and held and 100 % B for 2 min

Identification of Bioactive compounds within a crude extract

The actinomycete isolated from a local cave using the microbial trap method was grown from cryogenic samples on ISP2 agar (4 g/L yeast extract, 10 g/L malt extract, 4 g/L dextrose, and 5 g/L agar) plates for one week. A single colony was selected and grown in 50 mL ISP2 media for seed culture. A 25 mL aliquot of seed culture was added to 500 mL of media consisting of 15 g/L soybean powder, 10 g/L glucose, 10 g/L soluble starch, 3 g/L sodium chloride, 1 g/L potassium dibasic phosphate and 2 mL/L of a trace element solution containing 1 g/L ferrous sulfate heptahydrate, 8 g/L manganese chloride heptahydrate, 7 g/L copper sulfate hexahydrate, 2 g/L zinc sulfate heptahydrate. The

culture was shaken at 170 rpm and 30 °C for 7 days. Active HP 20 resin stored in water was added to each flask at a concentration of 200 mL/L and shaken for 3 hours. The biomass and resin were separated from the broth by centrifugation and extracted with equal volumes of methanol while shaking for 2 hours. The biomass and resin were separated and extracted again with equal volumes of acetone. The methanol and acetone supernatant were dried separately. Each solvent extract was evaluated for bioactive compounds using the 2D TLC method. The methanol extract was fractionated using LH 20 (hydroxypropylated cross-linked dextran, prepared by hydroxypropylation of sephadexG-25;GE Health Care Cat#17-0090-01) resin. Fractions containing compounds of interest determined by BIMS were combined after evaluation using TLC with UV and anisaldehyde stain. Pure compounds were obtained by preparative RP-HPLC. Structure was determined using 2D NMR.

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CHAPTER 5

SUMMARY AND FUTURE WORKS

The use of chemical perturbations has revealed a link between fatty acid synthesis and polyketide synthesis as both metabolic pathways rely on the precursors acetyl-CoA and malonyl-CoA.[1] Evaluation of the impact of a variety of antibiotics on the gene expression of the model pathogenic microbe *Burkholderia thailandensis* revealed that at sub-inhibitory concentrations many antibiotics have the ability to influence transcriptional effects rather than growth and survivability.[2] While many studies have focused on the metabolic responses using a variety of model species, there is little information regarding the maximum genomic dereplication through the use of chemical additives to less than ideal microorganism capable of producing secondary metabolites, such as, *A. hypotensionis*. Evaluation of the metabolic impact as a result of specific chemical perturbations revealed a variety of complex metabolic responses determined by the use of LC-MS and PCA. Expanding on the developed analytical and statistical analysis method for the development of a high through-put method to influence the maximum number of secondary metabolite genes could lead to the development of new chemical entities to address the growing concern of drug resistant pathogens.

A more complicated evaluation of chemical perturbations, is the use of co-culture between a variety of microbes and the producing organism. The induction of secondary metabolism as a result of co-culture is hypothesized to be the result of a stressor response

in which the producing organism attempts to inhibit the growth of the competing organism.[3] Evaluation of the metabolic impact of *S. coelicolor* revealed significant differences in the production of a variety of known secondary metabolites, including the pigmented molecules actinorhodin and undecylprodigiosin. Applying the same method to a microorganism known to produce the secondary metabolite apoptolidine, revealed that only one of the competing organisms was capable of inducing expression of the gene cluster responsible for apoptolidin production. Evaluating the metabolic impact of the chosen competing organisms with unknown streptomycete revealed a number of metabolic differences when evaluated by LC-MS and PCA. Multiple ions were observed to be present at increased relative concentrations, additionally, detection of new compounds presumed to be secondary metabolites not produced in mono-culture were also observed. While the current method is capable of detecting metabolic differences resulting from exposure to a competing organism, the amount of extract generated from each culture is non-conducive to isolation of purified compounds of significant quantity.

The technique of IMS has been applied to a variety of scientific fields over the last decade, including plant tissue[4], mammalian tissues[5], and microorganisms.[6] Imaging of mammalian tissues make up the bulk of the application, with 68% of the literature published since 2006, while microbes represent only 5% of the literature published over the same time period.[7] With the combination of 2-D TLC, bioautography, and IMS, the development of BIMS methodology facilitated the determination of compounds within a mixture and the metabolic impacts caused by the bioactive compounds. A variety of ions were observed using each of the sample preparation methods identified in Figure 4-5, some of which showed intensity differences for each of the antibiotics which act with

differing modes of action. While the method of combining imaging mass spectrometry and bioautography has successfully been developed, the current method appears to be at the limit of detection for driving the discovery of natural products. Increased sensitivity would result in an increase in the number of ions detected per region of interest.

The increase of pathogens resistant to current medicinal treatments has resulted in the drive to discover and develop new chemical entities capable of safeguarding the population against pathogens that were once thought to be eradicated or of no consequence. This type of analysis could lead to the detection of novel microbial metabolic responses capable of deactivating the bioactive molecule and provide insight into how to overcome resistance. Given the ability of the developed BIMS method to identify compounds and the metabolic responses to exposure of those compounds, this method could further be evolved to interrogate the mode of action (MOA) of various bioactive compounds and how the microorganism being investigated adapts to exposure of chemical entities. Metabolic analysis has the potential to identify the chemical responses capable of influencing resistance to cytotoxic compounds. Furthermore, comparison of the metabolic output for pathogens which are and are not resistant to antibiotics could provide insight into how pathogens develop resistant. If the process by which resistance is developed can be identified, there is the potential of extending the clinical relevance of antibiotics by inhibiting the process of resistance development. The generation of a library of ions produced as a response to a bioactive compound and the chemical classification of that ion within the same analytical experiment would significantly improve not only the rate of isolation of novel chemical entity vs the number of lead compounds detected, but also allow for the identification of compounds capable

of producing a previously unreported response. The application of ion mobility mass spectrometry (IMMS) has the ability to extend the analytical capacity of imaging mass spectrometry by identifying the biological class of molecule for the detected ions based on their drift time.

Following the development of a method capable of generating a library of ions and their biological class, the method could be applied to compounds with known MOA to determine unique bacterial responses for each mode of action. Expanding on this concept, the investigation of metabolic responses for isolated compounds within microbial extracts could lead to the isolation of compounds with currently unknown MOA. The same application could be applied to synthetic chemical scaffolds to identify potential starting structures and improvement or detriment of the biological impact as modifications to the scaffold are performed. In this manner, it could be possible to identify, not only new MOA, but also new chemical scaffolds to address the growing issue of antibiotic resistant pathogens.

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APPENDIX A

Media Components

Complex extracts were generated by the addition of 200 mL of activated HP20 resin suspended in water on the seventh day of culturing. Following a 3 hour period of shaking under the culture conditions, the broth was separated form the resin and micellia and discarded. The resin and micellia were extracted with 100 mL of mass spectrometry grade methanol for 1 hour. The methanol extract was seprated from the resin and micellia and dried down. LC-MS was performed on a Thermo TSQ triple-quad equipped with a Surveyor PC pump and Thermo Pal autosampler. Metabolites were separated on a 150 mm x 4.6 mm Waters C18 (5 µm Particle size) column. The auto-injector sample tray was held at 4 °C to minimize sample degradation and a sample size of 20 µL was injected. Chromatographic separation was achieved using a 30 minute gradient at a flow rate of 1 mL/min using a gradient mixer of 10 mM ammonium acetate in 95 % water and 5 % acetonitrile (mobile phase A) and 10 mM ammonium acetate in 5 % water and 95 % acetonitrile (mobile phase B). The mobile phase was held at 100 % A for 1 minute, followed by a linear ramp to 100 % B over a period of 29 minutes and held at 100 % B for 20 minutes, followed by a 5 minute gradient to return the system to 100 % A and allowed to equilibrate for 10 minutes. Metabolites were analyzed using positive and negative mode electrospray ionization. Parameters for analysis include capillary voltage of 4.5 kV, source temperature of 275 °C. The samples were analyzed at a concentration of 100 mg / mL.

KA medium

Glucose 10 g/L
Corn Steep Liquor 10 g/L
Soybean Powder 10 g/L
Glycerol 5 g/L
Dry yeast 5 g/L
NaCL 5 g/L
pH to 5.7 then add $CaCO_3$ 2 g/L
Autoclave

m/z	ionization mode	retention time	*m/z*	ionization mode	retention time
619.74	+	5.71	450.72	-	11.25
781.64	+	5.71	608.68	-	11.39
457.68	+	6.54	414.59	-	12.37
583.92	+	9.43	430.47	-	13.7
416.61	+	12.67	653.62	-	13.7
490.9	+	12.67	1309.95	-	13.7
454.54	+	12.67	677.07	-	14.64
474.81	+	12.67	712.98	-	14.64
432.55	+	14.15	456.53	-	15.11
700.93	+	14.15	955.02	-	15.25
460.56	+	15.27	472.68	-	16.52
927.22	+	15.27	940.98	-	16.52
599.78	+	15.53	765.01	-	17.38
437.72	+	16.93	794.99	-	17.38
942.93	+	16.93	910.95	-	17.38
796.83	+	17.29	490.69	-	17.93
492.8	+	17.91	938.92	-	17.93
554.72	+	23.72	633.24	-	18.39
466.61	+	25.85	1067.38	-	18.79
522.67	+	25.85	568.79	-	21.21
517.87	+	28.77	594.72	-	22.62
493.92	+	30.07	570.66	-	23.85
			506.83	-	24.03
			480.7	-	25.22
			637.64	-	26.08
			449.86	-	26.7
			598.79	-	26.7
			475.62	-	28.14
			613.51	-	28.14
			477.91	-	29.73

OA media

Glucose 10 g/L
Glycerol 5 g/L
Corn steep liquor 3 g/L
Beef extract 3 g/L
Malt extract 3 g/L
Yeast extract 3 g/L
$CaCO_3$ 2 g/L
pH to 7.2 and autoclave
Thiamine 0.1 g/L

m/z	ionization mode	retention time	*m/z*	ionization mode	retention time
408.51	+	2.39	594.7	-	8.29
486.71	+	3.18	422.63	-	10.49
526.69	+	6.43	487.71	-	10.49
430.66	+	10	550.72	-	10.49
406.8	+	11.16	404.73	-	11.28
444.64	+	11.16	503.72	-	11.28
452.72	+	11.63	438.66	-	11.97
490.81	+	12.93	679.84	-	11.97
672.81	+	13	478.6	-	12.87
603.99	+	13.61	677.07	-	14.68
679	+	14.62	712.97	-	14.68
716.97	+	14.62	408.59	-	16.44
599.74	+	15.52	438.61	-	16.44
437.72	+	16.82	600.71	-	16.88
492.82	+	17.73	402.71	-	20.67
412.72	+	18.63	594.8	-	22.72
428.67	+	18.63	530.21	-	22.72
584.86	+	20.47	570.88	-	23.77
701.02	+	20.47	596.8	-	24.38
628.83	+	22.13	482.9	-	24.74
596.78	+	22.13	688.87	-	24.74
554.78	+	23.78	472.73	-	27.45
672.86	+	24.65	588.91	-	30.45
424.8	+	34.69			
684.91	+	36.74			

WA medium

Yeast extract 0.8 g/L
Casamino acids 0.5 g/L
Glucose 0.4 g/L
K_2HPO_4 2 g/L
pH to 7.2 and autoclave

m/z	ionization mode	retention time	*m/z*	ionization mode	retention time
599.57	+	7.12	571.18	-	7.71
543.69	+	8.52	610.61	-	7.71
430.71	+	9.9	594.54	-	8.25
408.73	+	11.16	404.72	-	11.1
444.55	+	11.16	677.09	-	14.75
452.68	+	11.66	712.9	-	14.75
496.7	+	11.95	941.87	-	16.94
540.77	+	12.28	479.76	-	25.54
490.79	+	12.75	505.8	-	26.88
603.86	+	13.58	805.11	-	28.28
716.98	+	14.12			
942.88	+	15.42			
587.7	+	16.42			
495.87	+	24.98			
521.96	+	27.11			

BA media

Soybean powder 15 g/L
Glucose 10 g/L
Soluble starch 10 g/L
NaCl 3 g/L
K_2HPO_4 1 g/L
Trace element solution 2 mL/L
- $FeSO_4$ x 7 H_2O 0.01 g/L
- $MnCl_2$ x 4 H_2O 0.08 g/L
- $CuSO_4$ x 5 H_2O 0.07 g/L
- $ZnSO_4$ x 7 H_2O 0.02 g/L
- 1 drop of concentrated H_2SO_4

pH to 7.2 and autoclave

m/z	ionization mode	retention time	*m/z*	ionization mode	retention time
416.14	+	3.36	538.87	-	2.8
707.03	+	3.36	718.9	-	2.8
447.05	+	12.17	482.91	-	4.97
491.37	+	12.67	448.94	-	7.6
433.06	+	13.47	690.74	-	9.37
679.37	+	13.97	444.99	-	10.96
701.23	+	13.97	498.9	-	10.96
717.36	+	13.97	528.87	-	11.61
943.51	+	16.21	654.04	-	12.84
609.25	+	18.49	684.3	-	12.84
805.23	+	20.15	490.89	-	13.45
			480.99	-	14.79
			940.95	-	15.83
			795.06	-	16.63
			764.99	-	16.63
			913.05	-	18.22
			1186.77	-	19.59
			516.99	-	19.8
			1200.89	-	20.42
			913.01	-	20.81
			468.86	-	22.58

EA media

Lactose 50 g/L
Corn steep solids 5 g/L
Glucose 5 g/L
Glycerol 15 g/L
Soybean flour 10 g/L
Bacto-peptone 5 g/L
CaCO3 3 g/L
(NH4)2SO4 2 g/L
FeSO4 x 7 H2O 0.1 g/L
ZnCl2 0.1 g/L
MnCl2 4 H20 0.1 g/L
MgSO4 x 7 H20 0.5 g/L
pH to 7.2 and autoclave

m/z	ionization mode	retention time	*m/z*	ionization mode	retention time
416.17	+	2.6	430.91	-	2.95
702.05	+	2.6	682.81	-	2.95
474.13	+	3.76	649.87	-	9.55
707	+	4.91	450.87	-	11.46
508.14	+	6	474.71	-	11.46
417.08	+	11.88	487.1	-	12.55
475.33	+	12.64	466.87	-	13.56
433.05	+	13.65	490.93	-	13.56
679.33	+	13.9	713.21	-	13.81
701.4	+	13.9	478.97	-	14.5
445.21	+	14.51	464.04	-	14.96
518.2	+	14.51	514.02	-	14.96
533.25	+	15.13	911.79	-	16.19
442.24	+	15.52	941.2	-	16.19
493.27	+	17.8			

QB media

Starch 5 g/L
Glucose 6 g/L
Corn steep liquor 2.5 g/L
Proflo 5 g/L
Proflo oil 2 mL/L
pH to 7.2 and autoclave

m/z	ionization mode	retention time	*m/z*	ionization mode	retention time
416.24	+	2.64	482.95	-	5.65
522.15	+	2.64	562.74	-	5.65
620.05	+	5.89	597.08	-	7.96
508.23	+	7.11	516.86	-	9.44
621.24	+	7.98	594.78	-	9.98
742.98	+	9.86	740.74	-	9.98
581.01	+	11.16	578.83	-	11.1
423.13	+	12.93	770.79	-	13.38
503.07	+	12.93	439.01	-	16.12
579.09	+	12.93	435.96	-	16.52
438.21	+	16.25	508.98	-	16.52
493.26	+	17.47	419.95	-	18.29
558.26	+	20.47	466.89	-	21.61
451.16	+	22.89	594.97	-	21.61
573.18	+	22.89	574.03	-	22.76
754.99	+	24.22	411.91	-	24.02
496.29	+	25.45	435.88	-	24.02
			752.82	-	24.02

APPENDIX B

NMR Data for Chapter 2 – Chemical Perturbations to Induce Secondary Metabolite Production

NMR experiments were acquired using a 14.0 T Bruker magnet equipped with a Bruker AV-III console operating at 600.13 MHz. All spectra were acquired in 3mm NMR tubes using a Bruker 5 mm TCI cryogenically cooled NMR probe. Chemical shifts were referenced internally to the solvent used for analysis, DMSO-d_6, $CDCl_3$, or CD_3OD which also served as the ^2H lock solvents. For 1D 1H NMR, typical experimental conditions included 15 ppm sweep width, a recycle delay of 1.5 seconds and 32-256 scans depending on sample concentration. For 2D 1H-1H COSY, experimental conditions included 15 ppm sweep width, recycle delay of 1.5 seconds and 4 scans per increment. The data was processed using squared sinebell window function, symmetrized, and displayed in magnitude mode. Multiplicity-edited HSQC experiments were acquired using a J(C-H) value of 145 Hz which resulted in a multiplicity selection delay of 34 ms, a recycle delay of 1.5 seconds and 16 scans per increment along with GARP decoupling on ^{13}C during the acquisition time (150 ms). The data was processed using a π/2 shifted squared sine window function and displayed with CH/CH_3 signals phased positive and CH_2 signals phased negative. J_1(C-H) filtered HMBC experiments were acquired using a J(C-H) value of 9 Hz for detection of long range couplings resulting in an evolution delay of 55ms, J_1(C-H) filter delay of 145 Hz (34 ms) for the suppression of one-bond couplings, a recycle delay of 1.5 seconds and 128 scans per increment. The HMBC data was processed using a π/2 shifted squared sine window function and displayed in magnitude mode. Selective 1D NOE spectra were recorded using the Double Pulse Field Gradient Spin Echo (DPFGSE) technique (T.L.Hwang,

A.J. Shaka J. Magn. Reson. Ser. A, 1995, 112,275-279; K. Stott, J. Stonehouse, J. Keeler, T.L. Hwang, A.J. Shaka, J. Am. Chem. Soc., 1995, 117, 4199-4200; K. Stott, J. Keeler, Q.N. Van, A.J. Shaka, J. Magn. Reson. 1997, 125, 302-324). Experimental parameters for this experiment were similar to those for the standard 1D 1H experiment with the addition of 600 ms which was used for the mixing time.

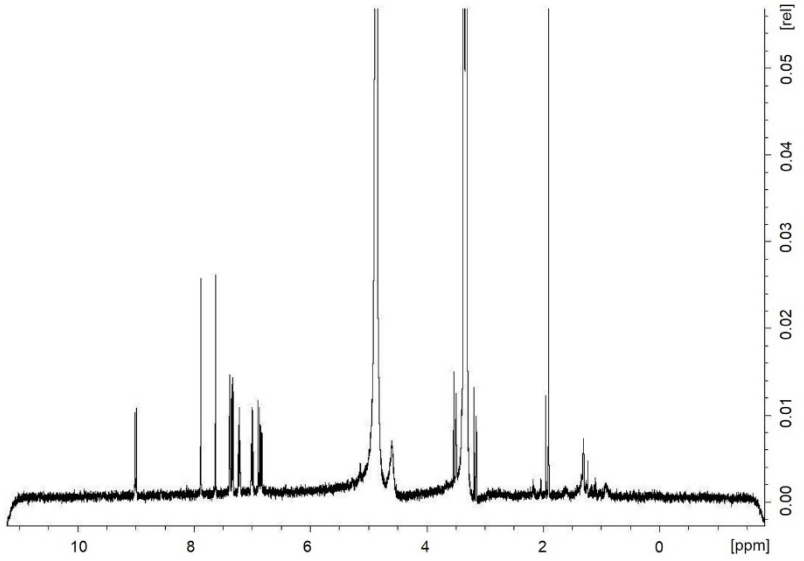


Figure B1. ^{1}H NMR of violacein in MeOD.

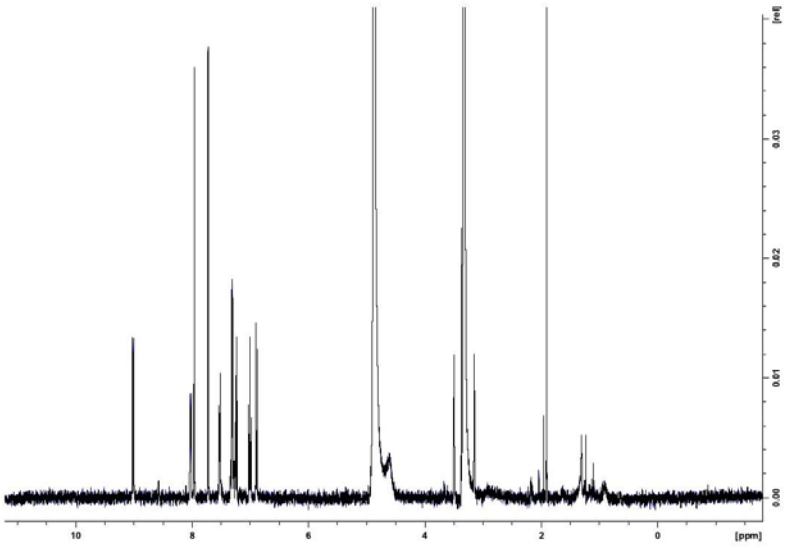


Figure B2. ^{1}H NMR of deoxyviolacein in MeOD.

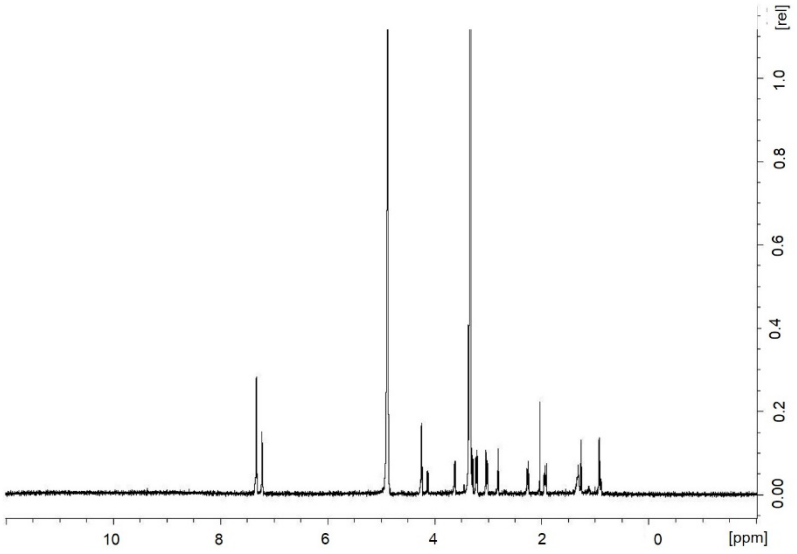


Figure B3. ^{1}H NMR of cyclo(phenylalanine-4-hydroxy-proline)in MeOD.

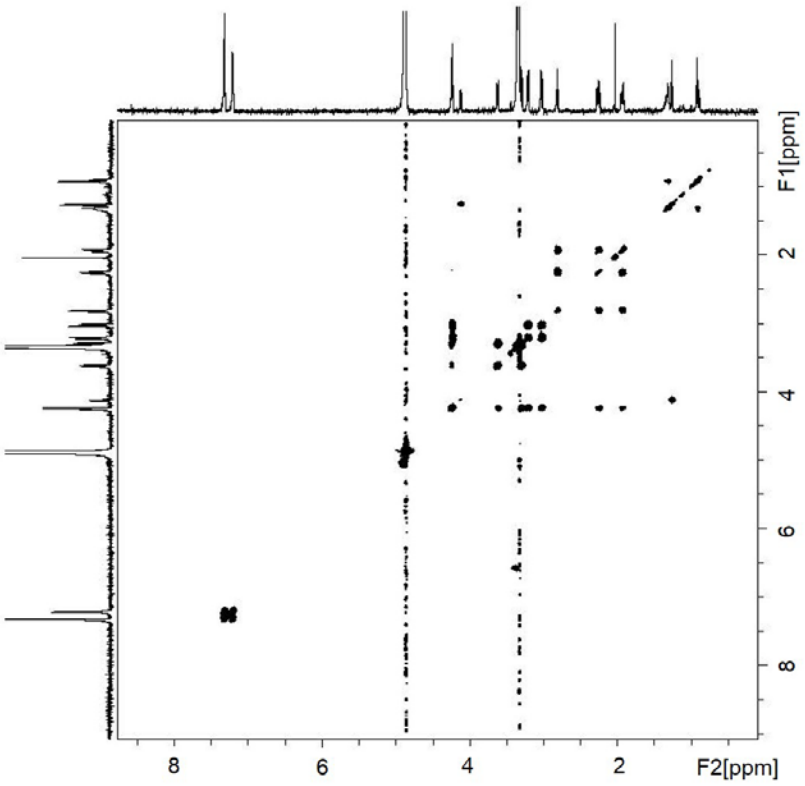


Figure B4. 2-D COSY NMR of cyclo(phenylalanine-4-hydroxy-proline)in MeOD.

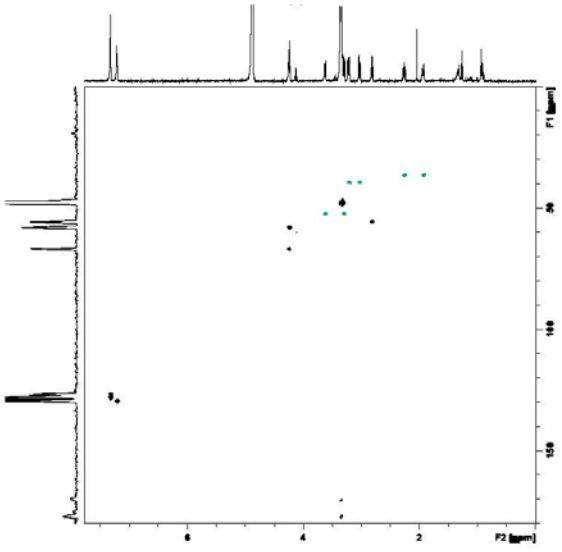


Figure B5. 2-D HSQC NMR of cyclo(phenylalanine-4-hydroxy-proline)in MeOD.

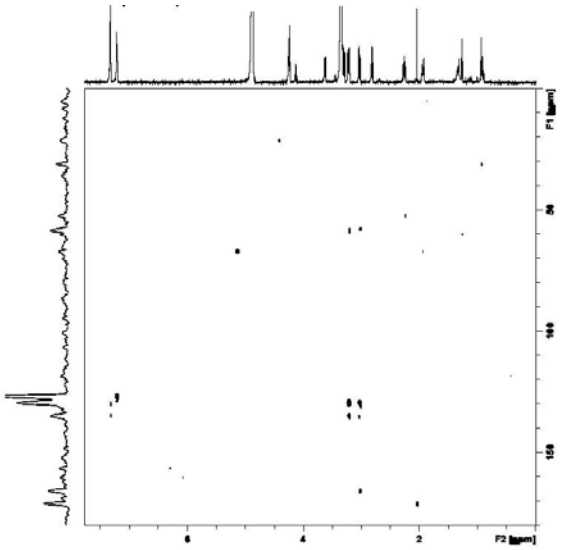


Figure B6. 2-D HMBC NMR of cyclo(phenylalanine-4-hydroxy-proline)in MeOD.

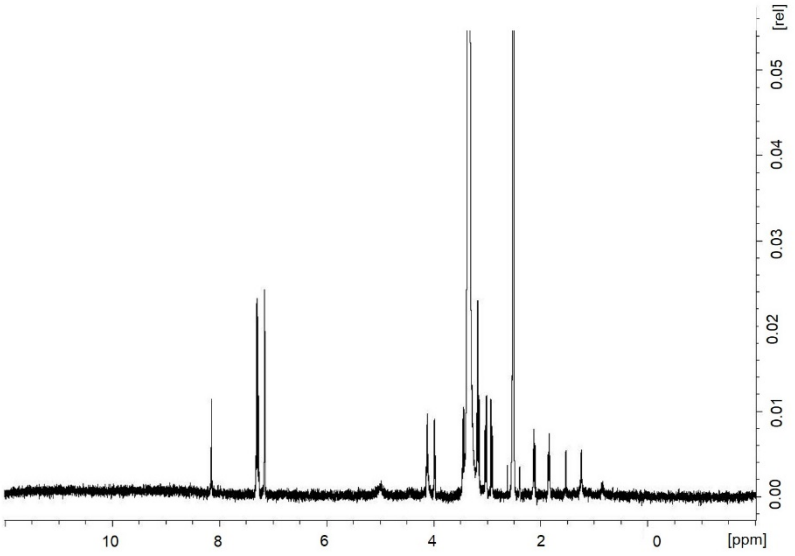


Figure B7. ^{1}H NMR of cyclo(phenylalanine-4-hydroxy-proline)in DMSO.

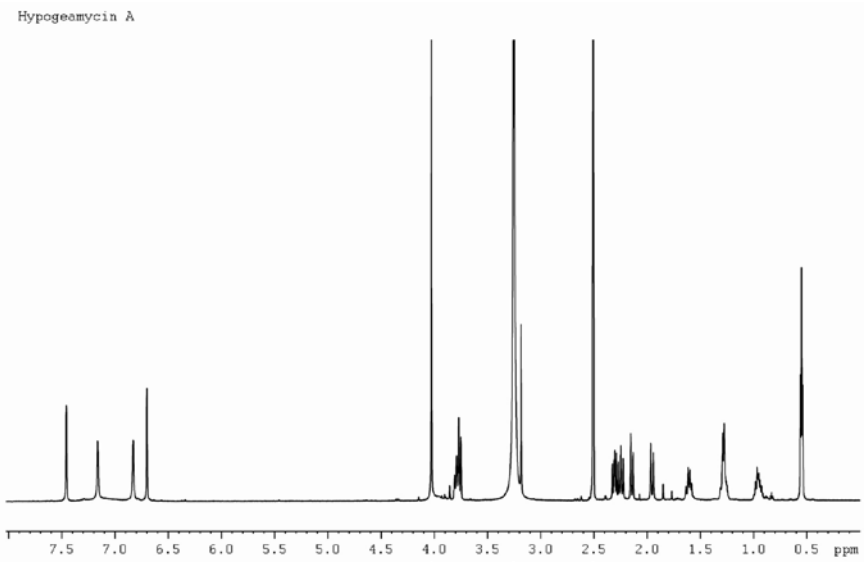


Figure B8. ^{1}H NMR of Hypogeamycin A in DMSO.

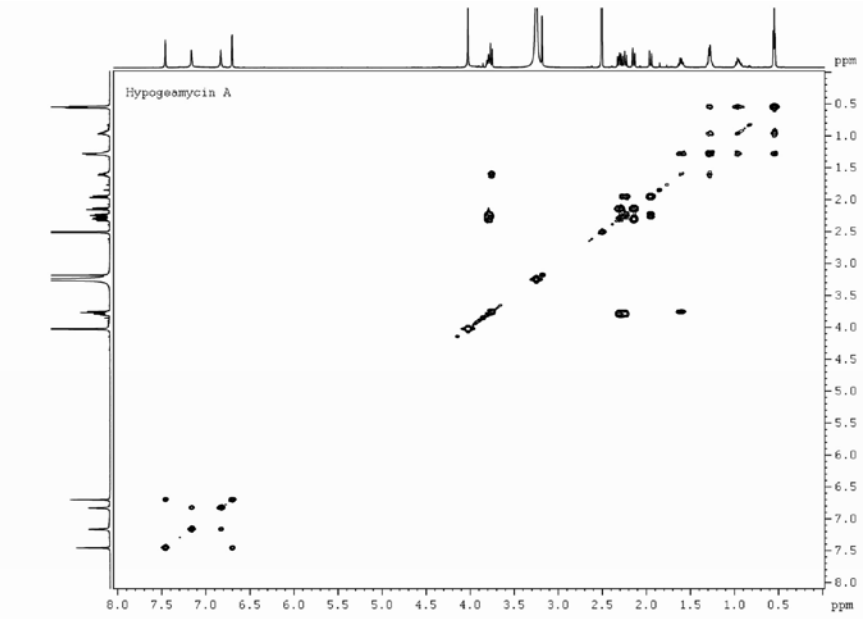


Figure B9. 2D COSY of Hypogeamycin A in DMSO.

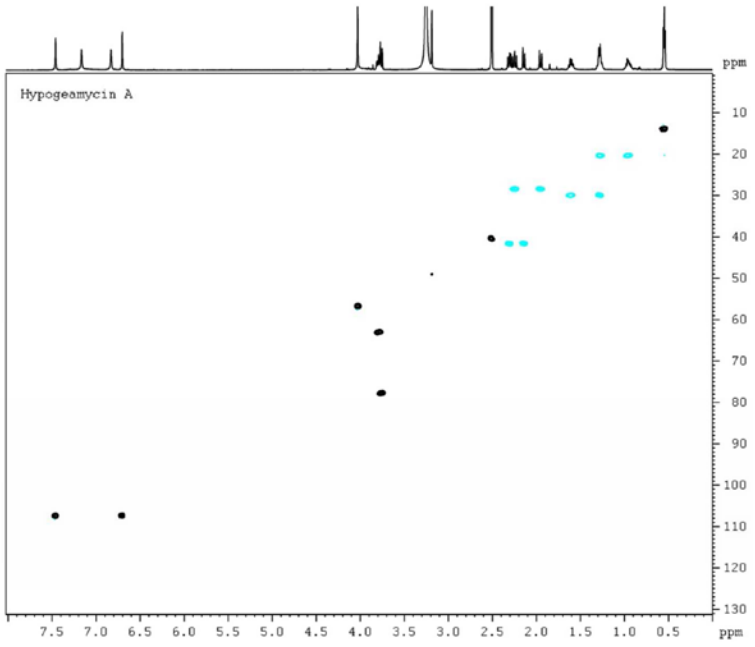


Figure B10. 2D HSQC of Hypogeamycin A in DMSO.

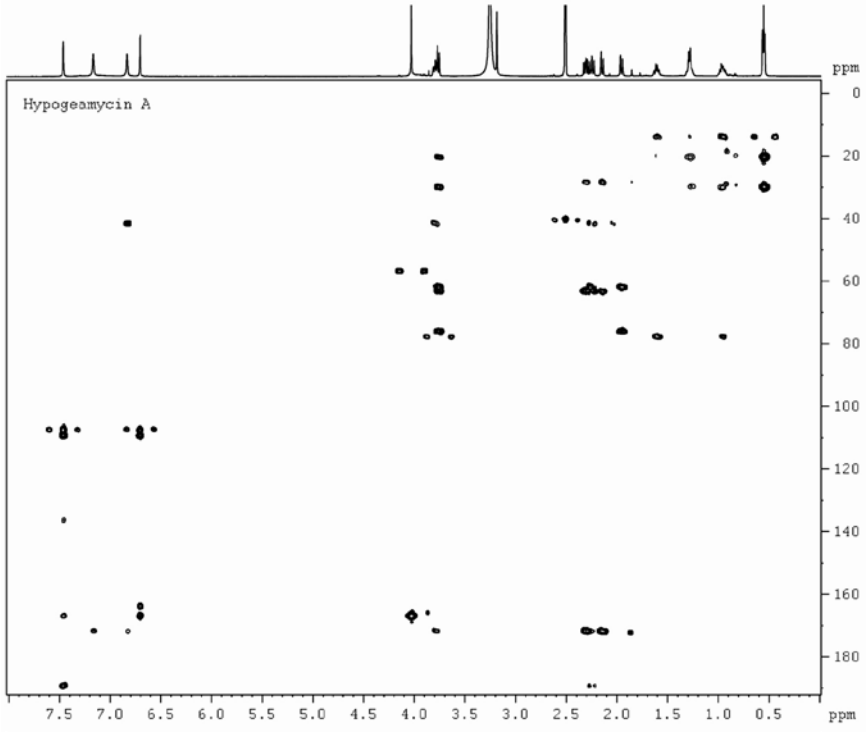


Figure B11. 2D HMBC of Hypogeamycin A in DMSO.

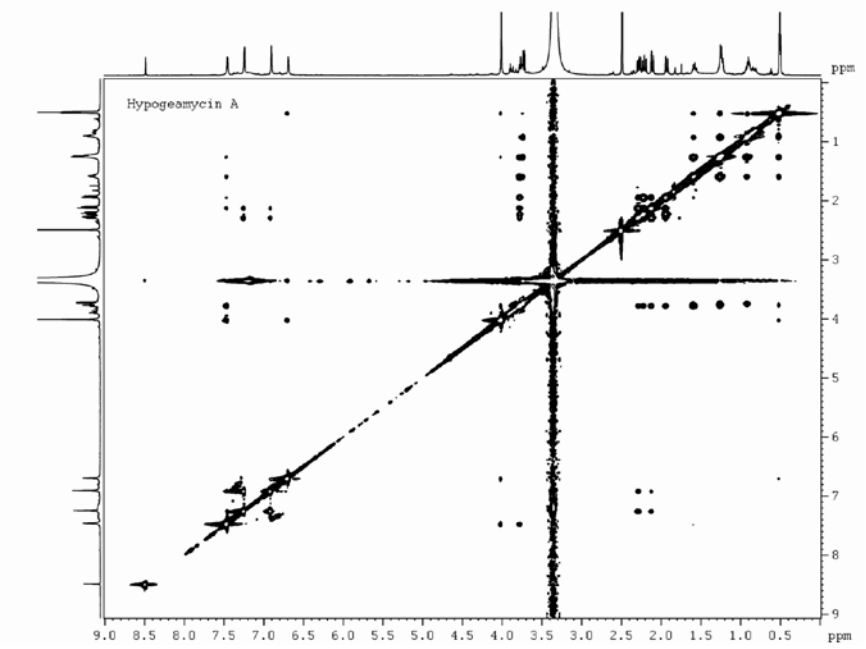


Figure B12. 2D NOESY of Hypogeamycin A (**1**) in DMSO.

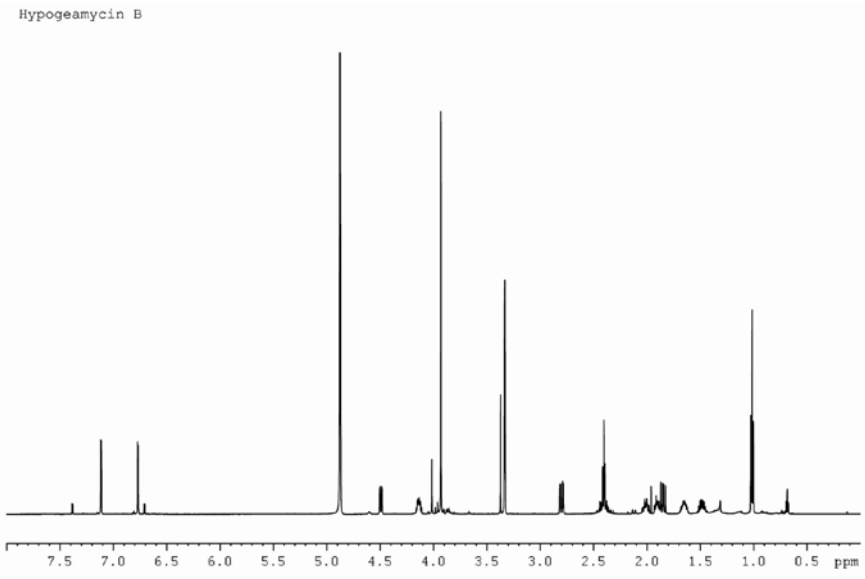


Figure B13. ^{1}H NMR of Hypogeamycin B in CD$_3$OD.

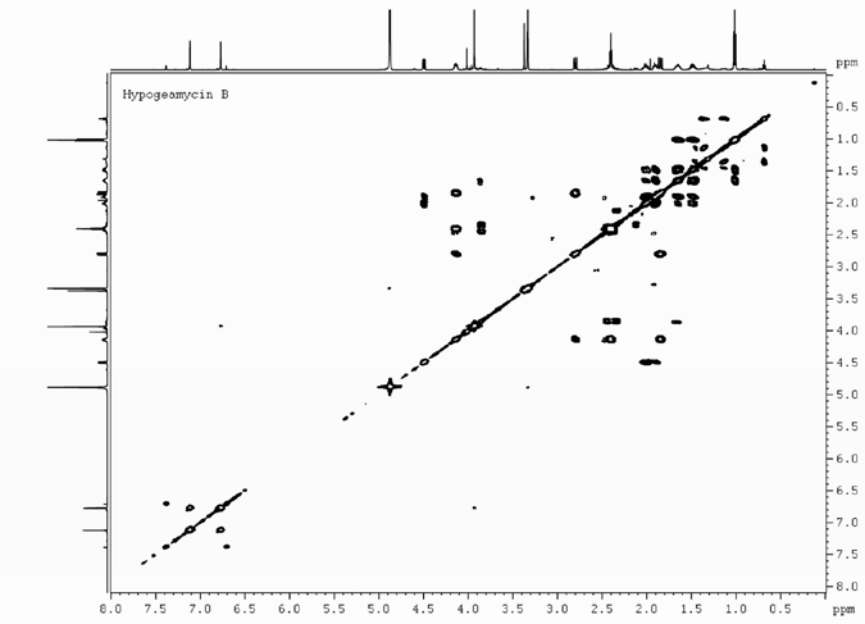


Figure B14. 2D COSY of Hypogeamycin B in CD_3OD.

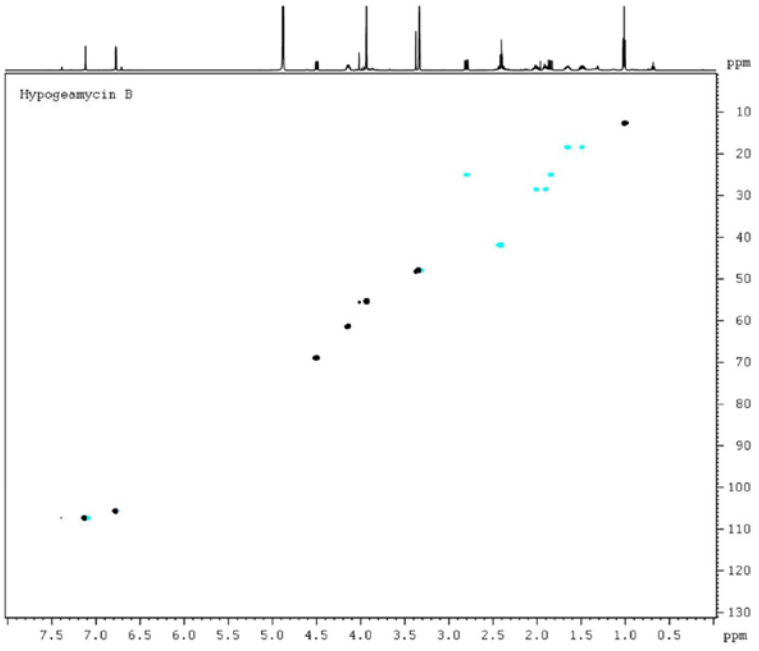


Figure B15. 2D HSQC of Hypogeamycin B in CD_3OD.

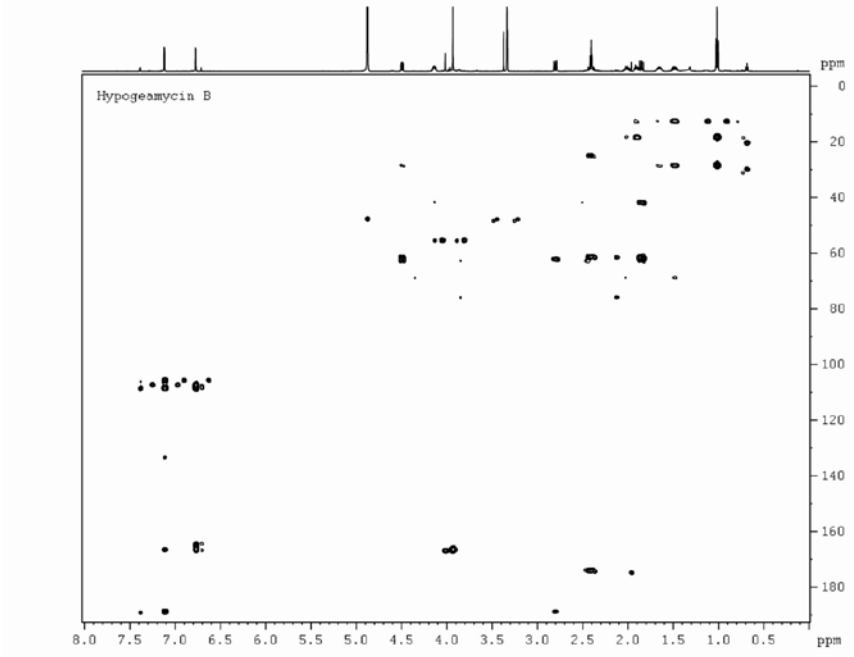


Figure B16. 2D HMBC of Hypogeamycin B in CD_3OD.

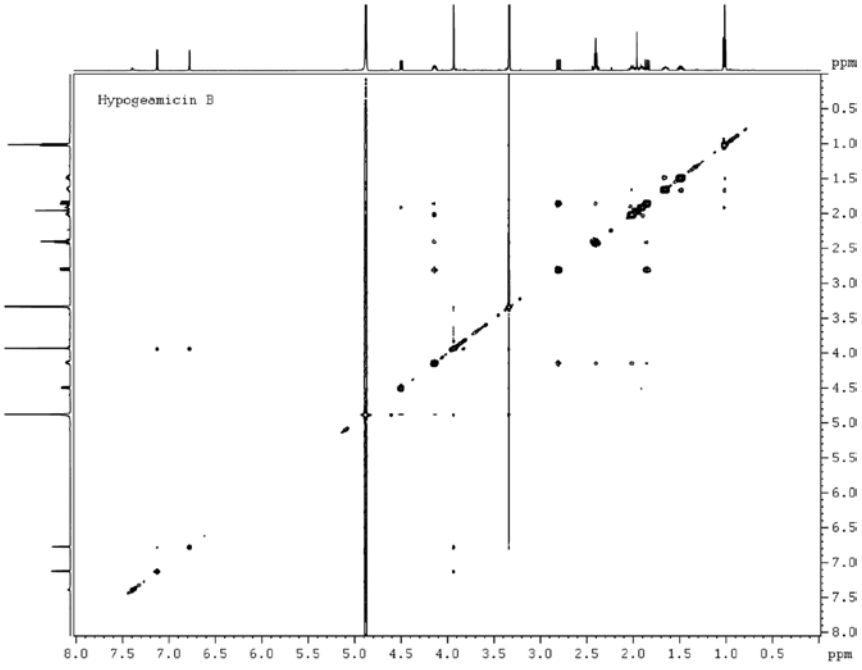


Figure B17. 2D NOESYof Hypogeamycin B in CD_3OD.

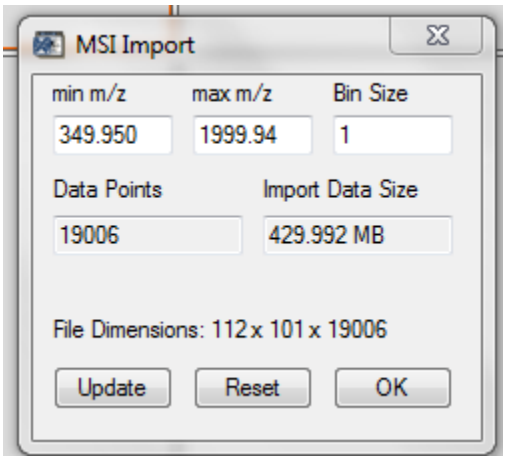
APPENDIX C

Data analysis for BIMS

1-1 Select the image file to be investigated by importing the associated files with the image experiment into Biomap.

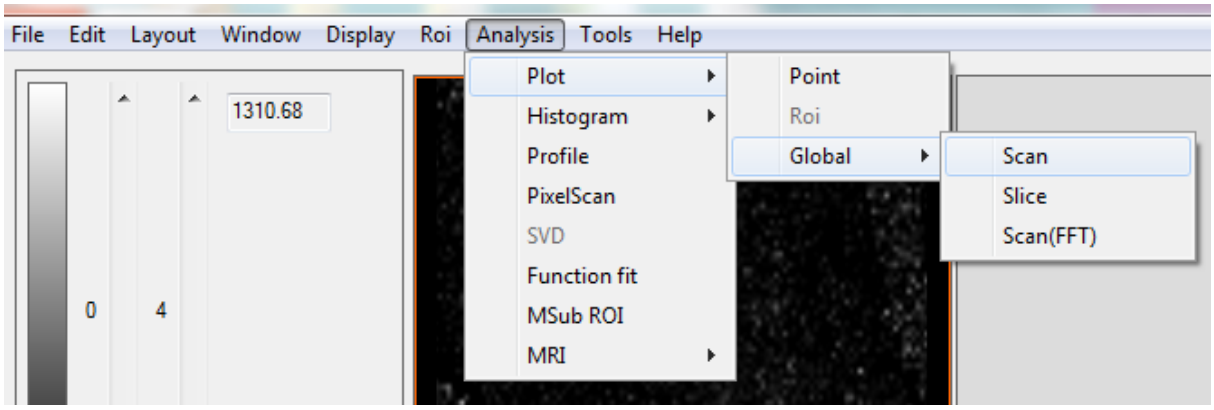
File >Import >MSI

Select the *m/z* range to be investigated with a Bin Size =1. For larger files, it may be necessary to import the image using a narrower *m/z* range and a Bin Size =1. Select OK when the parameters have been defined.

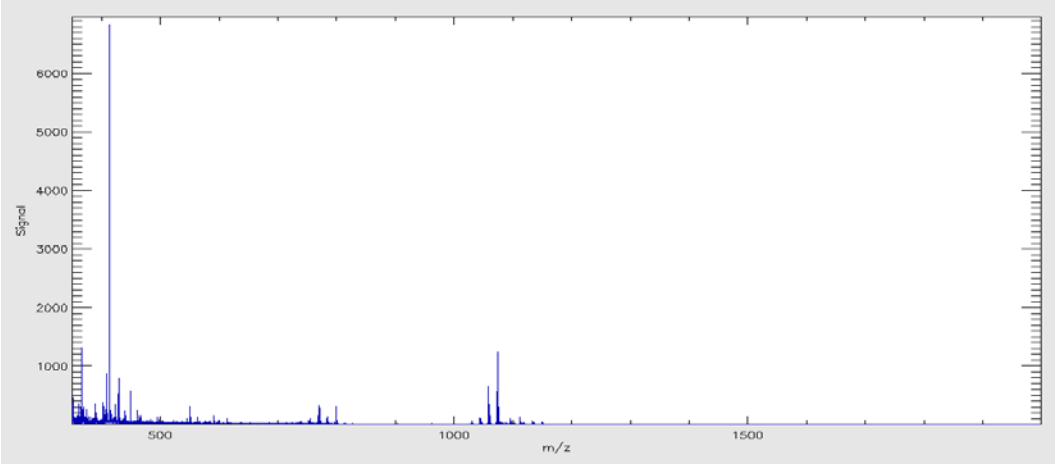


1-2 Generate mass spectrum for the entire sample imaged. Use the default settings for minimum and maximum intensity and select Done.

Analysis >Plot >Global >Scan >Done



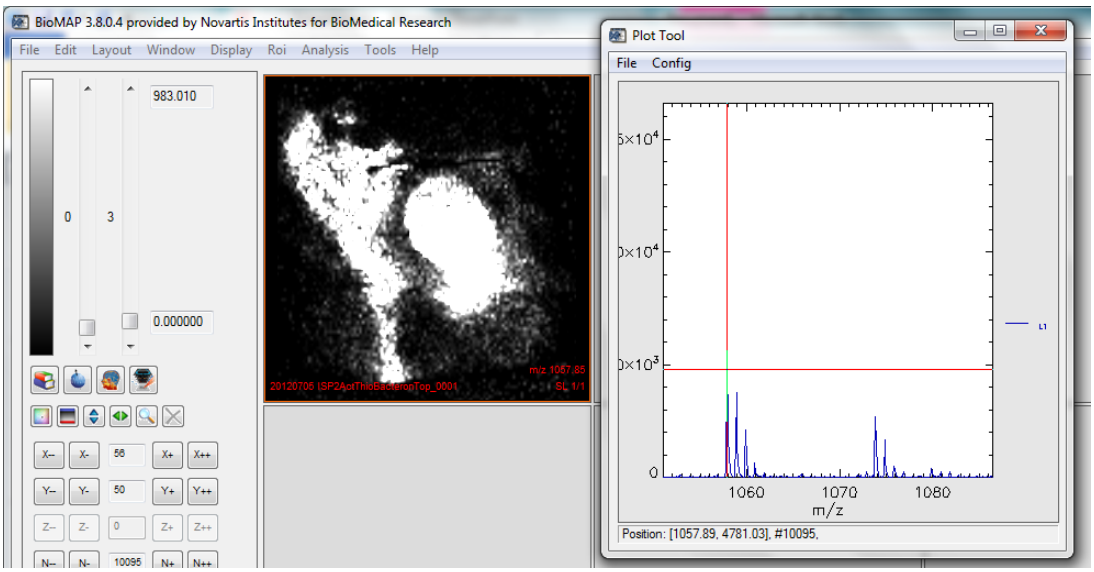
1-3 A mass spectrum will appear, within this plot, identify abundant ions. The y – axis can be scaled by holding Shift and the left mouse button while scrolling along the axis. The origin may be adjusted by holding Shift and the right mouse button while scrolling along the axis. The x – axis may be adjusted in the same manner. Holding the left mouse button allows selective zooming of an *m/z* range. Identify abundant ions and exit the spectrum.



1-4 Determine regions of interest (ROI) by determining 2-D location of abundant ions. Generate a mass spectrum by analyzing a single point within the image.

Analysis >Plot >Point

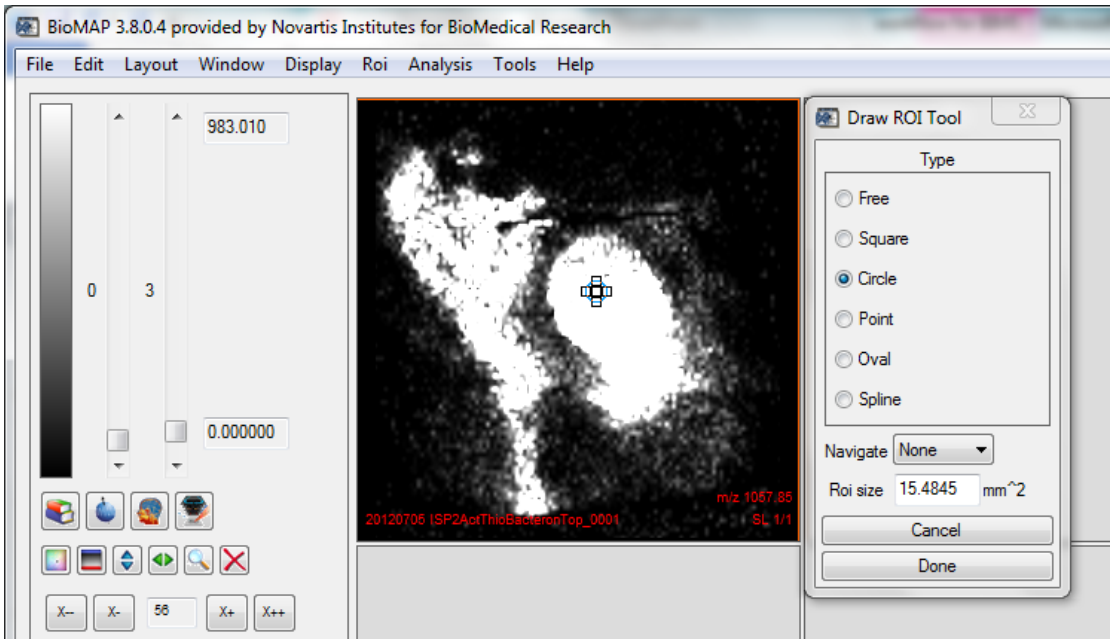
Select any point within the image using the left mouse button. Zoom in on the m/z range associated with the identified abundant ions. Use the right mouse button to generate a 2 – D location associated with the selected mass.



1-5 Define the ROIs to be sampled within the sample.

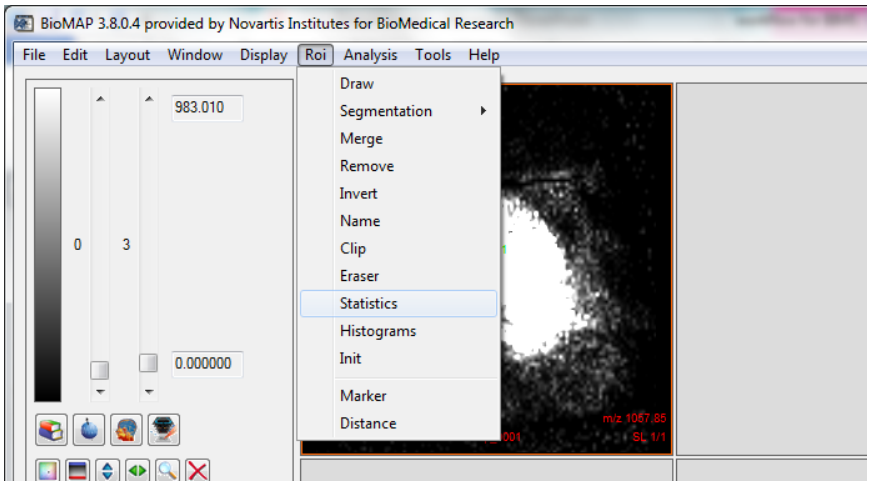
Roi >Draw

Select the shape and location of the ROI based on ion abundance which correlate to observed phenotypes using INT stain. ROIs were generated by drawing a circle within the center of the null growth region. For overlapping ROIs, evaluate each individually as Biomap will remove data from a previously defined ROI if a new ROI is drawn on top of such that the two overlap.



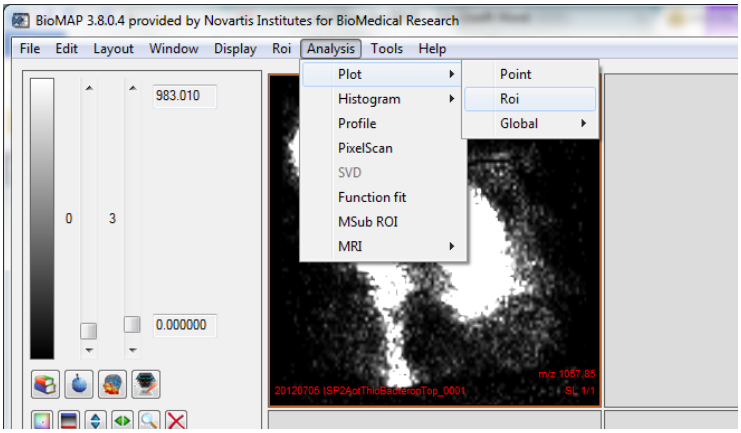
1-6 Ensure that each ROI drawn contains approximately the same number of pixels.

Roi >Statistics



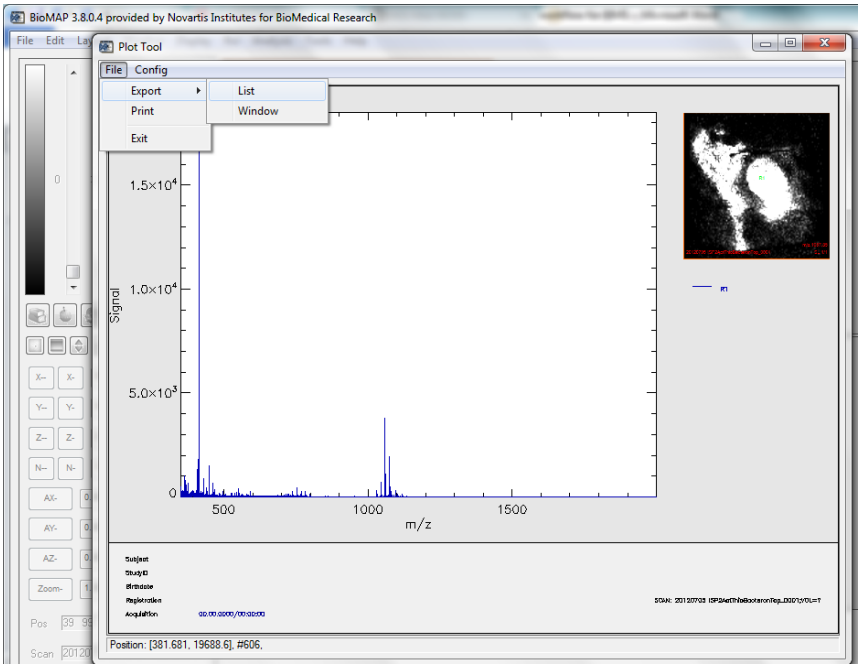
1-7 Generate mass spectrum for each ROI

Analysis >Plot >Roi



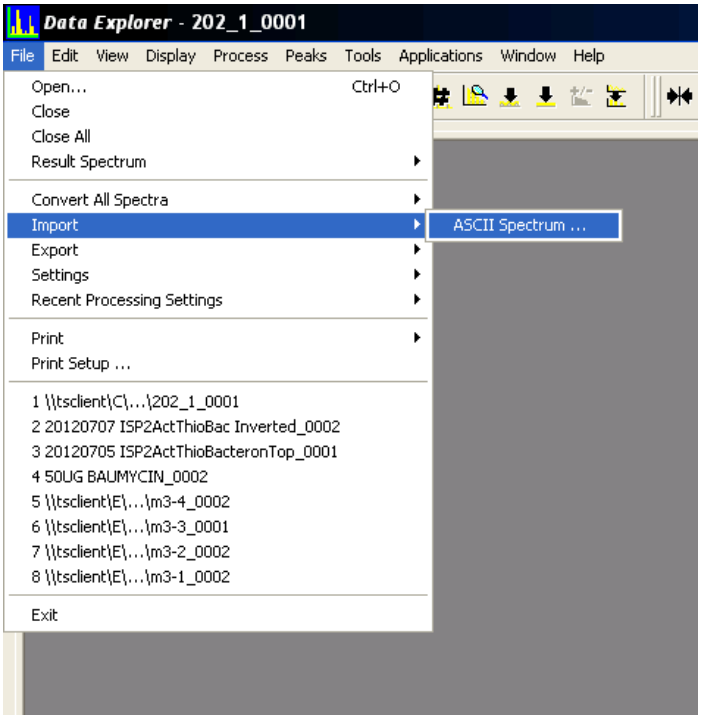
1-8 Extract the m/z values and intensities into a text file for analysis by Data Explorer.

File >Export >List >Export



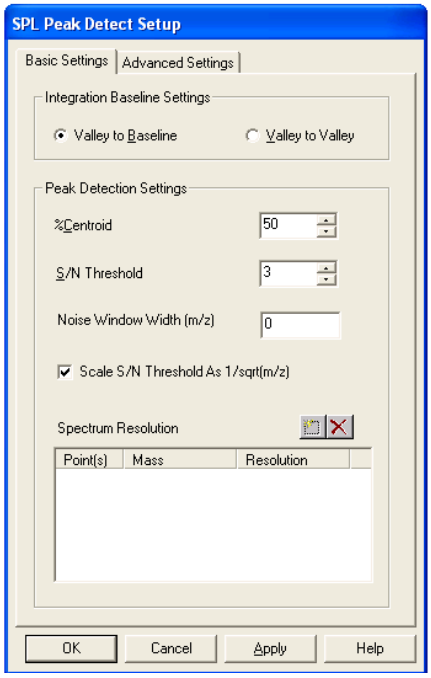
2-1 Import the text file from Biomap into Data explorer.

File >Import >ASCII Spectrum

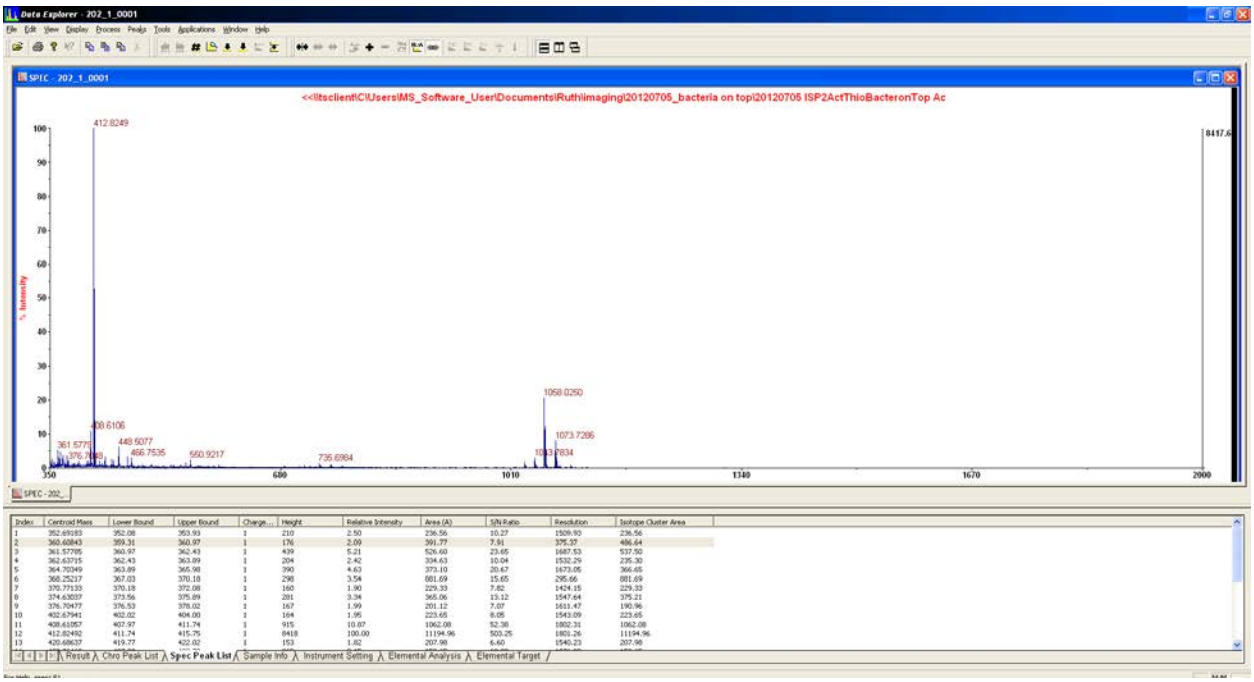


2-2 Pick peaks using s/n > 3 and > 0

Peaks >Pick Peaks



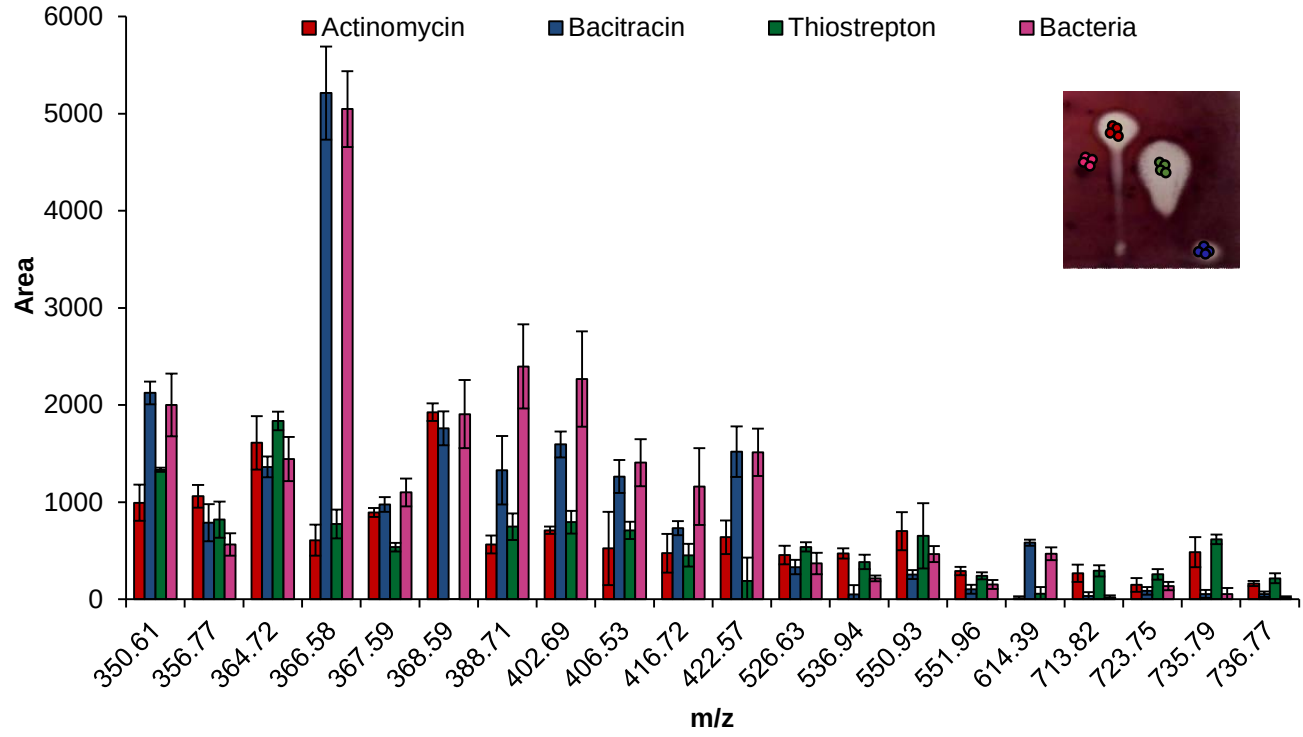
2-3 Export the peak list for each ROI to an Excel file (retain columns containing the centroid mass and area)

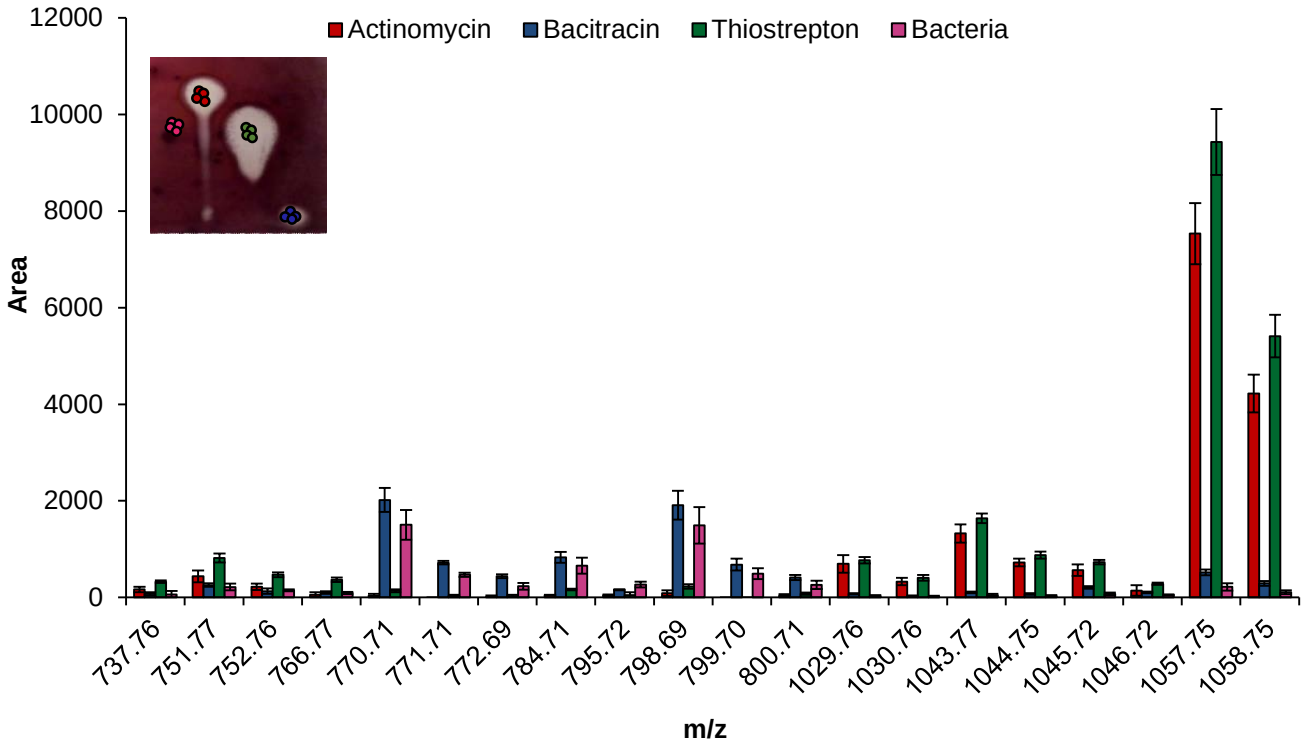


3-1 Compile the peak list for ions with a s/n > 3 in excel. Using data explorer, pick peaks using s/n > 0 and export the peak list for each ROI to an Excel file (retain columns containing the centroid mass and area). Populate the peak list with the area identified for each ion.

APPENDIX D

Comparison of Ion Abundance within ROIs for BIMS





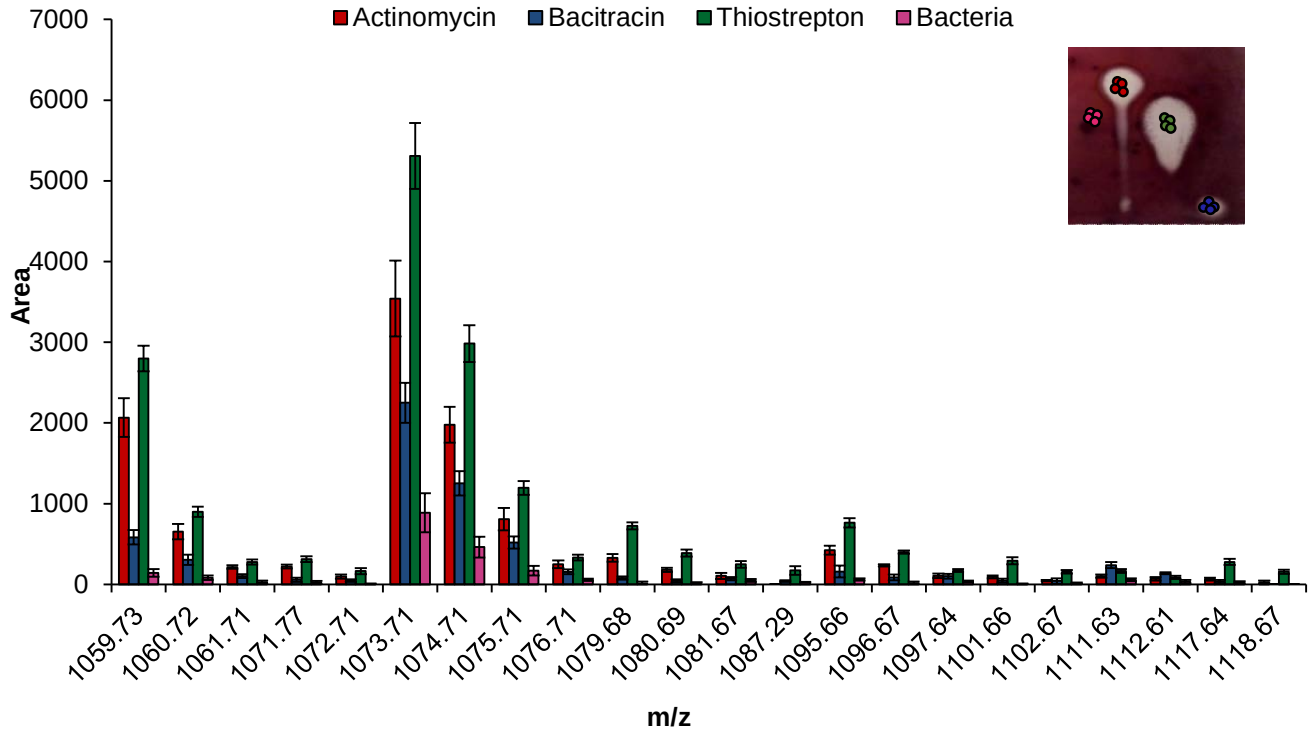


Figure D 1-3. (A) Intensity profile with INT stained sample (insert) to indicate the user defined regions of interest for healthy bacteria and null growth regions used for intensity comparison of selected ions with S/N $\geq$ 3 from sample with bacteria applied to the agar after 2-D chromatographic separation of actinomycin, bacitracin, and thiostrepton.

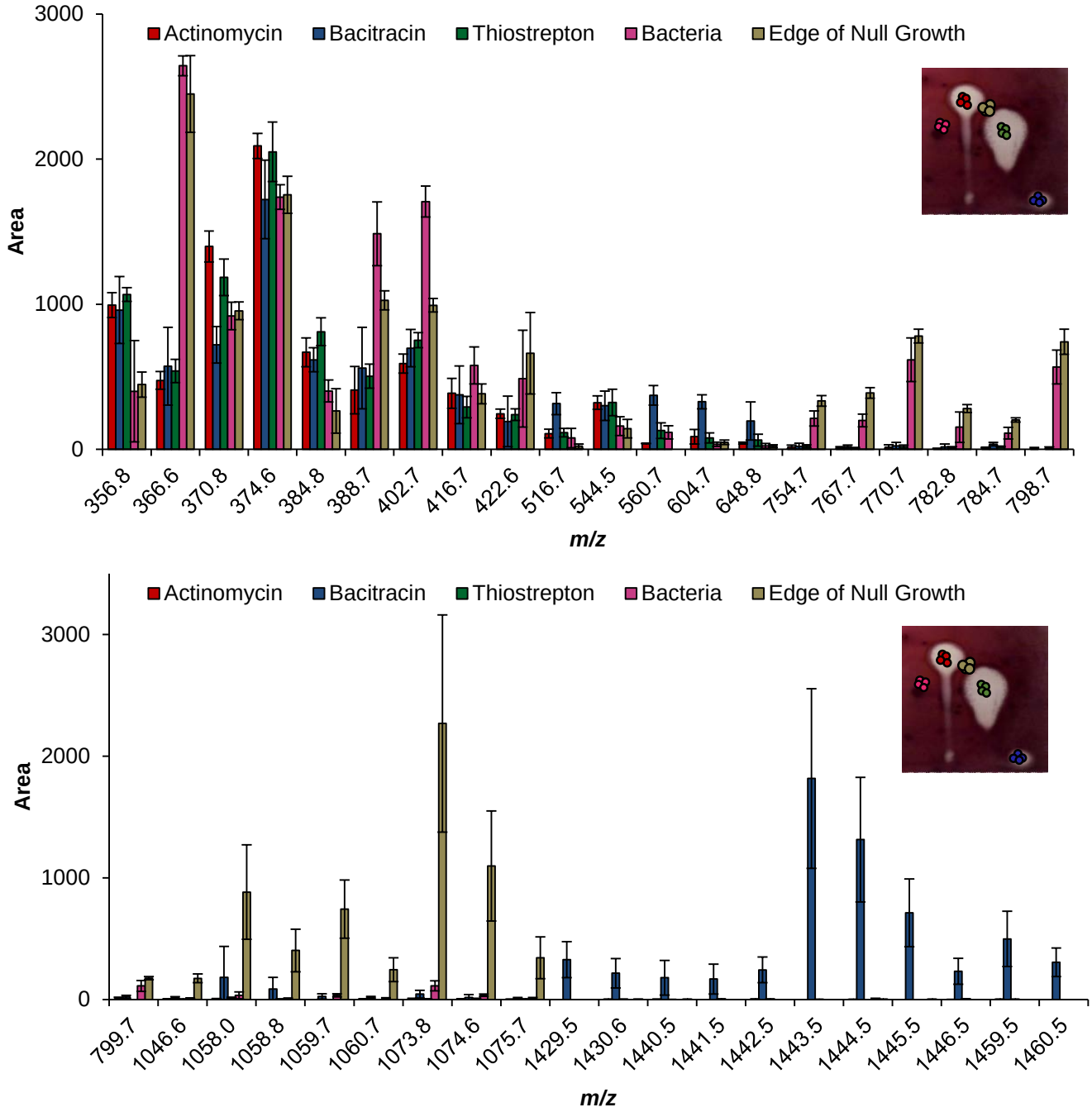


Figure D 4-5. INT stained sample (insert) to indicate the user defined regions of interest for healthy bacteria and null growth regions and intensity profile ions with S/N $\geq$ 3 from sample with bacteria embedded in the agar and inverted for sample mounting after 2-D chromatographic separation of actinomycin, bacitracin, and thiostrepton.

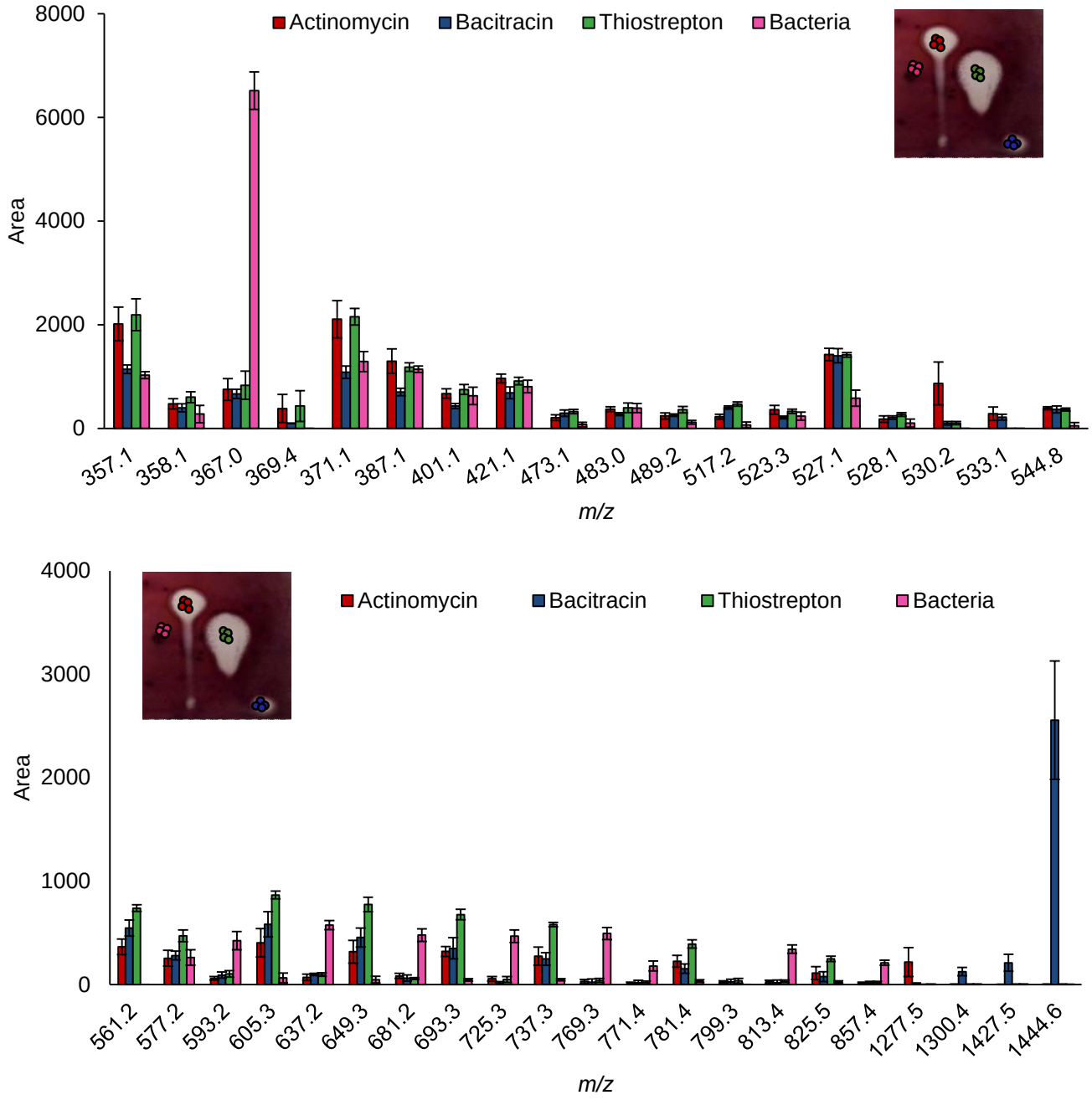


Figure D 6-7. INT stained sample (insert) to indicate the user defined regions of interest for healthy bacteria and null growth regions and intensity profile ions with S/N $\geq$ 3 from sample with bacteria embedded in the agar and inverted for sample mounting after 2-D chromatographic separation of actinomycin, bacitracin, and thiostrepton.

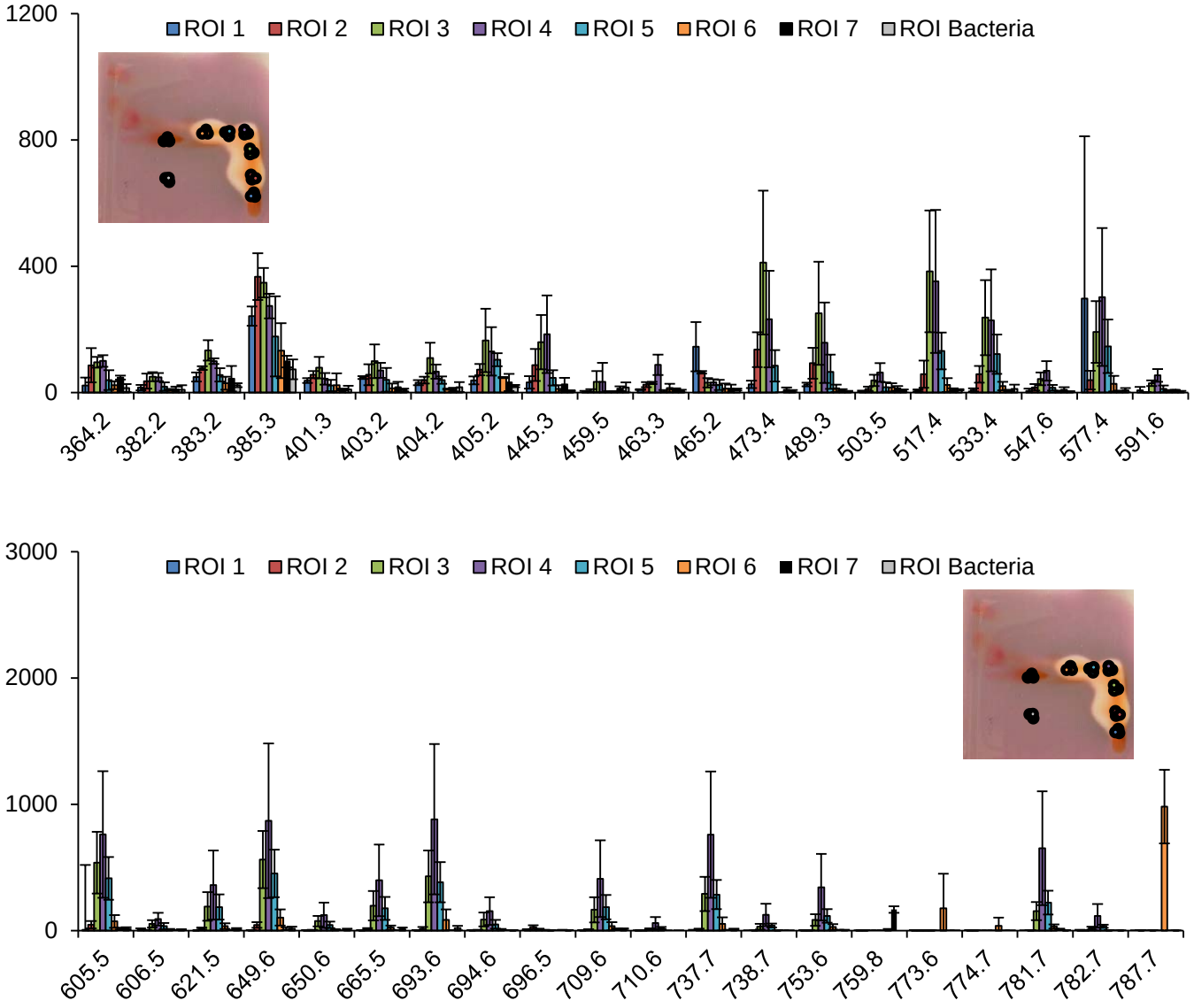


Figure D 8-9. INT stained sample (insert) to indicate the user defined regions of interest for healthy bacteria and null growth regions and intensity profile ions with S/N $\geq$ 3 from sample with bacteria embedded in the agar and inverted for sample mounting after 2-D chromatographic separation of actinomycete crude extract.

APPENDIX E

NMR Analysis for BIMS Isolated Compounds

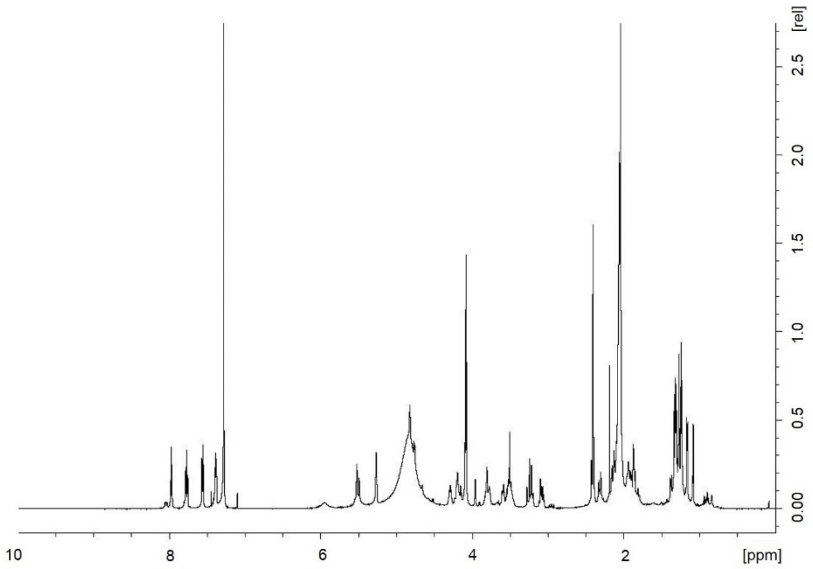


Figure E1. ^{1}H NMR of Baumycin analogue in $CDCl_3$.

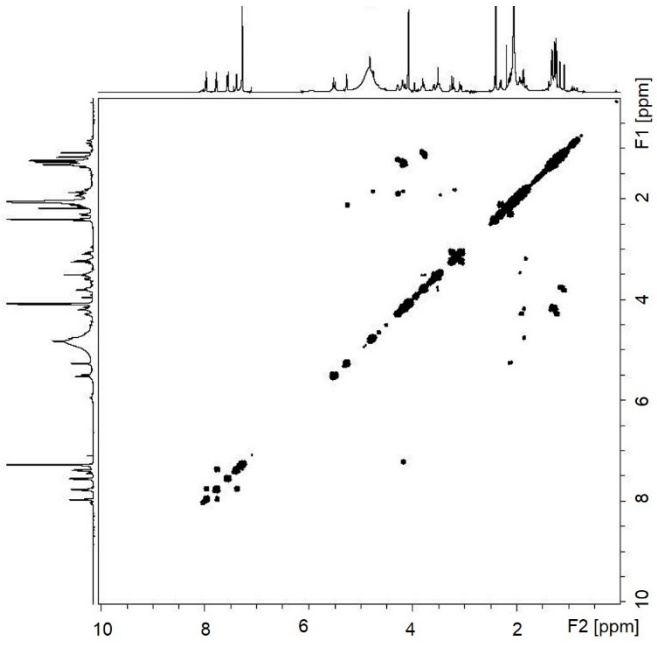


Figure E2. COSY NMR of Baumycin analogue in $CDCl_3$.

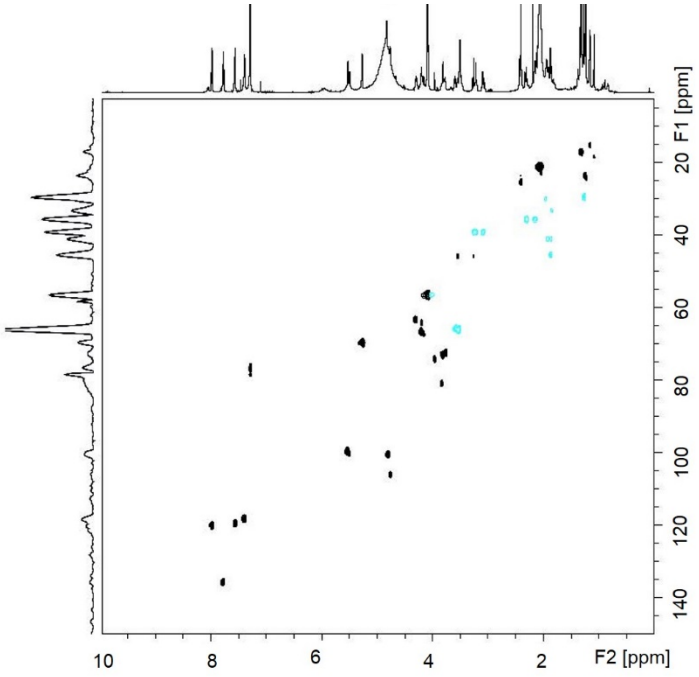


Figure E3. HSQC NMR of Baumycin analogue in $CDCl_3$.

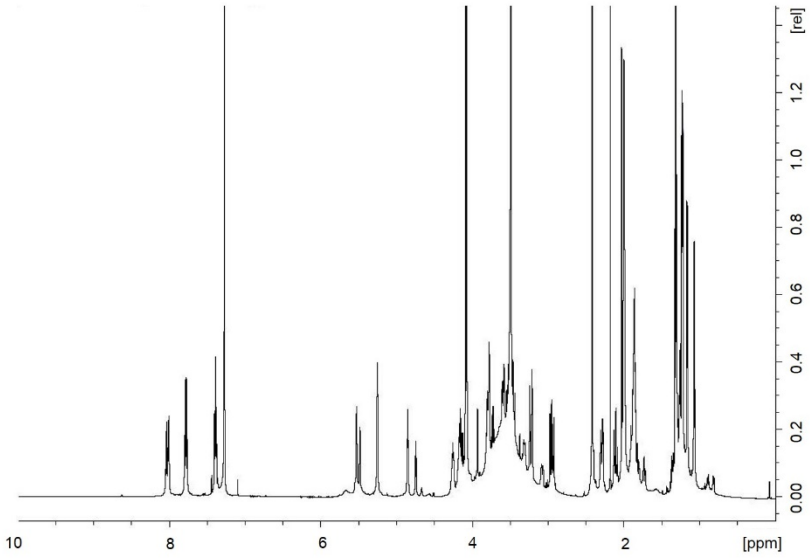


Figure E4. ^{1}H NMR of Baumycin in $CDCl_3$.

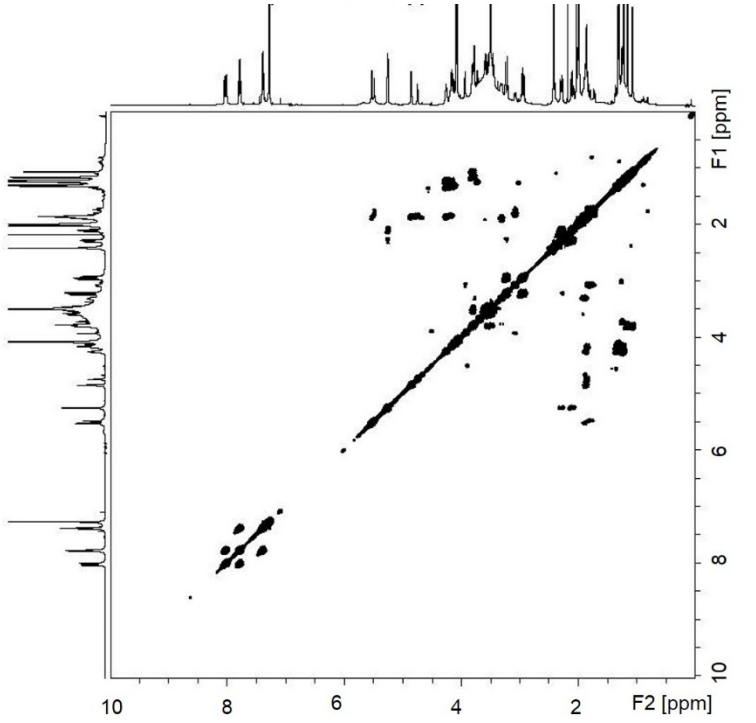


Figure E5. COSY NMR of Baumycin in $CDCl_3$.

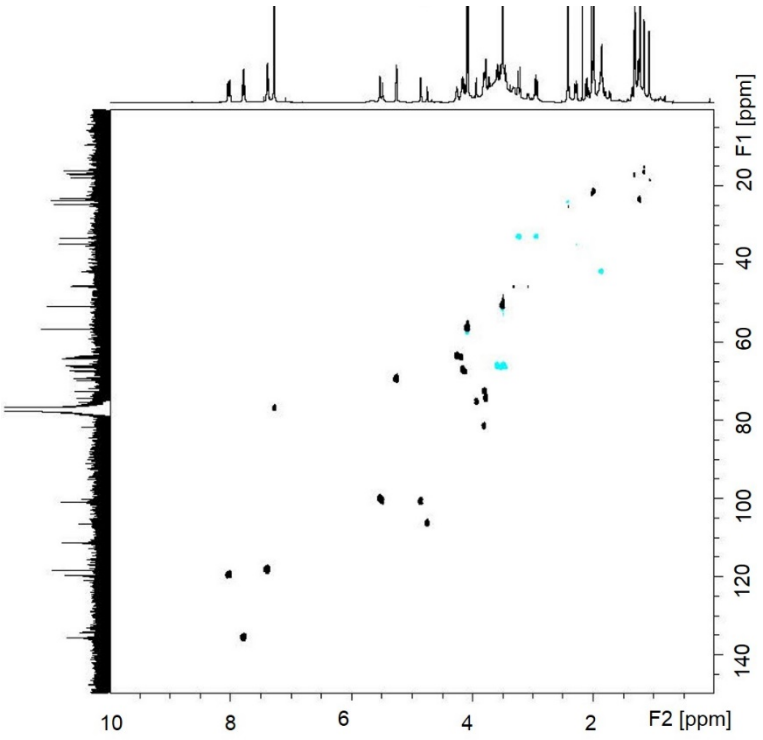


Figure E6. HSQC NMR of Baumycin in $CDCl_3$.

APPENDIX F

Curriculum vitae

C. Ruth McNees

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While earning my Ph.D. in chemistry at Vanderbilt University, my research focused on developing methodologies to induce secondary metabolism of actinomycetes. This was achieved through the use of chemical perturbations to alter protein expression and complex microbial co-culture systems which facilitated bacterial chemical communication. Evaluation of the complex microbial extract generated was performed via principle component analysis to determine lead compounds for isolation, utilizing HPLC, size exclusion, ion exchange, etc., followed by structure elucidation by 2-D NMR techniques. Additionally, the application of imaging mass spectrometry for the identification of ions in a TLC chromatographically separated sample has been developed and successfully implemented for use in determining lead compound isolation. Development of this method also facilitated the identification of ions associated with biological responses induced in the biofilm forming microorganism, *Bacillus subtilis,* upon exposure to various antibiotics.

Specialties Liquid chromatography separations, MALDI and ESI mass spectrometry, Metabolomics based studies via PCA, compound isolation and NMR based structure elucidation, mass spectrometry data analysis software (MarkerLynx, XCMS, Xcalibur), and Water's HPLC Empower software.

Education

Vanderbilt University **Nashville, TN**
Ph.D Department of Chemistry, defended in March 2015. Dissertation title: New Methodologies to Improve Natural Product Discovery from Actinomycetes. Supervised by Dr. Brian Bachmann.

North Georgia College and State University **Dahlonega, GA**
Bachelor of Science, Chemistry major advised by Dr. Brad Hurbert, Suma Cum Laude, degree conferred May 2006.

Research Experience

Vanderbilt University, PhD candidate in chemistry **2008-Present**
Method development for expression of genes associate with the production of secondary metabolites to be purified using multiple chromatography methods followed by structure elucidation based primarily on 2-D NMR techniques for compound identification. Development of MALDI-imaging mass spectrometry method for detection of biofilm formation associated molecules and bioactive lead compound identification.

Ciba Vision, Lab technician in research and development **2006-2008**
Primary responsibilities included development of new analytical methods utilizing HPLC, IR, and UV to characterize raw materials, in process materials, and final products. Writing

and implementing standard operating procedures for use in quality control following FDA guidelines under the guidance of Dr. Fred Meadows.

Furman University **2004**

Research Experience for Undergraduates. Develop efficient methods for the separation of inorganic based enantiomers using capillary electrophoresis employing various buffer additives with special focus on sulfated cyclodextrins while under the supervision of Dr. John Wheeler.

Teaching Experience

Vanderbilt University **2008-2013**

Teaching Assistant/ Laboratory Coordinator - Supervised laboratory sections organic chemistry courses: maintained safe laboratory environment, dispensed hazardous reagents for undergraduate experiments, graded and corrected reports and exams.

Vanderbilt University Research Experience for High School Students (REHSS) **2013**

Educated students on proper lab techniques for microbial isolation and preliminary identification from soil samples collected from a local organic farm, aided in soil characterization for soils collected from various crop regions

Vanderbilt University Research Experience for Undergraduates **2009**

Educated undergraduate students on the methods associated with actinomycete fermentation, compound isolation, and structure elucidation. The results of which were published in 2010.

North Georgia College and State University **2004-2006**

Teaching Assistant/ Laboratory Coordinator - Supervised laboratory sections of Introductory and Organic Chemistry courses: prepared laboratory reagents and equipment for teaching and testing exercises, graded papers, and lab record notebooks.

Awards

Recipient of National Science Foundation Summer Research Scholarship
CRC Press Freshman Chemistry Award
Roy Bottoms Scholarship for Excellence in Chemistry (North Georgia College)
Most Outstanding Senior Chemistry Student (North Georgia College)
American Chemical Society Award for Undergraduate Student of the Year (Northeast Georgia Region)

Presentations

C.R. McNees, B.J. Herbert, N.A.P Kane-Maguire, J.F. Wheeler, Enantiomeric Seperations of $M(Diimine)_3^{3+}$ Transition Metal Complexes Using Cyclodextrin-Modified Chiral Capillary Electrophoresis. Poster Presentation at 56th SERMAC (Southeastern Regional Meeting of the American Chemical Society), 2004.

C. Ruth McNees Highly Sulfated Cyclodextrin for Enatiomeric Serparation via Capillary Electrophoresis. Oral presentation at Annual Honor's Day Academic Conference for Original Undergraduate Research, 2005.

Publications

Bioautographic Imaging Mass Spectrometry: Simultaneous discovery of secondary metabolites and their consequences

C. Ruth McNees, Michal Kliman, Dagmara K. Derewacz, et al.
In preparation for *Nature Methods*.

Structuring microbial metabolic responses to multiplexed stimuli via self-organizing metabolomics maps.
Cody R. Goodwin, Brett C. Covington, Dagmara K. Derewacz, **C. Ruth McNees** et al.
Accepted by *Chemistry and Biology* March 2015

Structure and stereochemical determination of hypogeamicins from a cave-derived actinomycete
Dagmara K. Derewacz, **C. Ruth McNees**, Giovanni Scalmani, et al.
Submitted Sept. 2013 to *Journal of Natural Products*.

Classification of natural product classes by ion mobility mass spectrometry.
Sarah M. Stow, Nichole M. Lareau, Kelly M. Hines, **C. Ruth McNees**, et al.
Accepted June 2013 for publication by Wiley-Blackwell for inclusion in "Natural Products Analysis: Instrumentation, Methods, and Applications."

Antimicrobial drug resistance affects broad changes in metabolomic phenotype in addition to secondary metabolism
Dagmara K. Derewacz, Cody R. Goodwin, **C. Ruth McNees**, et al.
Proceedings of the National Academy of Sciences of the United States of America Volume: 110 Issue: 6 Pages: 2336-2341
DOI: 10.1073/pnas.1218524110 Published: FEB 5 2013

Light-Induced Isomerization of Apoptolidin A leads to Inversion of C2-C3 Double Bond Geometry
Brian O. Bachmann, **Ruth McNees,** Bruce J. Melancon, et al.
Organic Letters Volume: 12 Issue: 13 Pages: 2944-2947
DOI: 10.1021/ol1009398 Published: JUL 2 2010

References

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