Drew University
College of Liberal Arts

Nutritional Studies on the Production of the Antibiotic Platensimycin by *Streptomyces platensis*

A Thesis in Biochemistry and Molecular Biology

By
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Abstract: Given a recent lack of research and development of antibiotic compounds, there is a great need for new drugs (Fernandes 2006). Resistant pathogens are becoming more prominent and are leading to an increasing number of infections that are difficult to cure (Jang *et al*. 2010). Platensimycin and platencin are two novel antibiotic compounds that are natural products produced by the actinomycete *Streptomyces platensis* (Wang *et al*. 2006). These compounds operate by inhibiting bacterial fatty acid synthesis, which is a novel and therefore uncommon antibiotic target. Platensimycin specifically inhibits the condensation-elongation enzyme FabF, and platencin is a dual inhibitor of FabF and FabH, another condensation-elongation enzyme. These compounds are active against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), as well as numerous other problematic pathogens (Singh *et al*. 2006). Unfortunately, they have shown very poor *in vivo* pharmacokinetics and have not yet been marketed. Because of their method of action against fatty acid synthesis, they are an important lead in the current struggle associated with antibiotic discovery and need for new antibiotic compounds. Efforts are being made to manipulate the structure of these compounds through natural production and through chemical synthesis to potentially improve pharmacokinetics (Jang *et al*. 2010). Platensimycin has recently shown activity as a diabetes drug in mouse models, making the study of these compounds even more important (Wu *et al.* 2011). In an effort to learn additional information about the biosynthetic pathways of these compounds and their regulation as well as to develop an optimal production medium for *S. platensis*, we have engineered a soluble, chemically-defined production medium from the original Merck and Co. complex, insoluble

production medium (PM1). The chemically-defined production medium, PM7, contains g/L 30 glucose, 50 lactose, 5 MOPS (3-(N-morpholino)propanesulfonic acid) buffer, and 0.5 ammonium sulfate. We have also determined that aspartic acid and potassium chloride (KCl) are stimulatory to antibiotic production when added to the production medium.

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Appendix A: List of Abbreviations Used

ADHBA: 3-amino-2,4-dihydroxybenzoic acid
AHBA: 3-amino-4-hydroxybenzoic acid
Ala: alanine
CoA: Coenzyme A
Cys: Cysteine
DHAP: dihydroxyacetone phosphate
DMADP: dimethylallyl diphosphate
ent-CDP: *ent*-copalyl diphosphate
FAD: flavin adenine dinucleotide
FASI: type one fatty acid synthesis
FASII: type two fatty acid synthesis
GGDP: geranylgeranyl diphosphate
Glu: glutamic acid
GSK: GlaxoSmithKline
HGT: horizontal gene transfer
His: histidine
HPLC: high performance liquid chromatography
LCMS: liquid chromatography mass spectroscopy
IDP: isopentyl diphosphate
IR: infared radiation spectroscopy
MEP: methylerythritol phosphate
MOPS: 3-(N-morpholino)propanesulfonic acid
MRSA: methicillin-resistant *Staphylococcus aureus*
NMR: nuclear magnetic resonance
Nz: no zone
PM1: production medium 1
PRSP: penicillin-resistant *Streptococcus pneumoniae*
PTM: platensimycin
PTN: platencin
TCA: citric acid cycle
Thr: threonone
Tr: trace
VISA: vancomycin intermediate *Staphylococcus aureus*
VRE: vancomycin-resistant enterococci

Introduction:

Antibiotics and Resistance

In the early 1900's, infectious disease was the leading cause of death around the world (Fernandes 2006; Livermore 2011). This problem was combated by penicillin, the first antibiotic, in 1944 (Fleming 1944). An antibiotic is any compound that inhibits the growth of bacteria or fungi (Livermore 2011). Following the discovery of penicillin, there was a large surge of antibiotic discovery and marketing, resulting in over 200 new compounds between 1940 and 1970. This time was known as the "Golden Age of Antibiotics" and is credited with the near doubling of life expectancy over the 20th century.

In addition to the drop off in discovery, there has been an immense increase in resistance to the compounds that are currently in use. Unfortunately, in the time following the "Golden Age," discovery of new antibiotics dropped off considerably, involing the deployment of only two completely new classes of compounds form 1970 to 2005 (clatworthy *et al*. 2007) (Hogberg *et al*. 2010). Resistant pathogens are becoming more and more prevalent, resulting in an increase in the number of deaths associated with infectious disease. In addition to resistance, there are several less prominent factors contributing to the need for new antibiotic drugs, including newly emerging infections. The lack of new drug discovery coupled with the drastic increase in resistance and other factors has led to a new for new antibiotic compounds and the present situation better known as the "antibiotic crisis".

Every class of antibiotic drugs has at least one, and often more than one, pathogen that shows resistance against it; moreover, every major pathogen is resistant to at least one type of antibiotic (Allahverdiyev *et al*. 2013). This situation is a problem because it generates a myriad of diseases that are difficult to treat and are often easily spread. Some of these resistant infections are seemingly rewinding time to the pre-antibiotic era as they have taken numerous lives. One particularly problematic pathogen has been methicillin-resistant *Staphylococcus aureus* (MRSA) (Klevens *et al*. 2007). It causes between 40,000 and 50,000 deaths per year, and can be found in numerous regions around the world. It is especially dangerous because it is commonly found in hospitals, where patients are generally more susceptible to infections. Another difficult pathogen as of late is vancomycin-resistant *Enterococcus* (VRE) (Courvalin 2006). It is another frequent hospital infection and while it is not known to be particularly virulent, it still poses a threat to global health. The organism can persist for long periods of time. It has been known to serve as a reservoir of resistance genes and frequently passes them along to other organisms nearby. Numerous other resistant pathogens, including *Mycobacterium tuberculosis*, are becoming a problem and causing an increasing number of deaths throughout the world (Allahverdiyev *et al*. 2013).

There are two main sources of antibacterial resistance in pathogens (Fernandes 2006; Allahverdiyev *et al*. 2013). The first source is the presence of resistance genes naturally coded for in the genome. Bacteria are extremely adaptable, able to live in extreme environments, and have had an extraordinarily long evolutionary history. These factors mean that bacteria have experienced a myriad of chemicals and may have already

developed resistance to compounds for which we are just realizing the antibiotic potential. In addition, many bacterial species live in close distances to one another, causing competition for resources. Often, the production of natural product antibiotics are a result of this competition, which results in emerging resistance to cope with the development and deployment of antibacterial compounds. In 2012, microorganisms that were isolated in a cave for up to 4 million years were determined to have certain antibiotic resistance genes (Bhullar *et al*. 2012). Not only was resistance to a number of different classes of compounds observed, but there was also evidence of previously unseen methods of resistance, such as an inducible daptomycin hydrolase. Moreover, the mechanism of the observed resistance includes a specific hydrolase enzymes which inactives the compound by hydrolyzing a key ester bond. The presence of such resistance is even more interesting because the enzyme responsible is inducible, meaning it is only present upon exposure to daptomycin. A similar study was done on a permafrost sample in Yukon at the Bear Creek site (D'Costa *et al*. 2011). The sample was removed from far enough down that it had been isolated for 30,000 years. It also harbored numerous bacteria with resistance genes.

The second reason for innate resistance in bacterial genomes is due to the rapid reproduction of bacteria and the high number of mutations that occur in their genomes (Levy and Marshall 2004). Because bacteria reproduce so quickly, mutations arise very frequently. The high mutation rate increases the possibility of random mutations allowing for antibiotic resistance. The high rate of reproduction encourages the spread of such a mutation, leading very quickly to populations of resistant bacteria.

The problem of vast innate resistance is compounded by the ease at which resistance can be spread within a particular population of bacteria (Hogberg *et al*. 2010). Any species or strain with resistance to a certain compound can easily pass their resistance on to other species or strains through horizontal gene transfer (HGT). HGT involves direct transfer of genetic material to different organisms, outside of the traditional parent to offspring transfer, and regardless of species. There are three main methods of horizontal gene transfer: conjugation, transformation, and transduction (Madigan *et al*. 2012). Conjugation involves the transfer of genetic material, usually a plasmid, between two cells through a temporary physical connection between the cells. Transformation involves a cell taking up genetic material from its environment and incorporating it into its own genetic material. Transduction involves the injection of genetic material into a cell by a virus, resulting in a utilization of the new genetic material. Horizontal gene transfer also occurs via transposons, mobile genetic elements that have the capability to translocate themselves within genetic material, including chromosomes and plasmids (Madigan *et al*. 2012). All of these events lead to rapid exchange of genetic material among bacteria and the sharing of antibiotic resistance (Levy and Marshall 2004). The capability for transfer and movement of genetic material is a major problem involved in the spread of antibiotic resistance, and is a major cause of the resistance observed today.

The increase in resistance has also been exacerbated by a number of other factors, mostly related to human behavior (Allahverdiyev *et al*. 2013). One example is related to human interaction with antibiotic drugs, specifically the overuse, improper use, and lack

of completion of antibiotic treatment. Currently, doctors are prone to prescribing antibiotics for illnesses that may not be necessarily caused by a bacterial infection, such as the common cold. In addition, people taking antibiotics do not always finish their course; they often begin feeling better after a few days and discontinue their medicine before they are supposed to (Fernandes 2006; Hogberg *et al*. 2010; Levy and Marshall 2004). This process results in an artificial selection for bacteria that are more resistant to antibiotics. The more bacteria are exposed to antibiotics, the more resistance is selected for, and the more it increases and spreads (Levy and Marshall 2004). Normally, within one individual whose course of treatment is complete, the susceptible bacteria are able to return and replace the resistant ones, which decreases the spread of resistance. However, if antibiotics are widespread and ever present, the susceptible bacteria are never able to regain ground, and become prominent, and thus the majority of the population is resistant. This concept is highlighted by numerous studies that show a positive correlation between the amount of antibiotic use and the amount of resistance present in a geographic area (Hogberg *et al*. 2010). Moreover, insufficient antibiotic treatment has been shown to lead to resistance to antibiotic compounds other than the one that was used for treatment (Kohanski *et al*. 2010). Kohanski and colleagues showed that non-lethal antibiotic treatment leads to the release of reactive oxygen species which cause DNA damage and mutations. The increase in mutations lead to an increase in resistance, and a trend toward multidrug resistance. The numerous ways that antibiotic resistance can emerge make the need for new antibiotics even more pressing.

As seen in Figure 1, resistance to the marketed antibiotics occurs very rapidly (Clatworthy *et al*. 2007). Within a few years of the development of a new compound, resistance occurs. For example, it took around five years before the resistance of penicillin was observed whereas it took less than five years for development of resistance to linezolid compounds, and around two years for development of resistance in daptomycin.

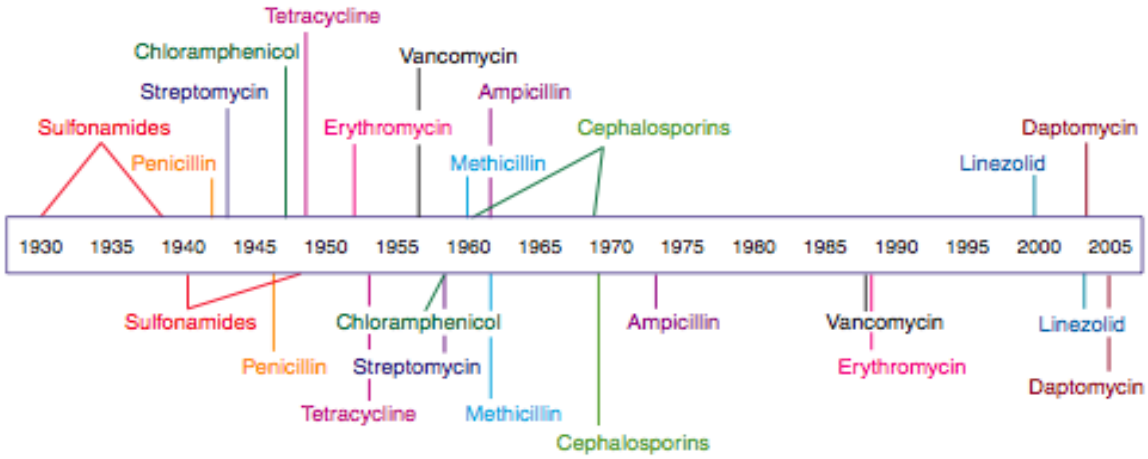

Figure 1: Timeline of Antibiotic Marketing Compared to Observed Resistance. Marketing date is represented on top and first observed resistance date is represented on the bottom. Resistance occurs shortly after introduction on the market, especially with newer drugs. (Image from: Clatworthy *et al*. 2007)

As explained, the rapid occurrence of resistance is a direct result of the presence of innate resistance in numerous genomes coupled with the capability of rapid spread associated with microorganisms (Allahverdiyev *et al*. 2013). Human health will always be in a constant arms race with these microorganisms. Overcoming resistance is a continual process; there will always be a need for the development of novel antibacterial drugs.

The Antibiotic Crisis and its Numerous Causes

The antibiotic crisis is the current predicament the world is facing in which new antibiotic drugs are desperately needed (Hogberg *et al*. 2010; Livermore 2011). This problem stems from the drop off in antibiotic discovery following the "Golden Age" coupled with a significant increase in resistance development. There are a number of other factors contributing to the crisis, including the emergence of new bacterial infections and an increase in food-borne pathogens. The only way to improve the state of the crisis is to investigate new antibiotic compounds

Lack of Antibacterial Drug Development

Despite the myriad of important and obvious reasons behind the need for new antibiotics, very few have been developed and not much new research is being conducted by large pharmaceutical companies (Demain and Spizek 2012). The number of new compounds with antibacterial properties has been declining steadily since the 1980's (Figure 2). Also, many of the new drugs being marketed are not completely new; they are slight modifications of compounds that have already shown success as drugs in that category. For antibiotics especially, lack of completely new structures is a problem because compounds with only slight modifications are less likely to be able to combat resistance for long enough to be significantly effective (Freire-Moran *et al*. 2011). Resistance is more likely to develop if a new compound operates with the same method of action as compounds for which resistance has already developed. These compounds may be effective at first, but are likely to become inactive relatively quickly.

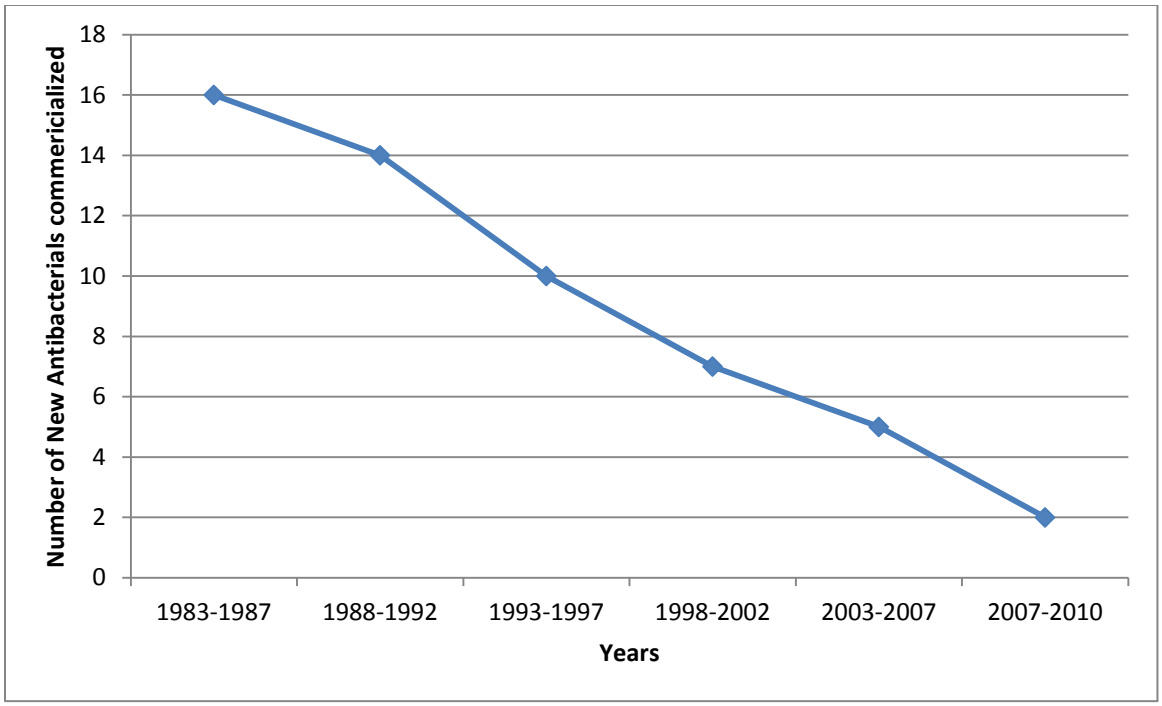

Figure 2: Summary of Antibiotic Commercialization**.** Number of new antibiotic compounds commercialized per four year interval from 1983 to 2010. Number steadily decreasing. Data from Demain and Spizek 2012.

There are a number of reasons behind the recent drop in new antibiotic discoveries (Malik 2008). One reason has to do with the reduced profit associated with antibacterial drugs. Pharmaceutical companies have realized that there is more money in marketing drugs that are taken every day over a lifetime, such as blood pressure medication, rather than those that are only taken on a temporary basis, like antibiotics. Most companies have shifted their interests accordingly, resulting in very few projects that are actually looking for new antibiotics (Fernandes 2006). Another reason for the lack of research is associated with the numerous mergers that have occurred among pharmaceutical companies (Malik 2008). Upwards of 40 major mergers took place between 1985 and 2005. The problem is that interests change and projects get dropped

when companies merge. Thus, the combined organization usually ends up with fewer projects than the sum of what each company had individually (Livermore 2011). A decrease in the total number of projects results in fewer projects that are focused on antibiotic development. The effects of the mergers are amplifying the already existent general lack of interest in antibiotic development. Additionally, after the golden age of antibiotics, new antibiotic compounds were deemed unnecessary because of the success that had been observed (Demain and Spizek 2012). It was not predicted that resistance and other problems would contribute to such a drastic need some 20 years later.

Another hurdle for antibiotic discovery is the increased length of time and complication of the process of drug trials necessary prior to marketing the drugs (Malik 2008). The time necessary for clinical trials doubled from 3 years to 6 years from 1982 to 2002 (Demain and Spizek 2012). It is estimated that is could take anywhere between 14 and 22 years to successfully get a drug on the market. This increased time also increases the funds necessary to market a drug to almost two billion dollars (Malik 2008). These increased costs have further encouraged the move away from antibiotic research for economical reasons. The time period necessary for this process reduces the time the drug can be marketed after a patent is issued before generic drugs are developed and marketed (Demain and Spizek 2012). Especially considering these costs, companies are further pushed toward developing drugs used for a longer time than antibiotics.

Assorted Other Factors Behind the Antibiotic Crisis

In recent years, the world has experienced a vast increase in newly emerging or re-emerging pathogens (DaSilva and Iaccarino 1999). Over the past four decades, at least 30 new diseases have emerged and spread rapidly. While not all bacterial, this group does include numerous serious bacterial diseases, such as Lyme disease and pathogenic *Escherichia coli*. New diseases will continue to emerge (Morens *et al*. 2004). Additionally, old diseases are spreading to new locations and causing severe infections in different groups of people around the world. One contributing factor is increased international travel. Frequent travel allows for pathogens to be easily spread to different continents, or carriers. The spread of disease trough travel is even more dangerous because people in distant populations may be especially susceptible to these new pathogens because they have not been exposed to such microbes.

Other contributing factors to the need for new antibiotics include high instances of antibiotics present in animal feed, the large increase in food-borne pathogens, and the drastic increase of deadly infections occurring in hospitals and nursing homes (Demain and Spizek 2012; Mole 2013). Many animal farmers use antibiotics in their feed as a way to increase growth of the animals (Mole 2013). In the United States in 2009, as high as 80% of the antibiotics sold went to animal feed. The continual levels of antibiotics present in farm animals allows for an increase in antibiotic resistant pathogens, including MRSA, which can be observed in the animals and in the humans that work closely with them. Food contamination has become a problem because of the increased processing

associated with food handling in current society. Over the last several years, there has been an increase in rapidly spreading infections common to hospitals and nursing homes that have claimed many lives (DaSilva and Iaccarino 1999). People in these environments typically have some type of medical problems than can compromise their immune systems, making them more susceptible to these infections. The larger number of people and their close proximity allow for rapid and continual spreading. New antibiotics are needed to combat these new sources of infection and disease spread.

Scientific Methods in Drug Discovery and Their Impact

Natural products are chemical compounds that are naturally produced by living organisms, normally as primary or secondary metabolites (Livermore 2011). Due to general biodiversity, especially in microorganisms, there is a vast number of natural products possible. Most of the successful antibiotics from the golden age, and numerous other biologically active compounds, were natural products and natural product derivatives (Singh and Barrett 2006). Microorganisms use a wide array of chemicals to survive in their environments, many of which have biological activity (Harvey 2007). While is it quite laborious to organize the microorganisms and screen the myriad of potential compounds for different types of potential activity, there is still a wealth of natural products that have yet to be explored. The abandonment of natural product screening is due to the expense and time necessary to complete such screening

The replacements for natural product screening are genomic observation accompanied by high throughput screening (Livermore 2011). Research consists of

elucidating the genomes of certain pathogens and observing all possible proteins that are absolutely necessary to survival. High throughput screening is then used to compare large compound libraries with these proteins to determine if any compounds in the library could potentially bind to and alter these critical proteins. One difficulty is that high throughput screening involves testing thousands of compounds at one time (Silver 2011). If a binding hit is generated from one screening, it is often difficult to determine which compound in the library is specifically responsible for the hit. Additionally, most compounds in the libraries are similar and were selected based on criteria believed to be associated with antibiotic compounds, including hydrogen-bonding capability. These methods have been unsuccessful because there really is no way to successfully predict what type of compounds will have antibiotic activity or compounds that could potentially bind to these protein targets because it limits the structure of the drug compounds.

Overall, high throughput screening has been, as yet, unsuccessful at yielding new antibiotics (Livermore 2011). Within SmithKline Beecham, now part of GlaxoSmithKline (GSK), five targets in a search for broad spectrum compounds were found with hits and were transferred to development. Two of these targets were not consistent across different species, making them ineffective as broad-spectrum targets. One target led to the discovery of a compound that is in a phase II drug trial, but the compound was found using other methods. The other two hits proved unhelpful as no antibiotics have been yielded by these methods in the 20 years they have been utilized. Another problem with this method is that compounds that bind to the highlighted target proteins are not guaranteed to be successful antibiotics (Fernandes 2006). For example,

in the cellular environment, they may not even be able to enter the bacterial cell or they may simply be removed before they are able to bind to their target. In addition, any compound that could be successful would have a high propensity for resistance development due to the specificity of the interaction and existence of only one binding target.

Drug Discovery

Due to the failure of engineered compounds in general, there has been a push back to natural product searches and screenings (Singh and Barrett 2006). Natural product screening is beneficial as it generally does not focus on a specific drug target and will thus lead to the observation of more compounds. Natural products are generally first screened simply for antibiotic activity by conducting a zone of inhibition assay against various organisms using the whole broth of the producing organism. Natural products are on average more complex and more likely to have antibiotic properties compared to engineered compounds (Demain and Spizek 2012). One new source of natural products being explored is marine microbes. Their exploration has resulted in numerous bioactive compounds including anti-cancer agents and antibiotics. Several have been successful enough to make it into drug trials. Another recently explored method of observing more natural product compounds is to alter the growth conditions of the organisms (Singh and Barrett 2006). Many prokaryotes produce different secondary metabolites in different environments. Thus, altering the growth conditions in the laboratory has the potential to bring out additional compounds that might have gone unnoticed.

Another strategy employing natural products that has been put to use is combinatorial chemistry (Fernandes 2006). This strategy involves the combination of altering gene expression in microbes that produce known antibiotic compounds and chemical modification of natural products in order to generate larger compound libraries or compounds with more efficacy or potency. In terms of chemical modification, there have also been studies that involve the addition of chemical groups common to drugs to natural products, in hopes that the addition will produce activity (Baltz 2008).

Screening Actinomycete for Antibiotic Compounds

One useful method of obtaining biologically-active compounds has been actinomycete screening (Genilloud *et al*. 2011). Actinomycetes are a group of filamentous Gram-positive bacteria containing a number of different species. Many of the species are common in terrestrial and marine soils, and are generally very widespread. They have been investigated due to their ability to produce numerous diverse and biologically-relevant compounds, including antibiotics such as cephamycin and caspofungin. The compounds produced have been known to have low toxicity, and to be effective at reaching and inhibiting their target (Baltz 2008). Unfortunately, it often takes searching through millions of different compounds to pinpoint the few that have biological activity; thus, a higher throughput method would be preferable.

The genome sequencing of actinomycetes indicates that most of the genes code for a substantial number of secondary metabolites, most of which are unknown (Genilloud *et al*. 2011). Having the potential to produce a vast number of compounds is

believed to be a general characteristic of actinomycetes. These secondary metabolites largely expand the number of compounds available to screen for antibiotic and/or types of biological activity. The problem is how to gain access to these compounds and utilize them (Baltz 2008). One method commonly used is to isolate the gene sequences that code for these secondary metabolites, attempt to predict their structures by analyzing the function of the genes present, ferment the bacteria in different media, and screen for the biological activity indicated by the predicted structure. This method, along with others like it, has led to the discovery of a number of biologically active compounds from actinomycetes, including Epoxyquinomicin C.

New screening methods to detect compounds that specifically inhibit a certain pathway or protein system have made it easier to analyze the wealth of compounds that can be gathered from actinomycetes (Singh *et al*. 2007). Previous methods that detect potential inhibitors using *in vitro* binding or inhibition studies were unsuccessful because many active compounds were ineffective in whole cell studies due to lack of ability to enter into the cell, making them ineffective drug candidates. Other methods going right to whole cell assays are also ineffective because it is often difficult to tell if the death of the bacterial cells is due to inhibition of a specific necessary protein or just general toxicity (Singh 2012). Employing bacterial strains expressing antisense RNA for a proposed target protein solves both the problems described above by allowing for isolation of a specific target in an *in vivo* assay. Introduction of antisense RNA results in decreased expression and thus smaller amounts of the target protein, making cells more sensitive to inhibitors of that protein (Singh *et al*. 2007). If a culture that was exposed to

antisense RNA for a specific target protein is more sensitive to a potential antibacterial compound than a wild-type strain, it can be concluded that the potential antibiotic compound acts on that target.

Targeting Fatty Acid Synthesis

When screening compounds for antibacterial activity, there are numerous targets that current drugs act on (Singh and Barrett 2006). These include cell wall production, various parts of bacterial ribosomes, several aspects DNA synthesis, and certain aspects of metabolism. One promising newer target has been bacterial fatty acid synthesis (Qiu 1999). Bacteria utilize type II fatty acid synthesis (FASII); due to the roles and assembly of the selected enzymes, they are significantly different from the enzymes found in the human fatty acid synthesis (type I fatty acid synthesis, FASI). The main difference between the two systems is that FASI is carried out by a large multifunctional enzyme whereas FASII is carried out by multiple smaller enzymes. The significant differences in the key enzymes of these pathways makes the enzymes present in FASII useful for the discovery and development of antibiotic compounds with extremely low toxicity (Manallack *et al*. 2008).

FabF is an elongation and condensation enzyme vital to the FASII pathway. The reaction carried out by FabF is very similar to a Claisen condensation (Figure 3). The acyl carrier protein (ACP) conducts a transfer of the growing chain to the FabF enzyme, as a linkage with a cysteine residue in the active site of FabF is formed, resulting in a thioester intermediate. The formation of the thioester intermediate results in a

confirmation change in the enzyme by which a phenylalanine residue swings away from the malonyl-ACP binding site, resulting in the open confirmation of FabF. In the open confirmation, manonyl-ACP binds to the enzyme and is decarboxylated, resulting in a carbanion. The carbanion is able to attack the thioester intermediate, regenerate FabF, and form a β-ketoacyl substrate. FabH is an elongation initiation enzyme responsible for the start of the FASII pathway (Wang *et al*. 2007). It is responsible for the catalysis of a similar Claisen condensation between acetyl-CoA and malonyl-ACP, resulting in the production of acetoacetyl-ACP. The acetoacetyl-ACP produced enters the pathway through a reaction with the FabF β-ketoacyl substrate.

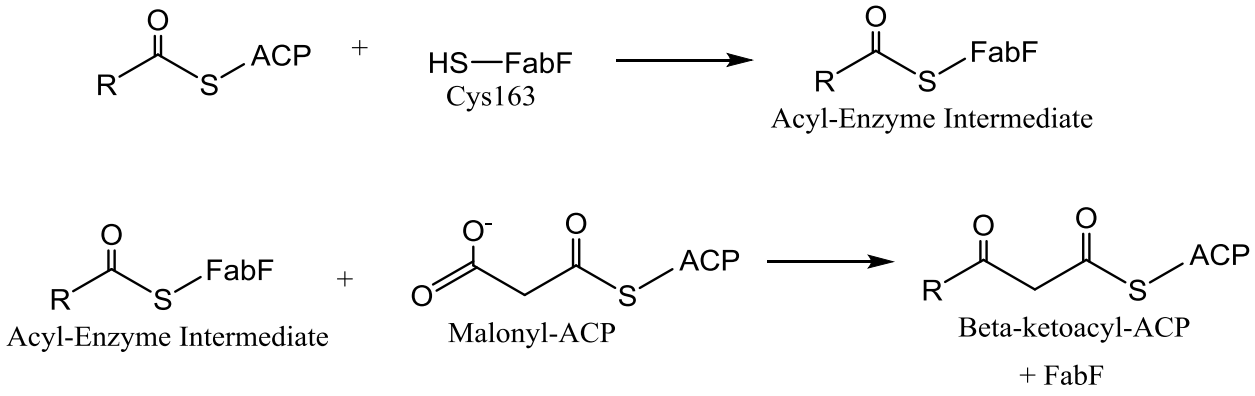

Figure 3: Reaction Catalyzed by FabF. Acyl carrier protein **(**ACP) transfers the fatty acid chain to Cys163 on FabF, forming the acyl-enzyme intermediate. The acyl enzyme intermediate reacts with malonyl attached to the ACP to form β-ketoacyl-ACP.

FabF along with FabH are well conserved among pathogenic species, making them good drug targets (Young *et al*. 2006). Two known antibacterial compounds that act on FabF and FabH are cerulenin and Thiolactomycin. Cerulenin inhibits FabF by forming a covalent linkage with the cysteine residue in the active site. Thiolactomycin

inhibits FabF and FabH binding of malonate by competitive inhibition (Qiu 1999). Because of the success of the FASII targets, numerous studies and assays have been developed and conducted to search for active compounds that work by inhibiting FabF and/or FabH (Young *et al*. 2006). The assays developed include antisense whole cell assays and some *in vitro* assays as well. These new assays are more beneficial than the previously used ones because they search for a specific target and reduce isolation of compounds that have unspecified toxicity rather than antibacterial activity. The new screening methods using FabF led to the discovery of platensimycin and platencin (Singh *et al*. 2006).

Discovery of Platensimycin and Platencin

Using the new high-throughput antisense RNA screening method, the compounds platensimycin and platencin were discovered by Merck and Co. to have potent antibacterial activity by way of inhibition of fatty acid synthesis (Singh *et al*. 2006). Platensimycin and platencin are produced by the actinomycete *Streptomyces platensis;* strain MA7327 is the primary platensimycin producer and produces small amounts of platencin as well, although strain MA7331 is the primary platencin producer (Manallack *et al*. 2008). These species were discovered by Merck in soil samples from Africa in 2005 and Spain 2009, respectively. They were tested using a fatty acid synthesis II pathway assay developed by Kodali and colleagues; the new pathway utilizes long-chain acyl-CoA rather than acetyl-CoA and malonyl-CoA to monitor the action of the pathway (2005). This change allows for a focus specifically on the actions of the elongation

enzymes (FabF and FabH) without the confusion of initiation and termination steps of the pathway.

Structures for these compounds were determined using a number of methods including X-ray crystallography, IR (infared radiation spectroscopy), 13C NMR (nuclear magnetic resonance spectroscopy), 1H NMR, HPLC (high performance liquid chromatography), and 2-D COSY and TOCSY NMR (Manallack *et al*. 2008). Platensimycin has a molecular formula of $C_{24}H_{27}NO_7$ and a molecular weight of 441 g/mol. It contains a 3-amino-2,4-dihydroxybenzoic acid and a C-17 pentacyclic enone with an ether ring joined by an amide bond (Singh 2012), as seen in Figure 4. Platencin is slightly smaller, but very similar to platensimycin with a molecular formula of $C_{24}H_{25}NO_6$ and a mass of 425.47 g/mol. The main structural difference is the presence of a ketolide portion lacking the cyclic ether ring seen in platensimycin, as seen in Figure 4.

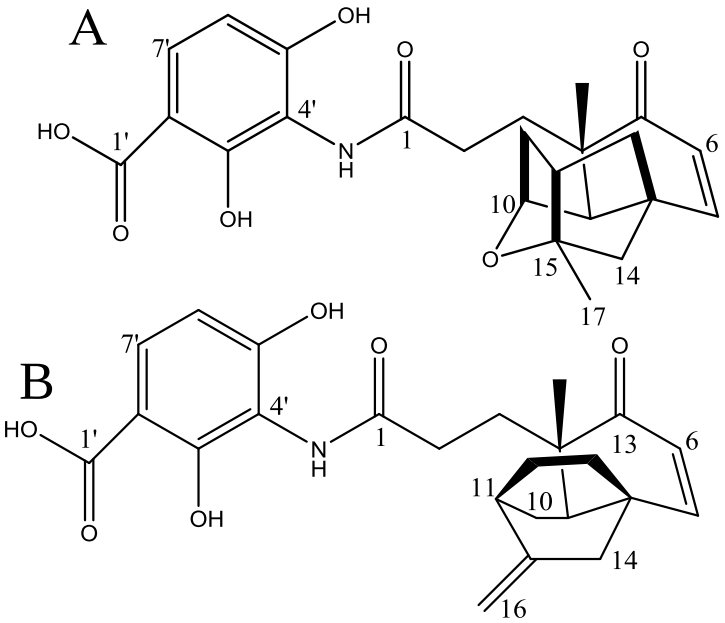

Figure 4: Structure of platensimycin and platencin. A: platensimycin. B: platencin

Importantly, these broad spectrum compounds are active with more potency than similar compounds against several Gram positive resistant pathogens including: vancomycin-resistant enterococci (VRE), penicillin-resistant *Streptococcus pneumoniae* (PRSP), and methicillin-resistant *Staphylococcus aureus* (MRSA) (Tiefenbacher and Mulzer 2009). Platensimycin has also shown activity against *Mycobacterium* species, despite their variations in the fatty acid synthesis pathway, including various strains of *M. smegmatis*, *M. bovis*, and *M. tuberculosis*. This activity is important as tuberculosis is a worldwide problem and is known for its frequent multi-drug resistance (Brown *et al*., 2009). Because platensimycin and platencin operate by inhibiting bacterial fatty acid synthesis, they have very low cytotoxicity (Allahverdiyev *et al*. 2013). Unfortunately, their pharmacokinetics are very poor, as successful *in vivo* treatment requires continuous intravenous injection. The compounds are cleared from the body and collected in the liver before they can successfully encounter and kill the pathogenic bacteria. Thus platensimycin and platencin are currently not clinically usable as antibiotics.

To confirm the method of action of these compounds, assays were conducted to rule out inhibition of DNA, RNA, and cell wall synthesis (Manallack *et al*. 2008). Through a direct binding assay, it was determined that platensimycin inhibits fatty acid synthesis in bacterial cells by inhibiting the condensing enzyme FabF. Inhibition by direct interaction with the protein was confirmed by observing increased inhibition of mutant bacteria that underexpress FabF and decreased inhibition of mutant bacteria that overexpress FabF (Jayasuriya *et al*. 2007). Platensimycin was found to bind to an acyl-enzyme intermediate of the FabF enzyme as part of the complex double displacement

mechanism (Figure 5). Platensimycin was tested for inhibition of FabH, another key condensation-elongation enzyme, but very little binding and inhibition were observed. In isolated binding assays, platencin was found to bind to an acyl-enzyme intermediate on FabF; however, it bound with lower affinity than platensimycin (Manallack *et al*. 2008). Interestingly, platencin was able to bind to an acyl enzyme intermediate of the FabH with more than four times the affinity seen in platensimycin.

The differences in inhibition of the two enzymes can be explained by the slight structural differences between platensimycin and platencin (Wang *et al*. 2006). Studies indicated that in this altered acyl state of FabF, the phenylalanine at amino acid position 400 (Phe400) swings out into an open confirmation, generating space for platensimycin to bind. In this confirmation, the benzoic acid ring in platensimycin is able to favorably stack with the phenylalanine in FabF, whereas in the closed confirmation, an interaction with platensimycin would create steric clashes with the phenylalanine. The benzoic acid ring of platensimycin may interact with the key catalytic residues of FabF, specifically the histidine at positions 303 (His303) and 340 (His 340) which are part of the histidine-histidine-cysteine (His-His-Cys) catalytic triad observed in the active site of many enzymes (Wang *et al*. 2006; Singh *et al*, 2006). Platencin also interacts with His303 and His340 through a salt bridge with its carboxylic acid portion, however, it lacks the oxygen in the pentacyclic ring (Singh 2012). In platensimycin, this oxygen participates in a hydrogen bond with the threonine at position 270 (Thr270), the lack of which is believed to contribute to platencin's lower affinity for FabF. While equally important and useful, platencin is not a major focus of this project. It shares a biosynthetic pathway with

platensimycin in the strain we utilize, *S. platensis* MA7327, but is only produced in minor quantities, as compared to platensimycin.

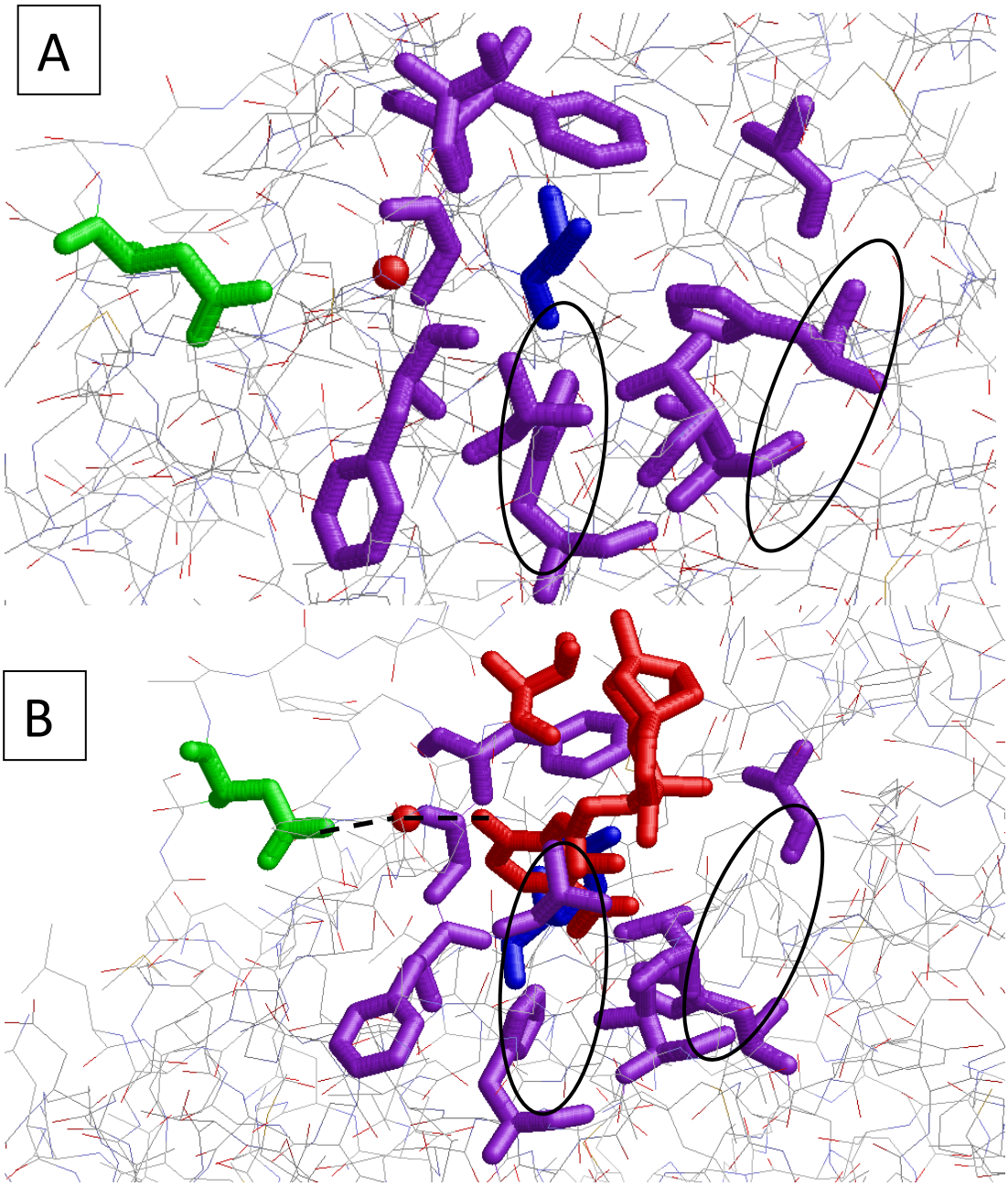

Figure 5: Active Site of FabF. Purple sticks are active site residues including: Alanine at position 309 (Ala309), threonine at position 307 (Thr307), Thr305, His303, His340, Gly399, Phe398, Phe400, Asp265, and Ala207. Red sphere is water. Green stick residue is Thr270. Blue stick is residue at position 163, Cys in A and Gln in B; part of mutation made to imitate acyl-enzyme intermediate. Ovals indicate changes in residues following mutation. A: Wild type active site. B: C163Q mutant active site with platensimycin bound; red sticks are platensimycin. Dotted lines represent hydrogren bond between platensimycin 5' oxygen, water, and Thr270. Adapted from Manallack *et al*. 2008

Platensimycin Analogs

Through analysis of *S. platensis* fermentation, numerous naturally produced platensimycin analogs have been discovered (Zhang *et al*. 2009). Exploration of the structure and activity of these analogs is a good resource to gain further understanding about the interaction between the antibiotic and its target. Further, studying analog structures can help pinpoint which contacts between platensimycin and FabF are most important for inhibition, which could assist in future drug design and screening.

While all but two of the analogs have not been more active or potent than the original compound, their structural differences provide insight into method of action and binding of platensimycin. The first of such compounds is known as platensimycin B_4 and was discovered with its methyl ester (Zhang *et al*. 2009). It is a glucoside of platensimycin at the 5' position on the oxygen off the aromatic ring; otherwise the structures are otherwise identical (Figure 6B). These compounds were observed for activity against *Staphylococcus aureus* in a cell-free assay; platensimycin B_4 was 320 times less active than platensimycin and the methyl ester was 80 times less active than platensimycin. All cell-free assays indicated that platensimycin B_4 is much less active than platensimycin. In a whole-cell assay against *S. aureus*, platensimycin B_4 showed 2500 times lower activity than platensimycin. The observed significant decrease in activity in a cell-based assay indicates that not only is the altered structure less active, it may also be less able to enter the cells. The crystal structure of the acyl intermediate of FabF from *Escherichia coli* in complex with platensimycin indicates that the 5' oxygen is

involved with a hydrogen bond with a water molecule that is also hydrogen bonded to the glutamic acid at position 265 (Glu-265) (Figure 5)(Wang *et al*. 2006). The addition of a glucose group would certainly disrupt this interaction, which would remove stability in the binding to the enzyme.

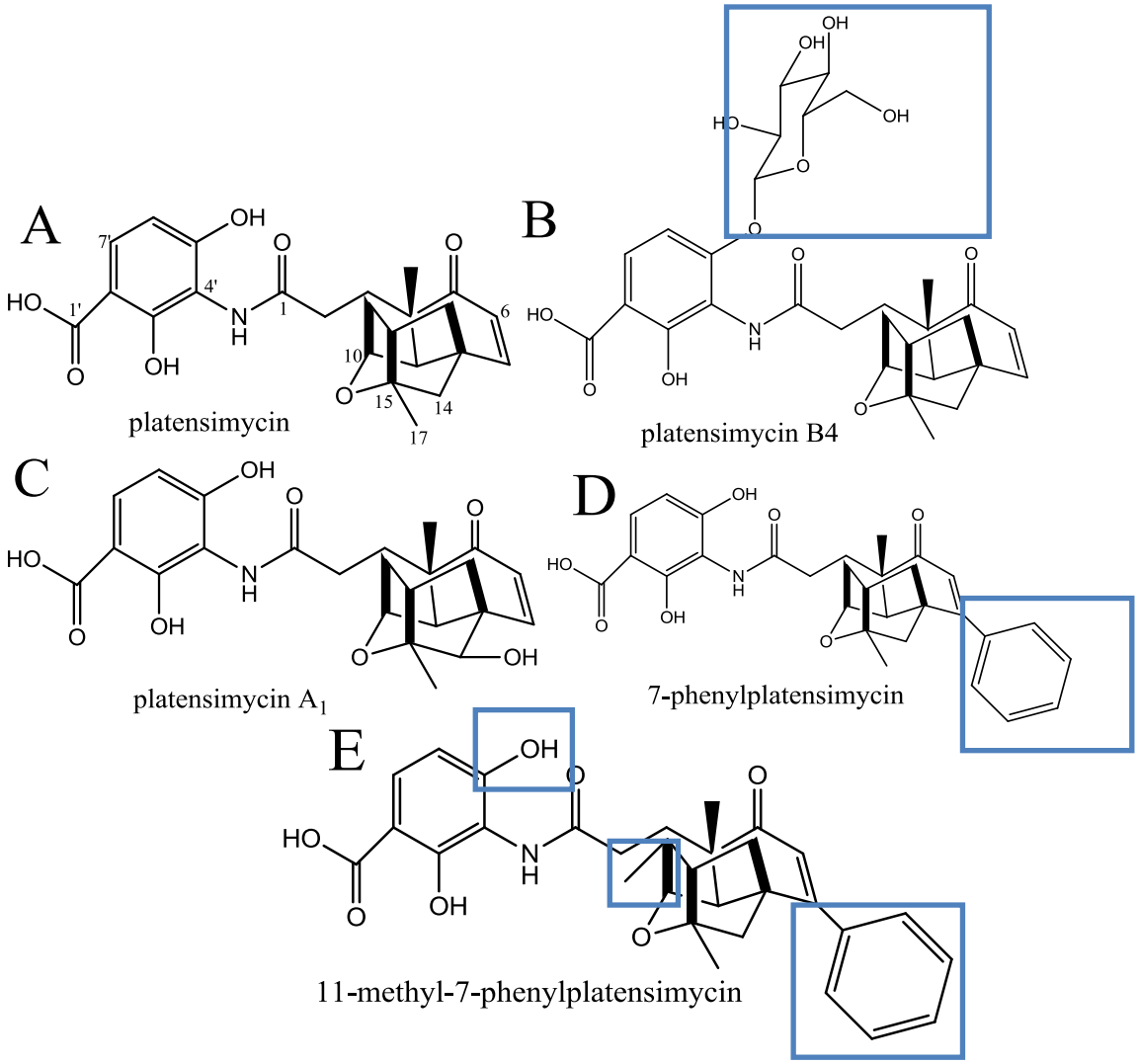

Figure 6: Analogs of Platensimycin. Boxes highlight differences from platensimycin. A:platensimycin. B: Platensimycin B_4. C: Platensimycin A_1. D: 7-phenylplatensimycin. E: 11-methyl-7-phenylplatensimycin

The second such structure is known as platensimycin A_1 and was also identified in 2009 (Singh *et al*. 2009). The only structural difference present is an additional hydroxyl group at carbon 14 on the tetracyclic enone group of platensimycin (Figure 6C); the methyl ester of this compound was isolated as well. These compounds were more active than platensimycin B_4 in both cell-free and cell-based assays using *S. aureus*, but were still significantly less active than the original structure. A structure of FabF bound with platensimycin A_1 was observed and indicates that the additional hydroxyl group results in the rotation of the inhibitor within the binding pocket, resulting in changes in several key interactions. The inhibitor is shifted away from the catalytic histidine at position 320 by 0.04 Å. The ether-oxygen hydrogen bond formed with Thr270 is increased by 0.27 Å and the hydrogen bond between the carbonyl at position 5 and the backbone nitrogen of the alanine at position 309 (Ala309) is decreased by 0.24 Å. It is unclear how the hydroxyl group results in this rotation, but it is clear that these interactions are specific and very significant to the inhibitory effects on the FabF enzyme and thus the efficacy of the compounds as antibiotics.

In addition to the discovery of new analogs, a significant effort has been put into synthesizing analogs of platensimycin to increase activity or to improve pharmacokinetics (Jang *et al*. 2010). The tetracyclic enone region of platensimycin could has the potential to act as an electrophile in an *in vivo* system, which would contribute to the poor pharmacokinetics (Shen *et al.* 2009). The potential electrophile activity led to an effort to attempt to modify this region of the compound. Several analogs were generated but only a few showed activity against wild type *S. aureus*, and in the cell-free FASII

assay. Activity experiments showed that the enone orientation is very important because it directs the location of the carbonyl that forms an interaction with the backbone amide of Ala309. The lack of activity of most of the enone modifications reflects the importance of the interaction with Ala309 for inhibition. Furthermore, the analogs that showed significant activity maintained the orientation of the carbonyl interacting with Ala309.

An alteration to the original structure that seems to be beneficial is the addition of a phenyl group at the 7 position (Jang *et al*. 2013). Two analogs with this added phenyl group, 7-phenylplatensimycin and 11- methyl-7-phenylplatensimycin (Figure 6D and E), showed better activity than platensimycin against MRSA and VISA; these are the only two analogs that have shown greater activity in any type of assay. Observations of the interactions between the inhibitor and FabF in the crystal structure indicate that the additional phenyl group would not interfere with the positioning of the carboyl and its alignment with Ala309 and may make this interaction stronger. The structure also indicates that this position is far enough away from the other key interactions that it would not disrupt them.

Platensimycin Activity as Diabetes Drug

Recent research has shown that platensimycin also has potential as a type 2 diabetes (diabetes mellitus) drug (Wu *et al*. 2011). This syndrome is characterized by hyperglycemia due to lack of insulin secretion and/or lack of insulin function (Berg *et al*. 2012). It can lead to complications with vision, joints, and kidney function. In 2010,

approximately 312 million people died as a result of type 2 diabetes (WHO 2013). Fatty acid synthase (FAS) is the mammalian equivalent to FabF, and has implications in diabetes and other metabolic problems (Radenne *et al*. 2008). Radenne and colleagues previously established that insulin is an activator of FAS by way of gene expression and glucogon is an inhibitor of fatty acid synthase. Recent studies showed that gene expression of fatty acid synthase was elevated in adipose tissue in individuals with type 2 diabetes and obesity, generating an association between diabetes and elevated activity of fatty acid synthase (Berndt *et al*. 2007). Studies involving deuterated platensimycin showed that when administered in humans, platensimycin is flushed to the liver and accumulates there before it is removed from the body (Singh 2012). The known accumulation in the liver and the association of increased activity of fatty acid synthase coupled with type 2 diabetes generated the idea to test platensimycin as a diabetes drug by way of inhibiting fatty acid synthase (Chakravarthy *et al*. 2005). This idea was especially significant because type 2 diabetes is commonly associated with FAS dysfunction, specifically in the liver. Platensimycin was determined to inhibit both mouse and human fatty acid synthase exclusively while not affecting sterol synthesis in either organism (Wu *et al*. 2011). Initial studies confirmed that platensimycin was collected in the liver and inhibited fatty acid synthesis only in the liver following oral and injection dosing in mouse models, even at doses up to 200 mg/kg. Because platensimycin was able to inhibit fatty acid synthase, it also inhibited fatty acid oxidation in liver cells but way of accumulation of malonyl CoA. This combination led a decrease

of liver triglyceride levels and increased glucose uptake after four weeks of treatment in mouse models.

Biosynthetic Pathways

An important wealth of information about these compounds and potential ways to improve them can be gathered by learning about their biosynthetic pathway and its regulation (Suzuki *et al.* 2006). Isotopic feeding studies using ^{13}C coupled with NMR revealed that both pyruvate and acetate are linked to the synthesis of the 3-amino-2,4-dihydroxybenzoic acid (AHDBA) portion of the antibiotic molecules, through a 3-amino-4-hydroxybenzoic acid (AHBA) intermediate (Herath *et al*. 2007; Herath *et al.* 2008). As seen in Figure 7, when carbon 1 on the acetate is labeled, the signal is seen in carbons 2' and 7' in platensimycin. When carbon 2 on the acetate is labeled, the signal is seen on carbons 1' and 6' in platensimycin and platencin. Interestingly, the acetate carbons are incorporated in a tail to tail manner, which is indicated by the signal results when carbon 1 and carbon 2 on the acetate are labeled simultaneously. The tail to tail incorporation suggests that this process is occurring in connection with the citric acid cycle. As seen in figure 7, when carbon 3 on the pyruvate is labeled, the signal is seen on carbons 2', 3', and 7'. When carbon 2 on the pyruvate is labeled, the signal is seen on carbons 1', 6', and 4'. When both carbons 2 and 3 are labeled, intact incorporation between carbons 1 and 2, carbons 3 and 4, and carbons 6 and 7 is observed. Because the acetate and the pyruvate are signaled on some of the same carbons, it can further be determined that the citric acid cycle may be involved because of the conversion of pyruvate to acetyl CoA by

the pyruvate dehydrogenase complex between glycolysis and the citric acid cycle (Berg *et al*. 2012).

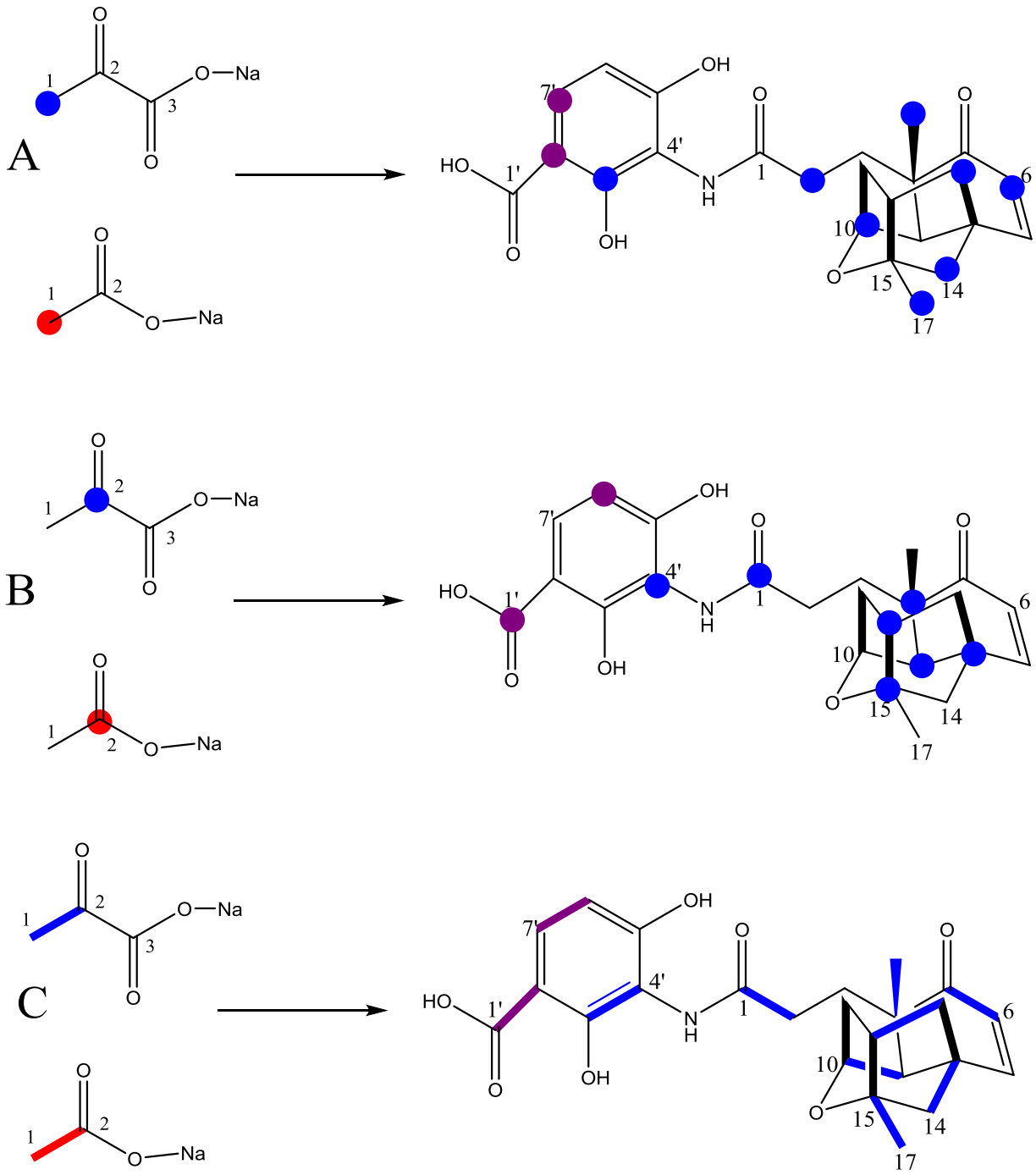

Figure 7: 13C Feeding Studies in Platenimycin. Acetate is colored red, pyruvate is colored blue, and positions that had a signal for both pyruvate and acetate are in purple. A: pyruvate and aceate carbon one labeled. B: pyruvate and acetate carbon two labeled. C: pyruvate and acetate carbons one and two labeled. Signal shows intact, tail to tail incorporation of acetate and pyruvate carbons one and two. Adapted from Herath *et al*. 2007.

Another streptomycete species, *Streptomyces griseus*, was found to synthesize AHBA from aspartate-4-semialdehyde and dihydroxyacetone phosphate (DHAP), a glycolysis intermediate (Suzuki *et al*. 2006). Using knockout studies, Suzuki and colleagues showed that only two genes were necessary for this synthesis, *gril* and *griL*. Upon the sequencing of the platensimycin (PTM) and platencin (PTN) gene clusters, genes homologous to *gril* and *griL* were characterized (PtmB1 and PtmB2; PtnB1 and PtnB2 respectively) (Smanski *et al*. 2011). Using knockout studies, these genes were shown to be responsible for AHBA synthesis in platensimycin and platencin. Mutants lacking these genes produced no platensimycin or platencin, but produced both platensic and platencinic acids, the other half of the platensimycin and platencin structures. In the knockout mutant strains, platensimycin and platencin production was rescued by the addition of AHBA, solidifying the conclusion that PtnB1 and PtnB2 are responsible for AHBA synthesis. These studies also indicated the presence of a separate gene to synthesize ADHBA from AHBA, which has been identied as PtmB3. Because of their homology to *gril* and *griL*, it is likely that they also utilize DHAP and aspartate-4-semialdehyde as starting material. The use of aspartate-4-semialdehyde was confirmed by acetate and pyruvate labeling studies. As laid out in Figure 8, between glycolysis and the citric acid cycle, pyruvate is converted to acetate, which enters the citric acid cycle (Berg *et al*. 2012). In the citric acid cycle, two molecules of acetyl CoA are incorporated into two molecule of oxaloaceate, which is also involved in the synthesis of aspartic acid (Berg *et al*. 2012) Aspartic acid is converted to aspartate-4-semialdehyde to serve as starting material for AHBA synthesis. The importance of aspartate-4-semialdehyde, and

thus aspartic acid, in this portion of the biosynthetic pathway makes it candidate for regulation of the synthesis of platensimycin.

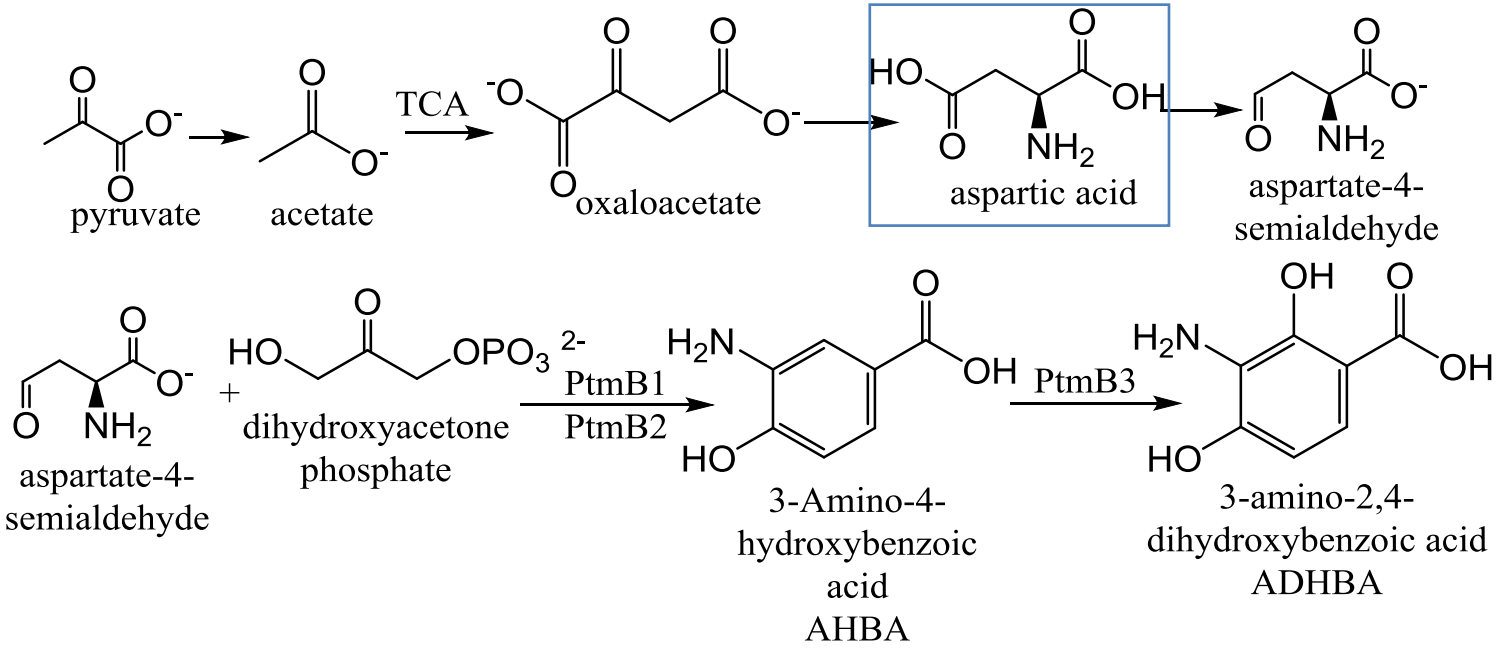

Figure 8: Biosynthetic Pathway of Platensimycin and Platencin: synthesis of the ADHBA moiety. Shows precursors and connection with the citric acid cycle; genes above arrows represent enzymes responsible for that step, Ptm refers to a gene found in the platensimycin gene cluster and ptn refers to a gene in the platencin gene cluster; TCA= citric acid cycle; box highlights incorporation of aspartic acid (Adapted from Herath *et al*. 2007, Smanski *et al* 2012, and Dairi 2005).

The proposed biosynthesic pathway of the platensic acid and platencinic acids is largely based on the genes located in the platencin and platensimycin gene clusters. Herath and colleagues determined that platensic acid was synthesized through an *ent*-kaurene intermediate and that platencinic acid was synthesized utilizing an *ent*-antiserene intermediate (Figure 9) (Herath *et al.* 2007; Herath *et al.* 2008). As seen in Figure 7, the labeling from pyruvate is also manifested in the tetracyclic enone moiety (Herath *et al*. 2007). Coupled with similar labeling studies using glycerol, Herath and colleagues hypothesized that this portion of the structure is synthesized via the methylerythritol phosphate (MEP) or non-mevalonate pathway in terpenoid synthesis. Activity of the MEP pathway was confirmed by a lack of labeling observed when cultures were grown on media containing labeled mevalonate. In addition, the platensimycin and platencin gene clusters contains genes homologous to genes involved in the MEP pathway (Smanski *et al*. 2012). As seen in Figure 9, this pathway utilizes pyruvate and glyceraldehyde-3-phosphate as starting material. The first reaction consists of the combination of pyruvate and glyceraldehydes-3-phosphate through a decarboxylation reaction catalyzed by 1-deoxy-d-xylulose 5-phosphate (DXR) synthase, which utilizes a thiamine cofactor (Dairi 2005). The MEP pathway produces both dimethylallyl diphosphate (DMADP) and isopentyl diphosphate (IPP), which are converted to geranylgeranyl diphosphate (GGDP) via GGDP synthase coded for by PtmT4 and Ptnt4 (Smanski *et al.* 2012). GGDP is then converted to *ent*-copalyl diphosphate (*ent*-CDP), via *ent*-CDP synthase coded for by PtmT2 and PtnT2. For platensimycin synthesis, the *ent*-CDP is converted to *ent*-kaurene via *ent*-kaurene synthase encoded by Ptm3. The

ent-kaurene synthase could also convert *ent*-CDP into *ent*-antiserene, explaining the small amount of platencin production associated with *S. platensis* MA7327; however, this hypothesis was disproved using a mutant strain lacking the *ent*-kaurene synthase. This strain produced no platensimycin and significantly higher amounts of platencin, indicating a completely independent mechanism for platencin synthesis. Exploration of the platencin gene cluster led to the discovery of an independent *ent*-antiserene synthase, which also utilizes *ent*-CDP as starting material, making this last common intermediate and a point of competition in the pathway. The *ent*-kaurene synthase is able to utilize more of the *ent*-CDP for platensimycin synthesis, explaining why platensimycin is the main product of *S. platensis* MA7327 and platencin is produced only in small quantities. One of the key enzymes in the MEP pathway, DXR synthase, is dependent on a thiamine cofactor, making that a compound of interest for regulation of production of platensimycin and platencin.

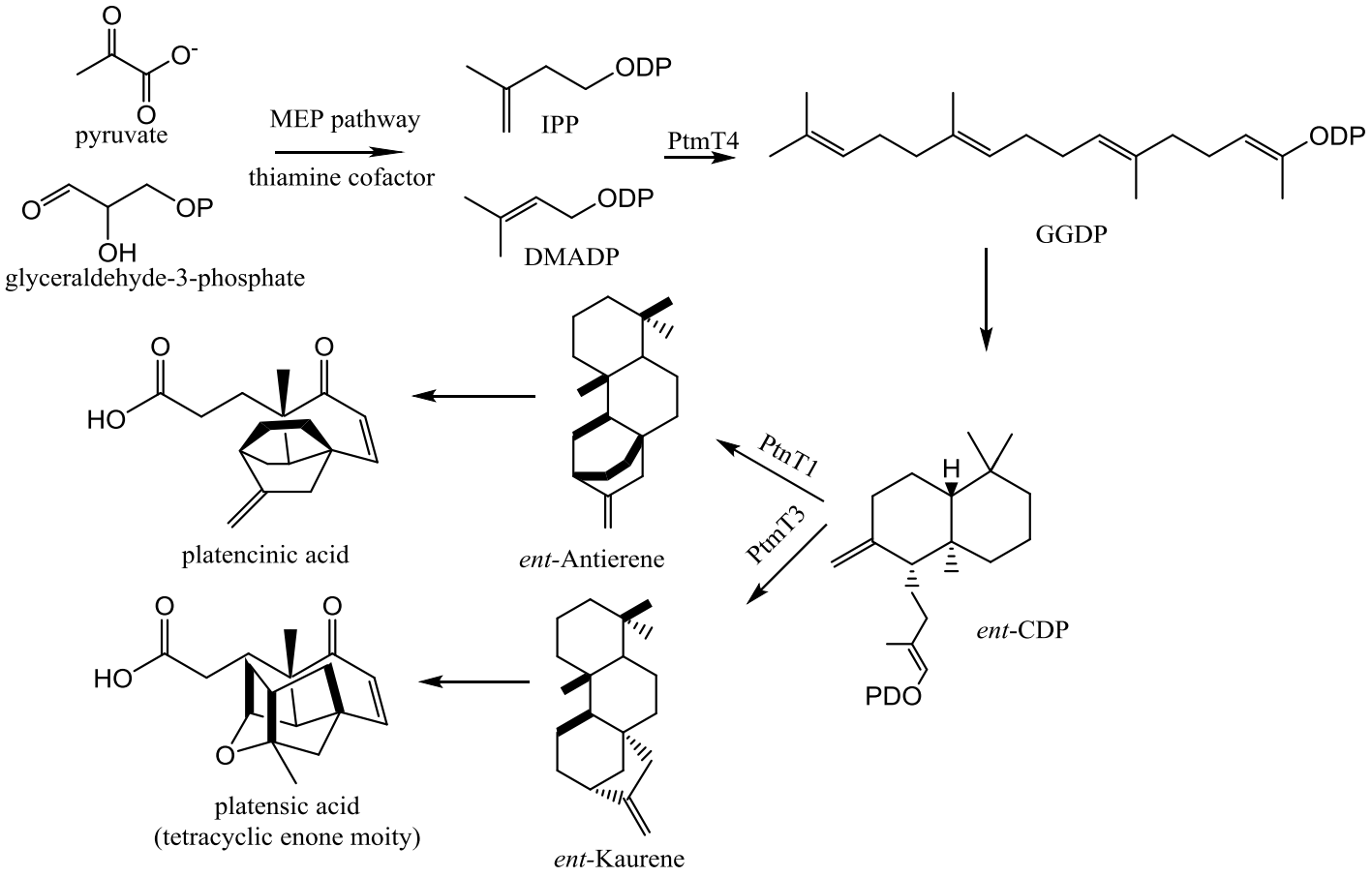

Figure 9: Biosynthetic Pathway of Platensimycin and Platencin: Synthesis of Platensic and Platencinic Acids. MEP is the methylerythritol phosphate; IPP is isopentyl diphosphate, DMADP is dimethylallyl diphosphate; GGDP is geranylgeranyl diphosphate; *ent*-CDP is *ent*-copalyl diphosphate. Shows precursors, connection with MEP pathway, use of thiamine, and independent intermediates in platencinic and platensic acid synthesis; genes above arrows encode enzymes responsible for that step; Ptm refers to a gene found in the platensimycin gene cluster and ptn refers to a gene in the platencin gene cluster. (Adapted from Herath *et al*. 2007, Smanski *et al* 2012, and Dairi 2005).

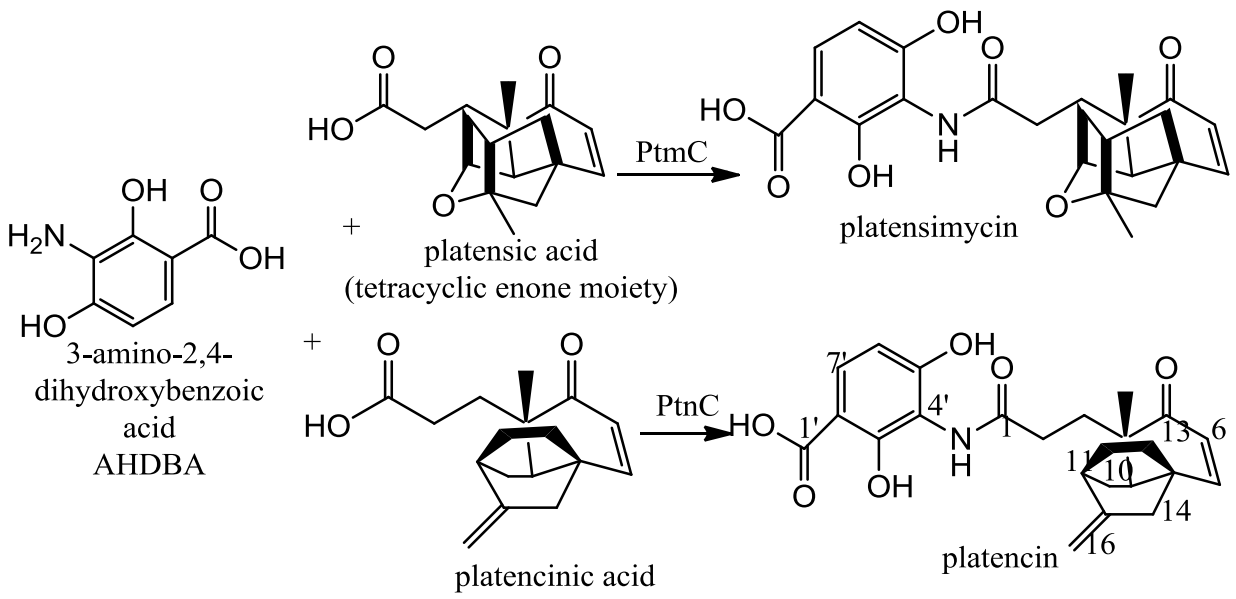

Figure 10: Biosynthetic Synthesis of Platensimycin and Platencin: Synthesis of Platensimycin and Platencin. Emphasizes that synthesis occurs through combination of platensic and platencinic acids with ADHBA, which are synthesized independently. Genes above arrows encode enzymes responsible for those steps, Ptm refers to a gene found in the platensimycin gene cluster and ptn refers to a gene in the platencin gene cluster (Adapted from Herath *et al*. 2007, Smanski *et al* 2012, and Dairi 2005).

Current Project

When Merck and Co. discovered platensimycin and platencin, they devised an insoluble, complex (i.e., not chemically-defined) production medium (PM1) that encouraged the production of platensimycin and platencin. PM1 contains (g/L) 5 Amberex, 40 yellow cornmeal, and 40 lactose. They devised a seed medium that is also complex, which is utilized to develop a seed culture. When the Demain group began this project at Drew University, they utilized these media. The overall goal of the project has been to learn more about the biosynthesis and regulation of the biosynthetic pathways of platensimycin and platencin, and to devise a production medium to maximize production of these compounds. While the compounds are not currently usable as antibiotics,

because they are cleared out of the body too quickly, learning about maximizing their production is a helpful contribution to the studies that are attempting to chemically modify the structures to improve pharmacokinetics and for the use of platensimycin as an anti-diabetes drug. Learning about the biosynthetic pathway and its regulation may also provide information on how to manipulate the products being produced, in an effort to find compounds with more favorable pharmacokinetics.

The first step in this procedure was the development of a soluble, chemically-defined production medium, starting from the complex, insoluble PM1. Chemical definition allows for absolute identification of all components and quantities present in the medium and therefore, the effects of additives. In addition, modification of the production medium can alter the distribution of secondary metabolites produced in the fermentation (growth of microorganism in liquid medium for the production of a specific compound). As mentioned above, actinomycetes have vast numbers of genes for the production of these metabolites, many of which are only produced under certain conditions (Wang *et al*. 2011). A chemically-defined medium ensures that the compounds being produced are consistent (Jose *et al*. 2010). The possibility of additional fermentation products is one reason for the continued monitoring of the presence of platensimycin using a mass spectrometric assay (developed previously by Drew student Christian Maggio with the help of Dr. Gullo, and currently by me using a mass spectrometric assay and high performance liquid chromatography (HPLC) and liquid chromatography mass spectroscopy (LCMS) analysis (with the help of Dr. Gullo). The challenge of developing a chemically-defined production medium was to minimize the

presence of complex and insoluble ingredients in the medium while still promoting suitable antibiotic production. These experiments were conducted by Dr. Demain's previous students, Sabrina Aluotto and Evan Martens. Throughout the media development process, chemical assays were carried out on fermentations to specifically identify platensimycin and platencin, and to quantify production. A summary of the media used and their ingredients can be found in Table 1.

The first goal tackled was to obtain solubility of the medium, which was accomplished with media PM2 and PM3 (Aluotto *et al*. 2013). These media mark the introduction of MOPS buffer to control pH during fermentation. PM3 introduced dextrin, which remained in every developed production medium until the chemically-defined medium was devised. Upon comparing PM2 and PM3, it was deemed that PM3 had better production (data not shown), so it was used as a starting point for further media development.

Table 1: Summary of Media Used and Contents

Medium	Ingredients g/L
Stock Culture Medium	30 Difco Trypticase Soy Broth
Seed Medium	20 soluble starch, 10 dextrose, 5 NZ amine type A, 3 beef extract, 5 Bacto Peptone, 5 yeast extract and 1 $CaCo_3$
PM1 (original Merck and Co. complex and insoluble Medium)	40 Yellow Cornmeal, 40 lactose, 5 Amberex
PM2 (Soluble)	26.5 Difco Trypticase Soy Broth (TSB) without dextrose, 10 soluble starch, 20 MOPS buffer
PM3 (Soluble)	40 dextrin, 40 lactose, 5 yeast extract, 20 MOPS buffer
PM4 (Semi-defined)	40 dextrin, 40 lactose, 5 Difco Vitamin-Free Yeast Base, 5 Difco Yeast Carbon Base, and 20 MOPS buffer
PM4A (Semi-defined)	40 dextrin, 40 lactose, 5 Difco Vitamin-Free Yeast Base, and 20 MOPS buffer
PM4B (Semi-defined)	40 dextrin, 40 lactose, 5 Difco Yeast Carbon Base, and 20 MOPS buffer
PM5 (Semi-defined)	40 dextrin, 40 lactose, 2 ammonium sulfate, and 20 MOPS buffer
PM6 (Semi-defined)	g/L 40 dextrin, 60 lactose, 20 ammonium sulfate, 20 MOPS buffer
PM6A (Semi-defined)	10 dextrin, 25 lactose, 5 MOPS buffer, and 0.5 ammonium sulfate
PM6B (Semi-defined)	10 dextrin, 20 lactose, 5 MOPS buffer, and 0.5 ammonium sulfate
PM6C (Semi-defined)	10 dextrin, 50 lactose, 5 MOPS buffer, and 0.5 ammonium sulfate
PM7 (Chemically-defined)	30 glucose, 50 lactose, 5 MOPS buffer, and 0.5 ammonium sulfate

The next goal was chemical definition; the first step was to replace the yeast extract present in PM3 using two different chemically-defined Difco supplements, resulting in media PM4, PM4A, and PM4B (Aluotto *et al*. 2013). PM4 had both components, Yeast Carbon Base and Vitamin-Free Yeast Base at 5 g/L. PM4A had only Difco Vitamin-Free Yeast Base and PM4B had only Yeast Carbon Base. Initially, Aluoto observed excellent antibiotic production with PM4, but with 28 different

components, further simplification was needed; thus PM4A and PM4B were tested. Experiments showed that PM4A supported better antibiotic production than both PM4 and PM4B, so PM4A was maintained (data not shown).

Because dextrin is only semi-defined (it contains glucose but the exact number of glucose molecules varies and is often unknown), PM4A is semi-defined, which was a huge improvement from the complex and insoluble PM1 (Aluotto *et al*. 2013). However, it was still unusable because of the numerous ingredients in Vitamin-Free Yeast Base. Simplification was attempted by substituting ammonium sulfate in place of the yeast base, which resulted in PM5. While there was production in PM5, increased production was desirable. Evan Martens determined that lactose stimulated production so its concentration was increased to 20 more grams of lactose per liter, resulting in PM6. Further work showed that a more dilute PM6, i.e., PM6A, resulted in comparable antibiotic production using significantly fewer materials so this medium was used moving forward.

The goals of my work have been to continue the development toward a chemically-defined medium, remove the crude nutrient carryover from the seed culture upon inoculation into the production medium, and evaluate the effects of amino acids and vitamins on antibiotic production. My experiments began in PM6A and started with an attempt to remove the complex and insoluble nutrient carryover that was observed when inoculating the production medium with the complex seed culture. We successfully reached the goals of a chemically-defined medium with PM7, and removal of the complex nutrient carry-over from the seed medium with a washing procedure. Following

the development of a successful washing procedure, the next goal of evaluating amino acids and vitamins was explored. I hypothesized that any compounds that have effects on production, stimulatory or inhibitory, may play a role in the biosynthetic pathway, growth of the culture, or serve as regulators of one or more of the steps. Because of the use of both thiamine and aspartic acid (aspartate) in the biosynthetic pathway (Figure 8 and 9), we hypothesized that these compounds would have stimulatory effects (Herath *et al*. 2007; Smanski *et al*. 2011).

Methods

Seed and Stock culture

All media were pH-adjusted to 7 using concentrated potassium hydroxide, and flasks were autoclaved at 121°C for 30 minutes prior to use (Aluotto *et al*. 2013). Ingredients for all media can be found in Table 1. Stock culture medium was inoculated with a sample of *S. platensis* MA7327 (obtained from the laboratory of Dr. Ben Shen at Scripps Research Institute of Florida), and incubated on the gyratory shaker at 220 rpm at 28°C for two days followed by storage in the refrigerator until use. Merck seed medium was inoculated with 0.5 mL of *S. platensis* stock culture and placed on the gyratory shaker at 220 rpm at 28°C for five days. Cultures were started in 250-mL Erlenmeyer flasks with 50 mL of medium. In later experiments, 125-mL Erlenmeyer flasks were used and the medium volume was reduced by one half.

Washing of the Seed Culture

Five-day seed culture was spun down in a sterile 50-mL conical tube using an international clinical one-speed centrifuge for 10 minutes (Falzone *et al*. 2013). The supernatant fluid was discarded, replaced with an equal volume of sterile water, and the pellet re-suspended. This process was repeated two more times. The culture was stored as a liquid in the refrigerator after the third addition of water.

Experimental Set up

All production media were made using distilled water (See Table 1); the pH was adjusted to 7.0 using concentrated potassium hydroxide (Aluotto *et al*, 2013; Falzone *et al*. 2013). Experiments were conducted in 250-mL Erlenmeyer flasks with 50 mL of medium or 125-mL Erlenmeyer flasks with 25 mL of media. All experimental flasks were autoclaved at 121°C for 30 minutes. Flasks were inoculated with 0.1 mL of washed seed culture per 5 mL of media. PM1 controls were used in all experiments, and PM7 controls were used in all additive experiments. Each experimental condition was evaluated using two identical flasks, prepared together on the same day. Any positive or negative results were repeated in additional experiments with new controls.

Additive Experiments: Vitamins, Amino Acids, and KCl

After the addition of the additives to the media, the pH was checked and re-adjusted to 7 if necessary. Vitamin solutions with 5 mg/mL vitamin concentration were made for each vitamin tested. Vitamins tested include: riboflavin, nicotinic acid, d-biotin, ascorbic acid, folic acid, thiamine, inositol, and B12. When 50 mL of production medium was used, 100 µL of vitamin solution was added to achieve 0.01 mg/mL final concentration. Amino

acids were initially evaluated at 1 mg/mL. Stimulatory amino acids were also tested at 0.25 mg/mL, 0.5 mg/mL, 3 mg/mL and 5 mg/mL. After adding the amino acids, the pH was adjusted to 7 using concentrated sodium hydroxide if necessary. Potassium chloride (KCl) was tested at a concentration of 100µg/mL. To evaluate effects of the additives, their zones of inhibition were compared directly to the PM7 control used in that specific experiment.

Agar Diffusion Assay

To test for antibiotic activity, zone of inhibition assays against *S. aureus* were carried out in Petri dishes (Aluotto *et al*. 2013). *S. aureus* stock culture (obtained from Dr. Joanna Miller from Drew University) was started by inoculating 0.5 mL of a previous liquid culture into 25 mL of TSB (mentioned above) and incubated at 35ºC for two days. 37 g/L of LB agar Miller was suspended in de-ionized water and autoclaved at 121°C for 30 minutes. After autoclaving, the mixture was allowed to cool enough to be touched to the forearm without burning and was rapidly inoculated with 2.5 mL of *S. aureus* stock culture for every 10 mL of agar. The inoculated agar was distributed into sterile Petri plates at 10 mL per plate.

To assay, 1% of the starting fermentation volume was removed from each experiment flask and placed into individual tubes. One paper disk, 6 mm in diameter, was dipped into each tube, dried briefly, and placed onto the agar plates. Plates were inverted and placed in the incubator at 35°C overnight.

Clear zones of inhibition around the disks were measured in millimeters across the diameter on two different sides and averaged, shown in Figure 12. Each plate was

read by two to three individuals, and the readings were averaged to generate a value for each flask. Values for identical flasks were also averaged, generating one value for each experimental condition. Conditions were compared by making a general comparison of the zone of inhibition size throughout the fermentation period as well as by comparing the highest zone of inhibition observed, regardless of which day it occurred. To compare the largest zone of inhibition, the average zone size of PM7 between the two flasks was used as a standard to generate relative zones of inhibition for each condition. The original measurement for each flask was divided by the PM7 average, and the values for each identical flask were averaged together to generate one value for each condition. Error bars representing range or standard error associated with the two values for individual flasks of each condition were incorporated. Summary of a calculation is shown in Table 2. Assays were conducted every one to three days, and zone sizes were compared throughout the numerous assays of the experiment. Conditions with overlapping error bars were deemed inactive, and those without were deemed potentially stimulatory or inhibitory respectively, and marked for further testing.

Table 2: Calculation Summary for Relative Zones of Inhibition Standardized to PM7 control.

Flask	Zone Diameter (mm) (measured)	Calculation	Relative Value	Average	Range
PM7	18	(18/18.5)	0.97	1	0.038
PM7	19	(19/18.5)	1.03		
Aspartic Acid	21	(21/18.5)	1.14	1.16	0.038
Aspartic Acid	22	(22/18.5)	1.19		

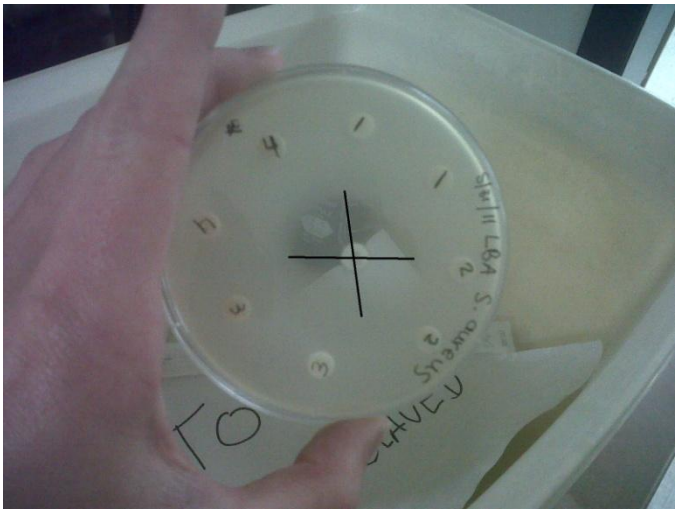

Figure 11: Image of Plate with Zone of Inhibition. Black lines indicate diameters of the zone that were measured.

Mass Spectrometric Assay

PM7 fermentations were pH-adjusted to below 5 using concentrated hydrochloric acid. The PM7 fermentation was extracted three times with an equal volume of ethyl acetate. The ethyl acetate solution was evaporated off using a Buchi rotary evaporator, and the remaining contents were re-suspended in the original volume of the fermentation using a mixture of 50% methanol and 50% ethyl acetate. This mixture, along with the remaining aqueous liquid, was tested for antibacterial activity using the agar diffusion assay. The solvent was evaporated using a rotary evaporator, and the remaining contents re-dissolved in 1 mL of methanol for injection into the liquid chromatography mass spectrometer (LCMS). A summary of the procedure can be found in Figure 13.

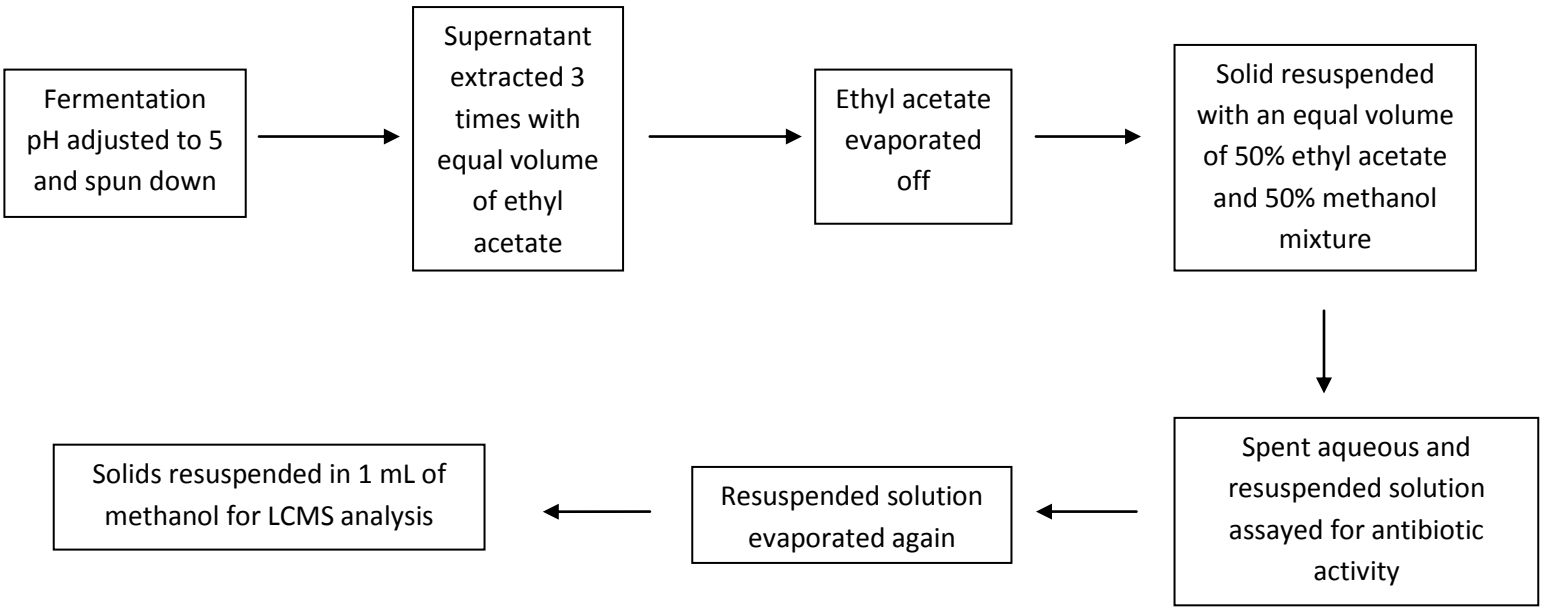

Figure 12: Summary of Chemical Procedure for Extraction of Platensimycin

LCMS was performed on a Waters Micromass LCT spectrometer interfaced with an Agilent 1100 HPLC (Falzone *et al.* 2013). Chromatography was performed on an analytical Echelon, C18 4μm, 100 x 4.6 mm column using a gradient system from 10% acetonitrile/aqueous 0.1% formic acid to 75% acetonitrile/aqueous 0.1% formic acid. All samples were run for 15 minutes at a flow rate of 1.5 mL/minute. Detection of the compound was performed using the Agilent 1100 HPLC diode array detector and the Waters Mass spectrometer. Peaks were identified by retention time and the presence of the platensimycin molecular ion. Concentrations were determined by comparing UV peak area to a platensimycin reference standard.

Results

Success of Semi-defined PM6A

Data comparing PM6A and PM1 are shown in Figure 13. Importantly, semi-defined PM6A supported suitable levels of antibiotic production, although not as well as PM1.

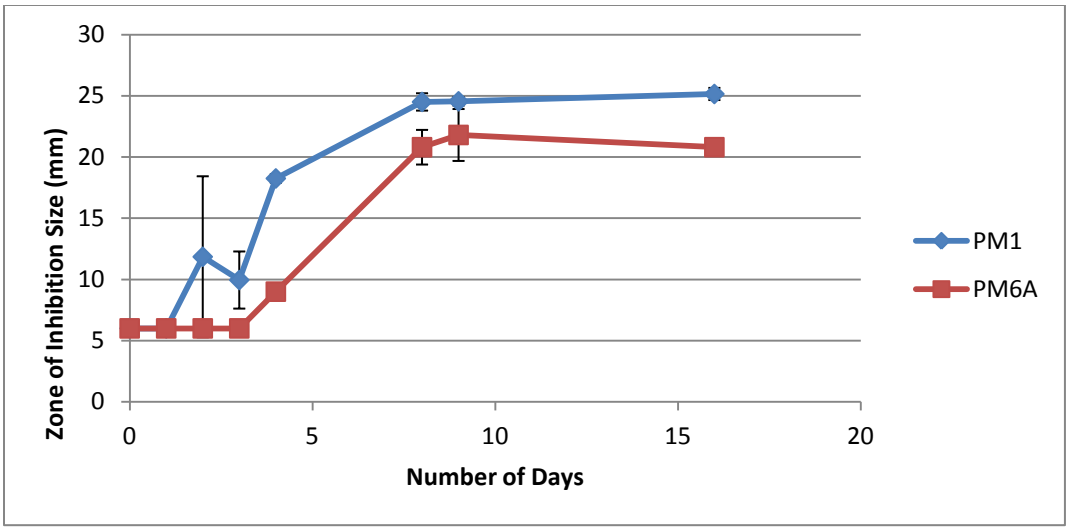

Figure 13: Comparison of Zone of Inhibition Sizes Between PM6A and PM1. Comparison of zones of inhibition between PM1 and semi-defined PM6A over the course of a 10 day fermentation, assayed on days 2, 3, 4, 5, 8, 9, and 10. Error bars represent range of standardized values between flasks of the same experimental condition. Lack of error bars indicates identical values between flasks. PM6A supports lower but suitable production.

Elimination of Crude Nutrient Carryover

After the establishment of the semi-defined production medium, PM6, there was an attempt to eliminate the crude nutrient carryover from the seed medium. Eliminating the crude nutrients was important because the presence of the crude nutrients in the inoculum would prevent chemical definition in any future media developed. Previous experiments attempted to minimize the crude nutrient carryover by reducing the volume of culture used to inoculate, also reducing the number of organisms added (Personal Communication, Evan Martens 12/15/13). This attempt was unsuccessful as reduced inoculum yielded less antibiotic production, especially in the semi-defined production media (data not shown).

The next attempt was to remove the nutrients from the seed culture by washing the cells. A washing procedure was developed by Dr. Demain involving centrifugation of the seed, washing the cells twice with sterile distilled water, and suspending the washed cells in sterile distilled water. Zone of inhibition assays on PM1 and PM6A fermentations using the washed seed showed that the washed seed resulted in good levels of antibiotic production throughout the experiment, as seen in Figure 14. The success of the washed seed was established by comparing antibiotic production in the same media generated when inoculated with the same amount of washed and unwashed seed culture. The raw data are shown in Table 3 as an example. The numbers for each condition on the graph were generated by averaging the values between the flasks for each day, as explained in the Materials and Methods. The error bars represent the range between the identical flasks. For the washed seed flaks, the error bars are small indicating low variability.

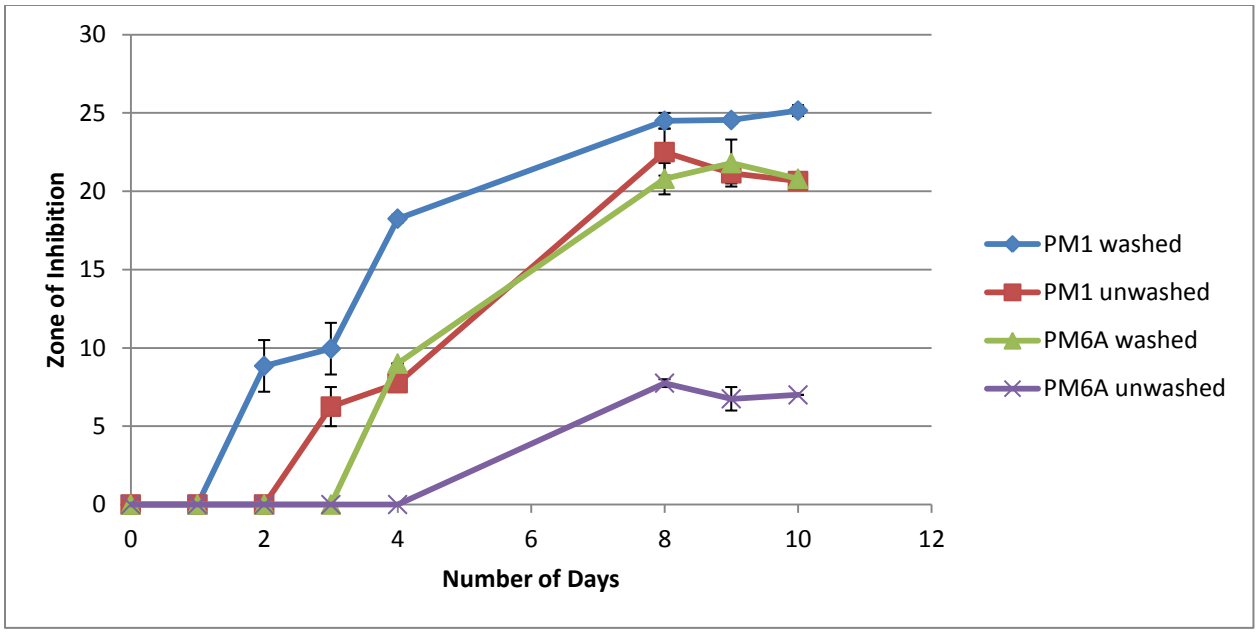

Figure 14: Comparison of Washed and Unwashed Seed Culture. Zone of inhibition diameters over the course of 10 days of growth sampled on days 0, 1, 2, 3, 4, 8, 9, and 10 comparing equal volumes of washed seed culture and unwashed seed cultures in PM1 and PM6A. Error bars represent range values between flasks of the same experimental condition; lack of error bars indicates identical values between flasks.

Table 3: Raw Data from Experiment Comparing Washed Seed in PM1 and PM6A.

Flask	Condition	0 days	1 day	2 days	3 days	4 days	8 days	9 days	10 days
1	PM1 washed	Nz(6)	Nz(6)	7.2	8.3	18	25	24.3	25.5
2	PM1 washed	Nz(6)	Nz(6)	10.5	11.6	18.5	24	24.8	24.8
3	PM1 unwashed	Nz(6)	Nz(6)	Nz(6)	Tr (7)	8	24	21.8	20.7
4	PM1 unwashed	Nz(6)	Nz(6)	Nz(6)	7.5	7.5	21	20.5	20.5
5	PM6A washed	Nz(6)	Nz(6)	Nz(6)	Nz(6)	9	21.8	23.3	20.7
6	PM6A washed	Nz(6)	Nz(6)	Nz(6)	Nz(6)	9	19.8	20.3	20.9
7	PM6A unwashed	Nz(6)	Nz(6)	Nz(6)	Nz(6)	Nz(6)	7.5	Tr(7)	Tr (7)
8	PM6A unwashed	Nz(6)	Nz(6)	Nz(6)	Nz(6)	Nz(6)	8	7.5	Tr (7)

All measurements in mm.
Nz means no zone and is assigned a numeric value of 6 mm for the size of the disk
Tr means a trace zone was observed and is assigned a numeric value of 7 mm

Establishment of Chemically-defined PM7

The next goal was to eliminate or replace the dextrin from the semi-defined production medium to obtain a completely chemically-defined one. Previous experiments in PM6C showed that when dextrin was removed, the medium did not support production. Because of the necessity of dextrin, chemically-defined ingredients that could replace it were explored. Experiments were done by visiting scientist Emily Friedman and Drew undergraduate students Emmanuel Crespo, Samantha Golden, and Vasyl Nayda using PM6C to examine the effect of additives from the seed medium (soluble starch, dextrose, NZ amine type A, Difco beef extract, Difco Bacto Peptone, Difco yeast extract and $CaCo_3$) on production in PM6C (Personal Communication 12/15/13). The hope was that determining which compounds from the crude and insoluble seed medium were the most stimulatory could help generate ideas of chemically-defined ingredients to be incorporated into the chemically defined medium. Of these compounds, soluble starch was the most active so attempts were made to replace it and dextrin with a defined sugar. Maltose, cellobiose, glucose, and additional lactose were evaluated by Emily Friedman, Emmanuel Crespo, Samantha Golden, and Vasyl Nayda in PM6C (Personal Communication 12/15/13). Out of the compounds tested, glucose had the most stimulatory effect presumably because it is a component of soluble starch. Further, the addition of glucose to PM6C lacking dextrin led to recovery of the antibiotic production. Adding glucose was the basis for establishment of chemically-defined PM7 which contains (g/L): 30 glucose, 50 lactose, 5 MOPS buffer, and 0.5 ammonium sulfate. Figure 15 shows that PM7 was successful at supporting antibiotic production despite its

variability over five separate experiments and lack of overlapping error bars with PM1. The development of the PM7 is a valuable advance because it allows the use of a chemically defined medium with no insoluble particulates.

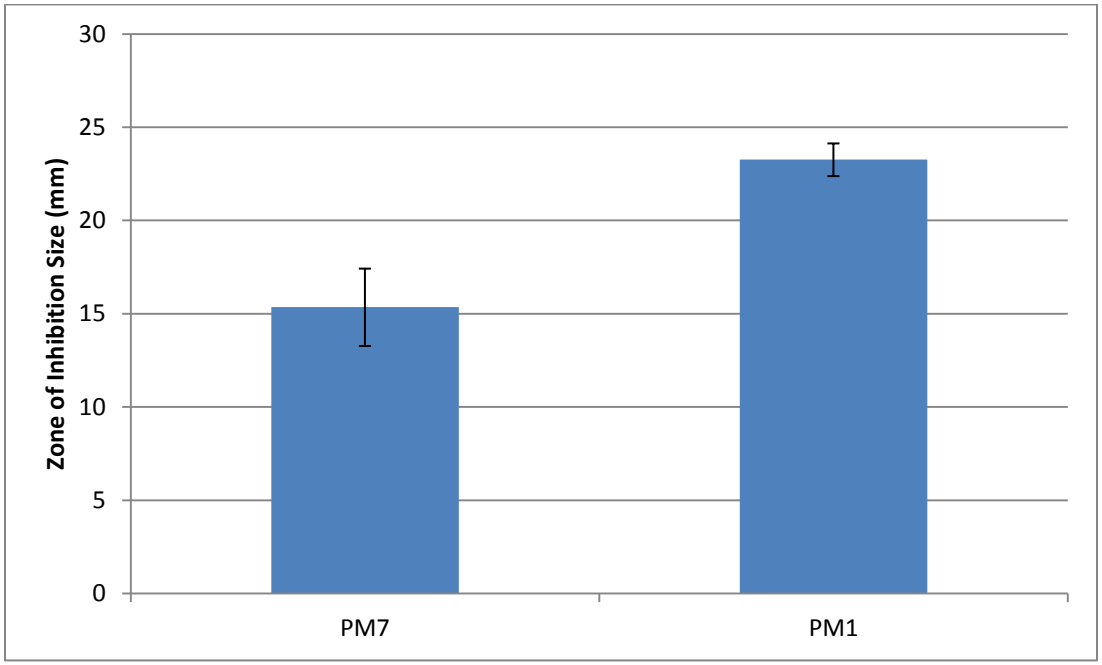

Figure 15: Antibiotic Production in Chemically-defined PM7. Largest zone of inhibition comparison between PM7 and PM1 averaged over five separate experiments; error bars represent range of values within data set. PM7 has lower but suitable production.

Mass Spectrometric Assay on Antibiotic Production in PM7

After the establishment of PM7, a mass spectrometric assay was performed. While the zone of inhibition assay is able to detect antibiotic activity in the fermentation, this detection is not specific for platensimycin or platencin; zones of inhibition could potentially be coming from additional antibacterial compounds in the fermentation. A mass spectrometric assay serves to specifically detect platensimycin and platencin, using

both mass spectroscopy and UV absorption signals. It also has the potential for quantification of these compounds to be determined. Mass spectroscopic assays were carried out on each new production medium developed in order to ensure that platensimycin and platencin were still being produced. Due to acquiring new instrumentation, a new procedure for the detection of the compounds using liquid chromatography mass spectroscopy (LCMS), rather than high performance liquid chromatography (HPLC), was developed. To accommodate the new equipment, a dose-response curve using a pure sample of platensimycin was generated using the UV absorbance peak area of the LCMS; the spectra are shown in Figure 16. Because of the carboxylic acid portion of the molecule, formic acid was included in the mobile phase.

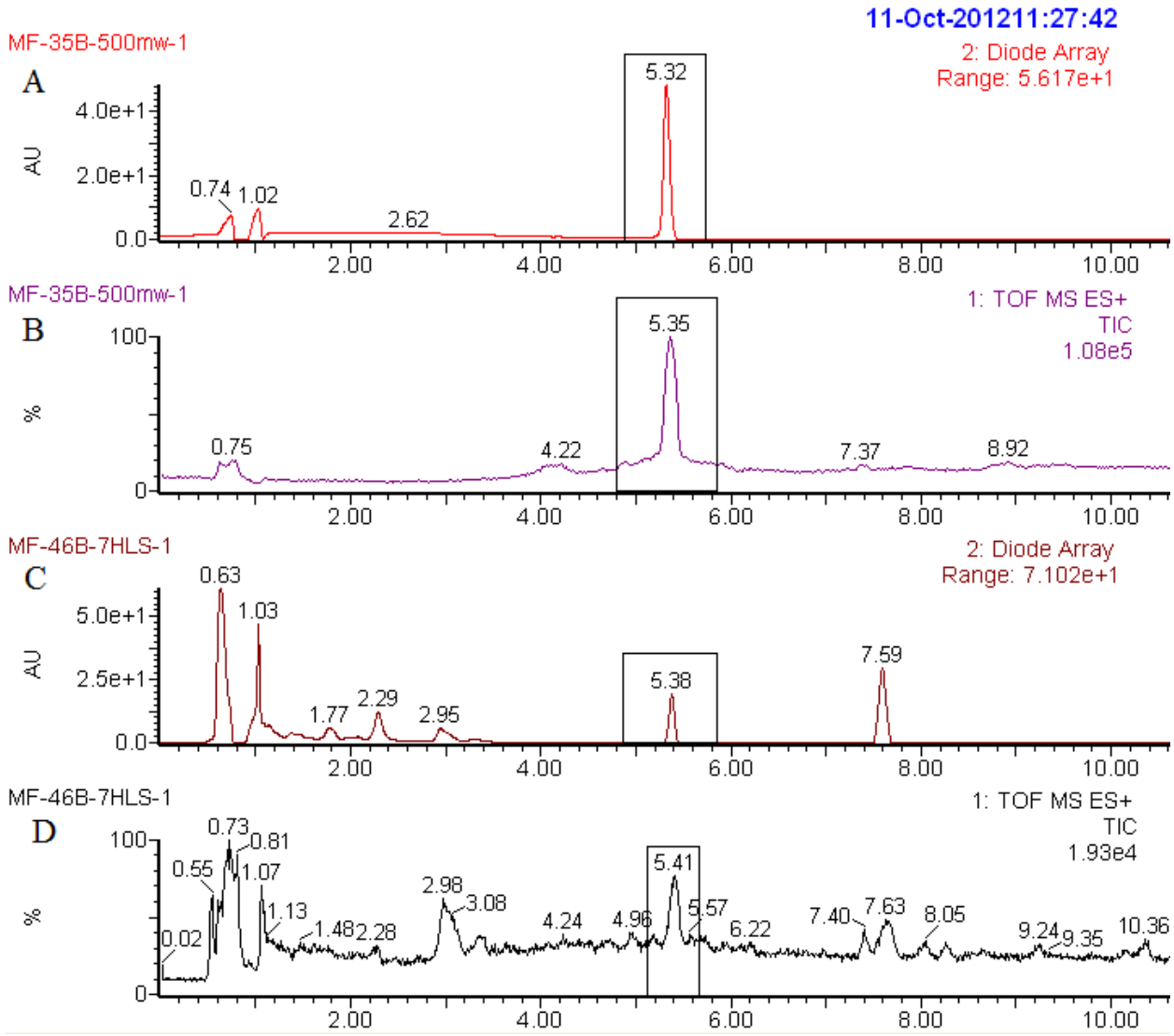

Figure 16: UV and Mass Spectroscopy Data Comparing Platensimycin Standard and PM7 Fermentation. 500 µg/mL standard used. Mass 442 highlighted. Observed from 190 to 600 nm. Data based on 40 mL PM7 fermentation. Boxes show platensimycin signal. A: Absorption of platensimycin standard. B: Mass spectroscopy of platensimycin standard. C: UV absorption of PM7 fermentation. D: Mass spectroscopy of PM7 fermentation. Platensimycin signal seen (boxed) in both UV and mass spectrum of PM7 fermentation.

There were some inconsistent results between the mass spectrometric assay data and the biological agar diffusion assay, as fermentations with significant zones of

inhibition did not always yield high concentrations of platensimycin and platencin, especially in PM7. This discrepancy in the assays further reinforced the lack of specificity of the zone of inhibition assay, indicating there could be other compounds in the fermentation broth that inhibit bacteria growth but are not platensimycin. The PM7 mass spectrometric assay was especially troublesome because there is an additional compound that comes out very close to the platensimycin peak on the UV spectrum with a mass spectroscopy peak at 463 (data not shown). This additional peak is so close to the platensimycin peak that it is very difficult to separate the peaks enough to isolate the platensimycin peak to acquire the area, which is needed for accurate quantification. Both the mass spectrum and UV show that platensimycin is in fact present in PM7 fermentations, which was the intention of the experiment (Figures 16 and 17). Figure 17 zooms in on the region of the mass spectrum associated with the platensimycin UV peak and allows for identification of platensimycin by comparing the mass spectrum of PM7 to the mass spectrum from the standard. Mass spectra show the mass of the compound present as a mass plus one peak (M+1), meaning the peak will be one mass unit higher than the actual peak. For platensimycin, the mass is 441 g/mol so the peak is expected at 442 M/Z. Importantly, the 442 peaks are observed at the same time, 5.480 minutes in fermentation and 5.421 minutes in the standard, further confirming the presence of platensimycin. As seen, the spectra do line up for a positive conformation of platensimycin.

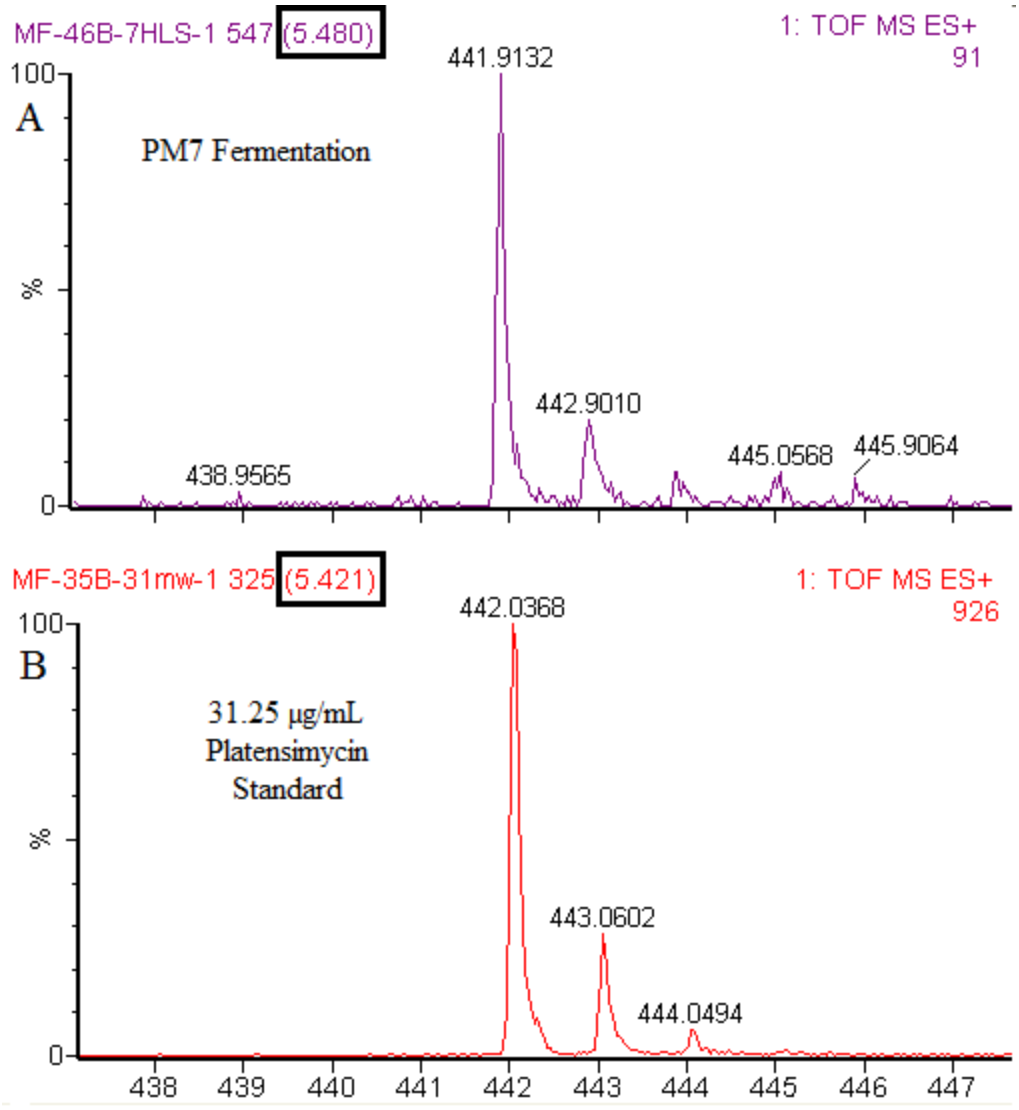

Figure 17: Mass Spectrum of PM7 and Platensimycin Standard. A: PM7 fermentation, data based on 40 mL PM7 fermentation. B: 31.5 µg/mL Platensimycin Standard; box in label indicates time that peak was observed, time is consistent between the standard and the fermentation samples.

The presence of platensimycin was also confirmed in PM1 (not shown). Platencin was not detected in the PM7 fermentation, as its mass peak, 426, cannot be seen on the mass spectrum. Lack of platencin was not surprising, as platensimycin is the ii9major product in this strain (Smanski *et al*. 2011). While the mass spectroscopic assay

is useful and necessary for specifically identifying the presence of platensimycin in fermentations, the lack of quantification ability makes it unfeasible to be used as a regular assay. The procedure also prevents continuous assays and monitoring throughout the fermentation. The agar diffusion assay only requires removal of 0.25 mL of the fermentation whereas a mass spectrometric assay would require the removal of a significantly larger volume. Removing large amounts of volume throughout the experiment would diminish the fermentation before the end of the experiment, limiting the number of assays that could be carried out.

Concentrated versus Dilute PM7

After confirming the production of platensimycin in PM7, the next goal was to look into stimulating antibiotic production in PM7 before moving on to experiments involving additives. A previous higher concentrations of ingredients in PM6 (g/L: 40 dextrin, 60 lactose, 20 MOPS buffer, and 2 ammonium sulfate) was used to generate a concentrated version of PM7 to compare to the current PM7 already in use (g/L: 10 dextrin, 15 lactose, 5 MOPS buffer, and 0.5 ammonium sulfate). The results of numerous experiments indicated that the more concentrated PM7 yielded no more antibiotic production than the dilute PM7 (data not shown). Later experiments continued using the original PM7 due to lack of stimulation of the concentrated version.

Testing Additives: Vitamins

The next goal was to observe the effects of vitamins as additives to PM7 to determine whether any had effects on antibiotic production: stimulatory or inhibitory.

Each vitamin-d-biotin, riboflavin, B12, nicotinic acid, ascorbic acid, folic acid, thiamine, and inositol-was tested at 10 µg/mL. As shown in Figure 18, d-biotin and riboflavin showed stimulatory effects on production, producing larger zones of inhibition than PM7 throughout the fermentation period. Figure 18B shows that their largest zone of inhibition size was larger than that of PM7 (both 33% larger) and their error bars did not overlap with PM7. Stimulation was confirmed in additional repeat experiments (data not shown).

Figure 18A shows that for B_{12} at 10 µg/mL concentration, neither of the identical flasks had zones, so there were no error bars on the B_{12} data points. Therefore, there is no overlap in PM7 throughout the fermentation or on the largest zone of inhibition, with a largest zone size 60% smaller than PM7 (Figure 18A and B). Drew undergraduate student Emmanuel Crespo evaluated B12 at 10 percent of its original concentration (i.e., 1 µg/mL) and found it to be inactive at this concentration (data not shown) (Personal Communication 12/15/2013). Figure 18B also shows that nicotinic acid did not have an effect on antibiotic production as its zone sizes are comparable to PM7. Figure 19A and B shows that the same is true for ascorbic acid, folic acid, thiamine, and inositol. The variability shown by large error bars in PM7 in this experiment was noted and the experiment should be repeated.

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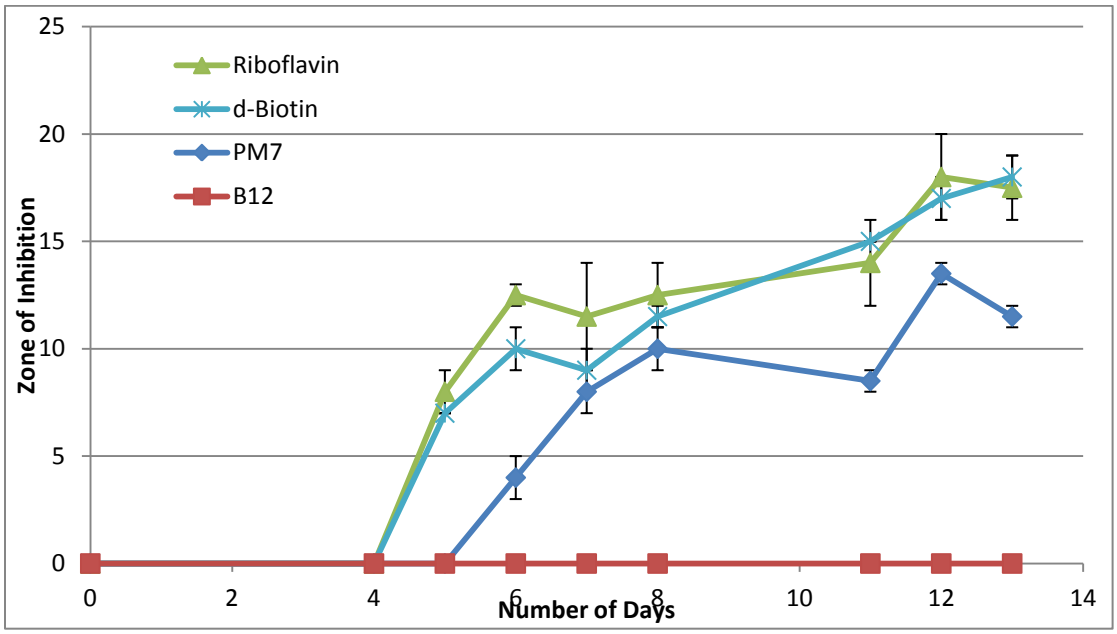

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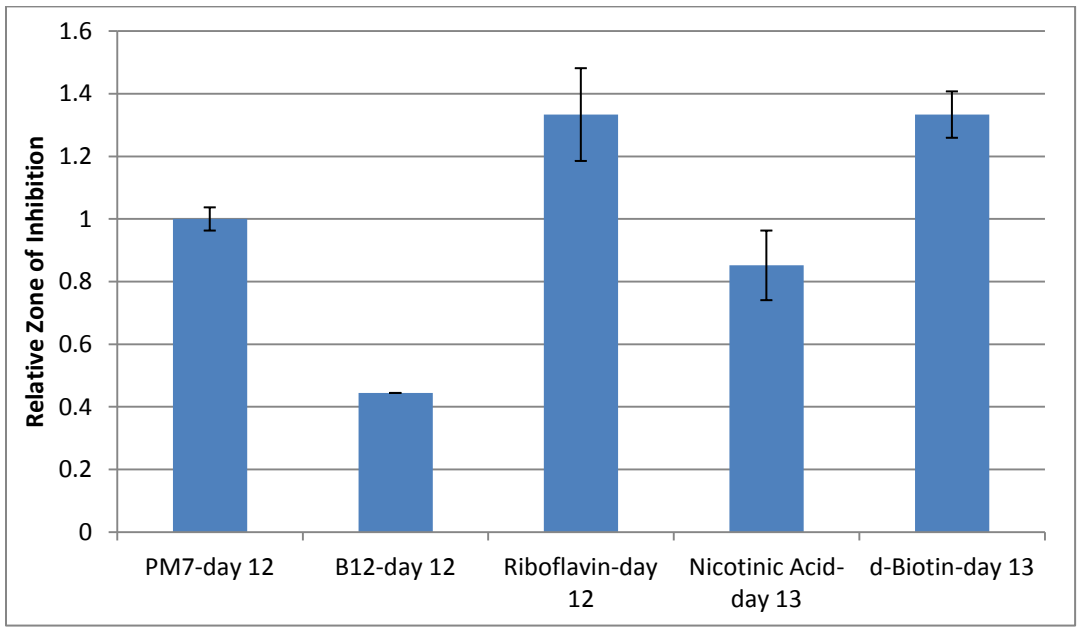
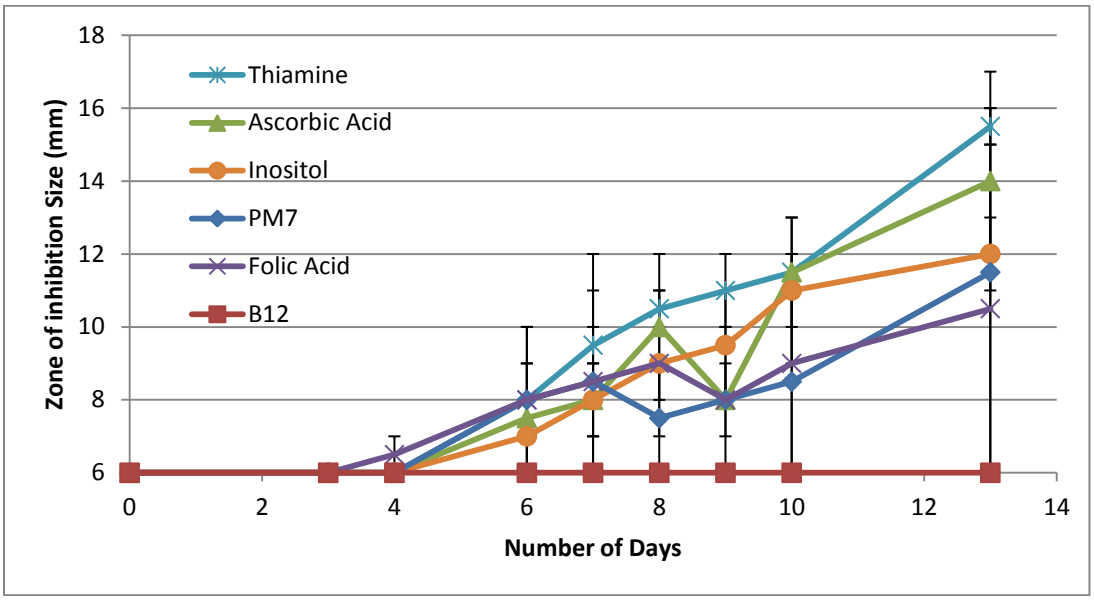


Figure 18: Effects of B_{12}, Riboflavin, Nicotinic Acid and d-Biotin. Error bars represent range of standardized values between flasks of the same experimental condition; lack of error bars indicates identical values between flasks. A: Comparison of zones of inhibition of PM7 with added vitamin at 10 µg/mL over the course of 14 day fermentation assayed on days 0, 4, 5, 6, 7, 8, 11, 12, 13, and 14. B: Measure of the relative largest zone of inhibition size normalized to PM7 as standard of comparison. Ribioflavin and d-biotin are stimulatory, B12 inhibitory.

A:



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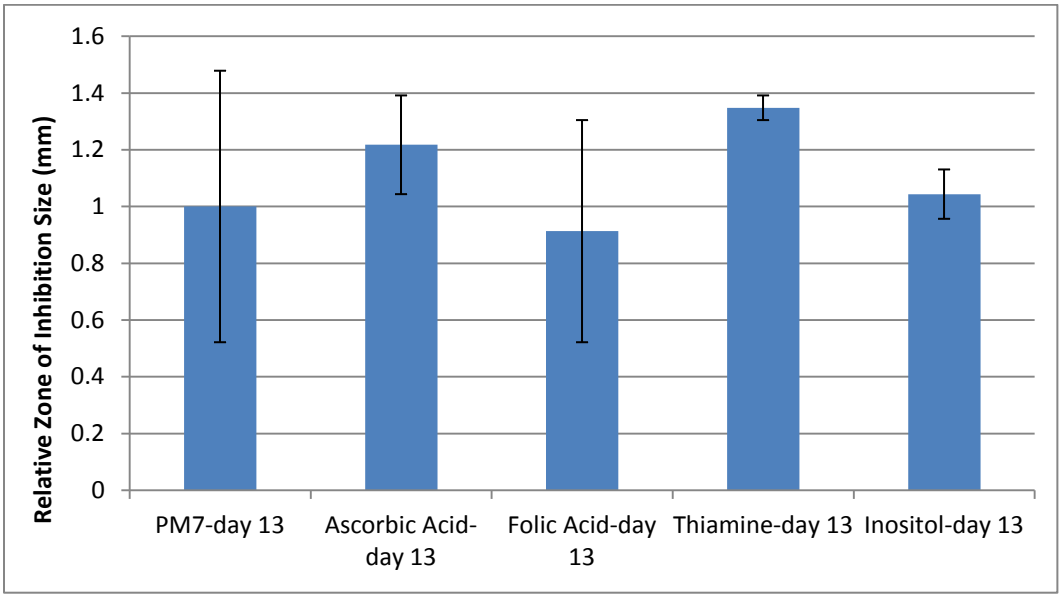

Figure 19: Effects of Ascorbic Acid, Folic Acid, Thiamine, Inositol. Error bars represent range of standardized values between flasks of the same experimental condition; lack of error bars indicates identical values between flasks. Vitamins tested at 10 µg/mL. A: Comparison of zones of inhibition of PM7 with added vitamins over course of 13 day fermentation assayed on days 0, 3, 6, 7, 8, 9, 10, and 13. B: Measure of the relative largest zone of inhibition size normalized to PM7 as standard of comparison. All amino acids inactive due to error bar overlap.

Additive Testing: Amino Acids

Amino acids were evaluated for potential effects on platensimycin production in PM7. Tests were done at concentrations of 1 mg/mL. Initial experiments indicated that histidine and alanine were potentially stimulatory (Figures 20 and 22). The largest zone of inhibition with histidine was 33% than that of PM7, and the zone size associated with the average of two assays for alanine was 38% greater than that of PM7 (Figures 20 22). Argining, serine, and leucine seemed to inhibit production, as their largest zone of inhibition diameters were lower than that of PM7 by 21%, 19% and 24% respectively) (Figures 20, 21B and 22). The inhibitory effect of serine was confirmed in an additional experiment completed by undergraduate students Drew Stenger, Sana Siddiqui (Drew University), and visiting student Carrie Perkins (Vassar College) (data not shown). Cysteine, methionine, arginine, threonine, tyrosine, valine, glycine, and phenylalanine were inactive as their relative maximum zone diameters had overlapping error bars with PM7 (Figures 20, 21, and 22). Amino acid and vitamin results are summarized in Table 4.

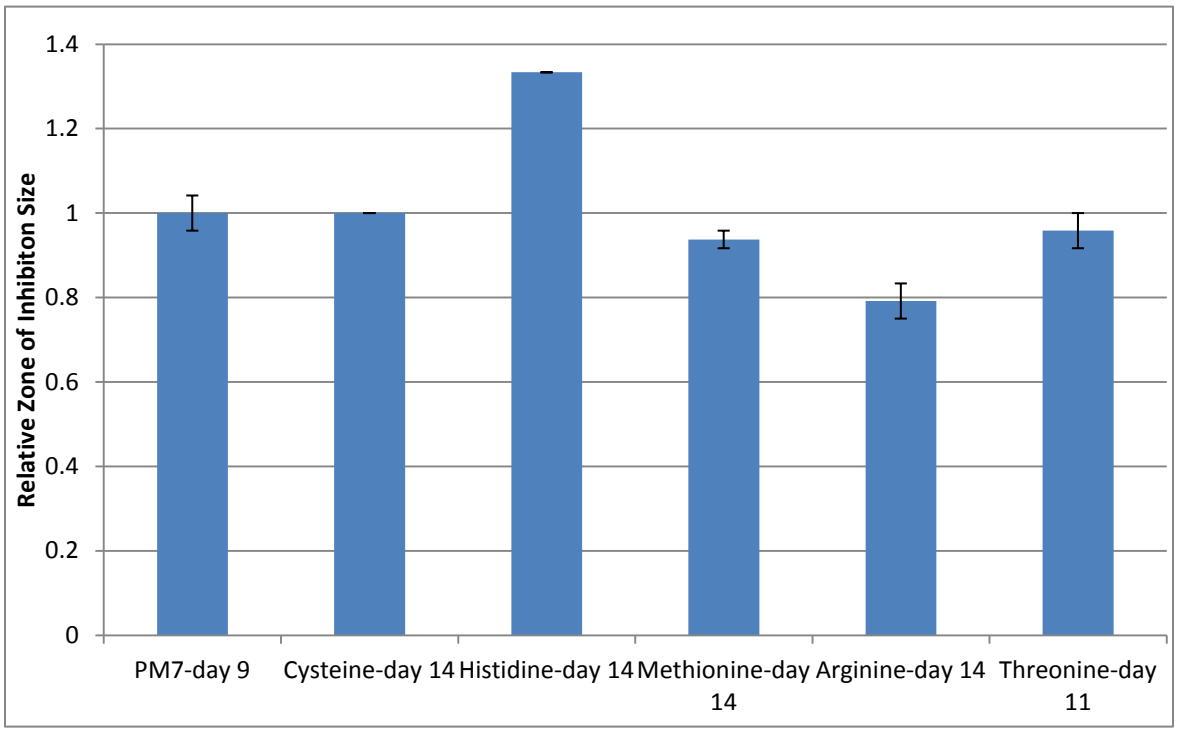

Figure 20: Effect of Histidine, Cysteine, Methionine, Arginine, and Threonine. Measure of the relative largest zone of inhibition size using the size of PM7 as standard of comparison. One mg/mL amino acid concentration used. Error bars represent range of standardized values between flasks of the same experimental condition; lack of error bars indicates identical values between flasks. Histidine was stimulatory and arginine was inhibitory.

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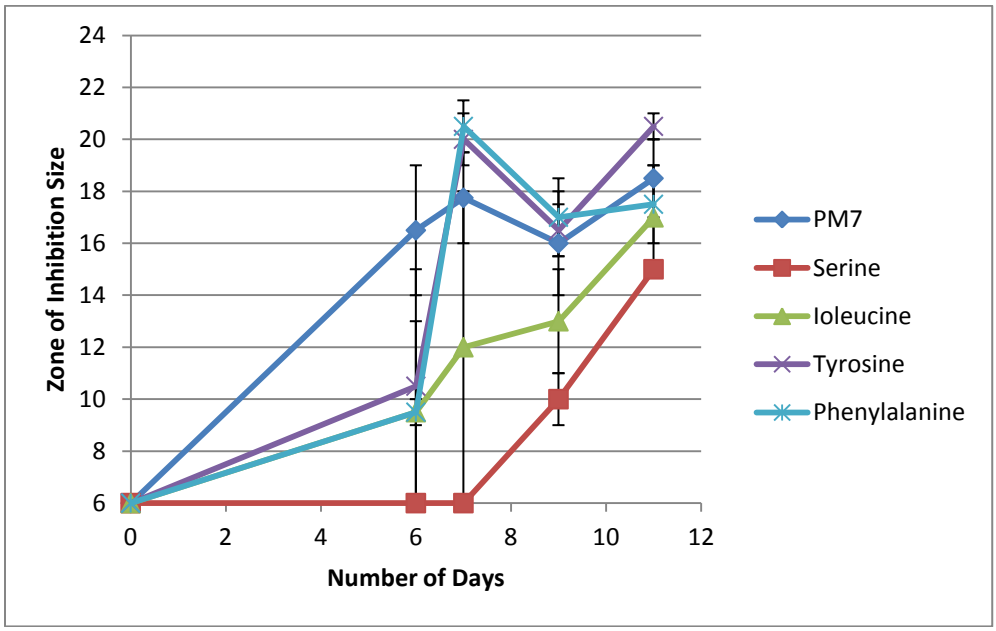

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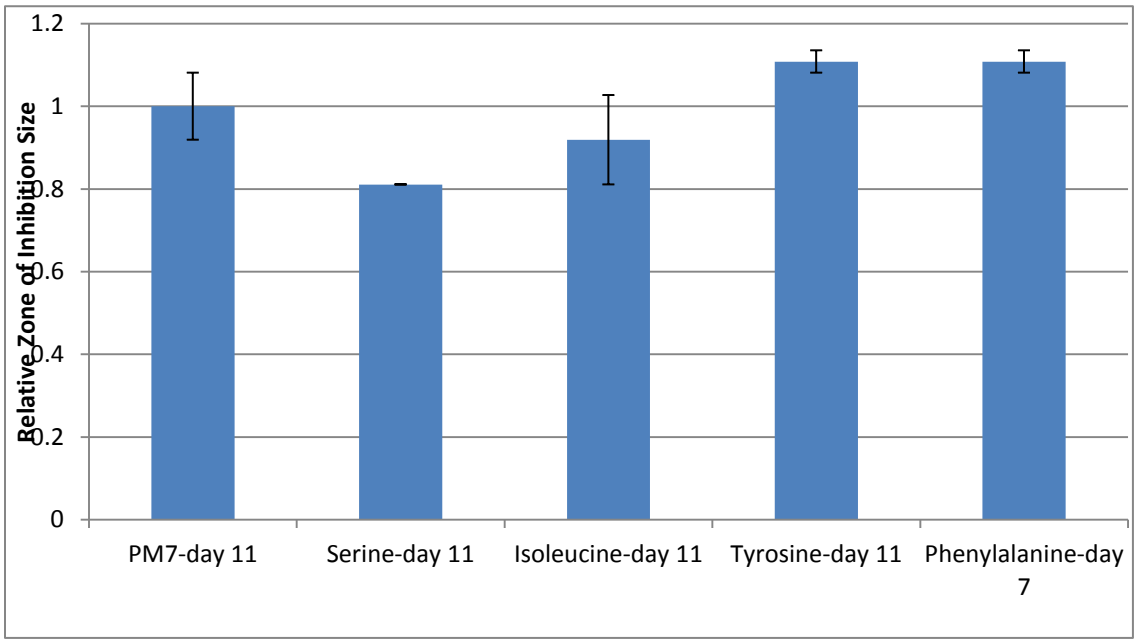

Figure 21: Effects of Serine, Isoleucine, Tyrosine and Phenylalanine. 1 mg/mL amino acid concentration used. Error bars represent range of standardized values between flasks of the same experimental condition; lack of error bars indicates identical values between flasks. A: Comparison of zones of inhibition of PM7 with added amino acids over the course of 11 day fermentation assayed on days 0, 6, 7, 9, and 11. B: Measure of the relative largest zone of inhibition size using the size of PM7 as standard of comparison. Only serine was inhibitory.

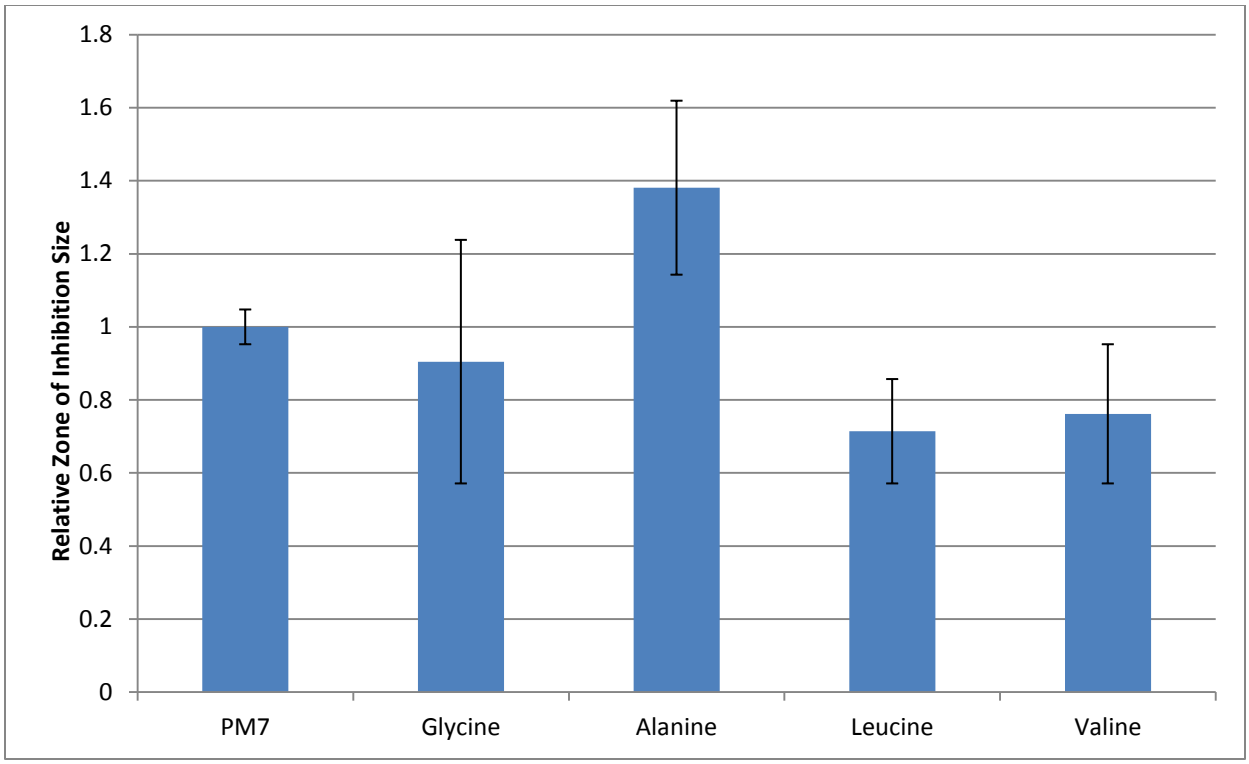

Figure 22: Effects of Glycine, Alanine, Leucine, and Valine. Normalization of the relative zone of inhibition averaged between two assays using the size of PM7 as standard of comparison. Error bars represent range of standardized values between flasks of the same experimental condition; lack of error bars indicates identical values between flasks. Alanine was stimulatory and leucine was inhibitory.

The remaining amino acids, tested by Drew Students Sana Siddiqui, Carrie Perkins, and Drew Stenger, will be discussed in the Discussion; the one amino acid tested by them that stood out was aspartic acid; it displayed a stimulatory effect on production in most experiments, as expected based on its role in the biosynthetic pathway. Data from an experiment verifying this effect conducted by me are shown in Figure 23.

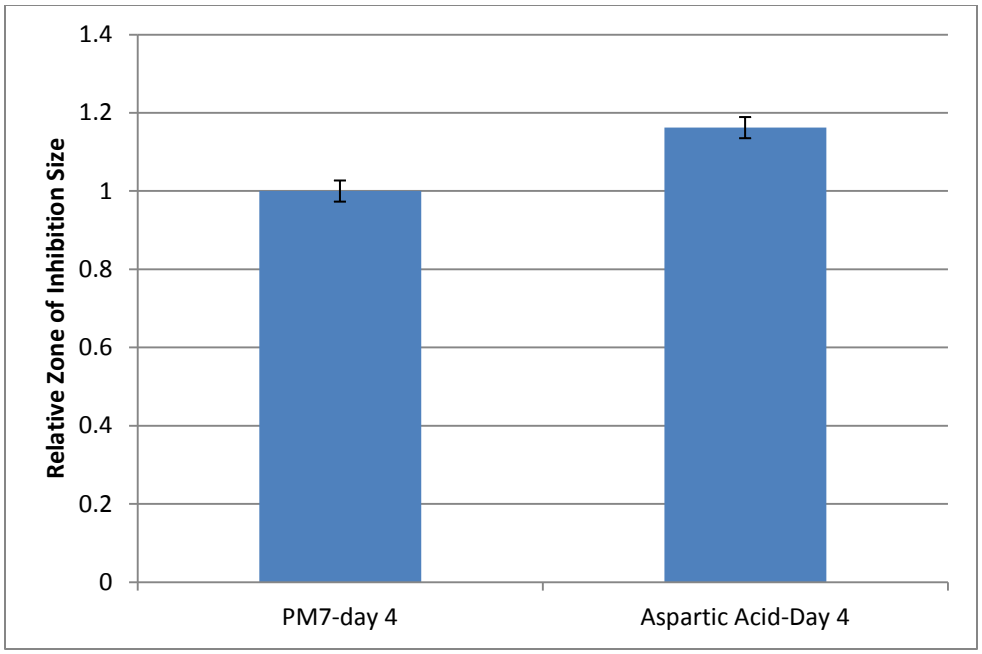

Figure 23: Stimulatory Effect of Aspartic Acid. Measure of the relative largest zone of inhibition size using the size of PM7 as standard of comparison. Error bars represent range of standardized values between flasks of the same experimental condition; lack of error bars indicates identical values between flasks. 1 mg/mL aspartic acid used.

Stimulatory Effect of Potassium Chloride (KCl)

Inorganic compounds were also tested to evaluate their effects on antibiotic production. Drew undergraduate Vasyl Nayda was responsible for testing some inorganic compounds; he tested potassium chloride (KCl), magnesium sulfate, manganese sulfate, zinc sulfate, and found that KCl was stimulatory at 100 µg/mL (Personal Communication, 12/15/2013) (data not shown). The effect of KCl was tested and confirmed several times, and the data from such a verification experiment conducted by me are shown in Figure 24. The maximum zone of inhibition diameter for KCl did not have overlapping error bars with PM7, with zone sizes around 12% higher (Figure 24). A summary of the results and concluded status of each additive is found in Table 4.

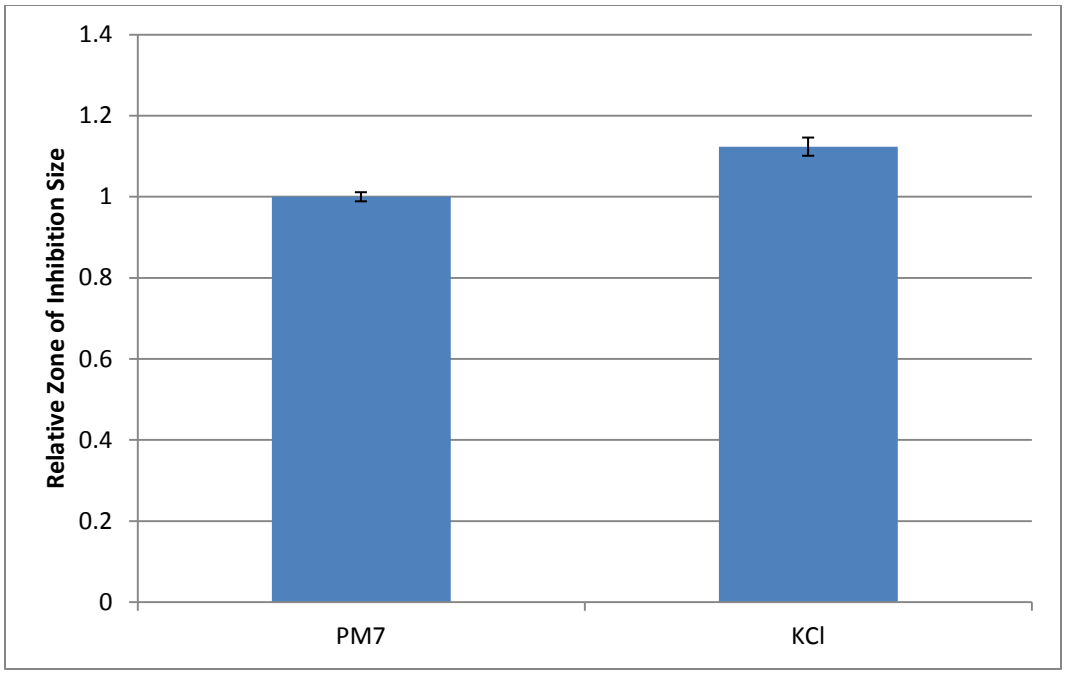

Figure 24: Stimulatory Effect of KCl. Normalization of the relative largest zone of inhibition size using the size of PM7 as standard of comparison. Error bars represent range of standardized values between flasks of the same experimental condition; lack of error bars indicates identical values between flasks. 100 µg/mL KCl used.

Table 4: Summary All Tested Additives, Including vitamins, amino acids, and KCl.

Stimulatory	Inhibitory	Inactive
• d-Biotin (unconfirmed) • Riboflavin(unconfirmed) • Histidine (unconfirmed) • Alanine (unconfirmed) • Aspartic acid • KCl additives showing positive or negative results only one time or are otherwise of interest for retesting are marked unconfirmed	• Serine • Arginine (unconfirmed) • Leucine (unconfirmed)	• Nicotinic acid • Ascorbic acid* • Folic acid • Thiamine* • Inositol • Pantothenic Acid • B12 • Valine • Tyrosine • Phenylalanine • Cysteine • Methionine • Arginine • Threonine • Glycine • Isoleucine

* Due to the large zone sizes observed with this additives and the large variability in the PM7 controls, these additives should be repeated.

Combination of Stimulatory Additives and Altering Concentrations

After testing all the amino acids at least once, the effects of combinations of stimulatory additives were evaluated. Because the stimulatory effects of both KCl and aspartic acid were observed several times, their combination was tested first. Aspartic acid was also evaluated at different concentrations, 3 mg/mL and 5 mg/mL (3x and 5x). Figure 25 shows the 3x aspartic acid had overlapping error bars with PM7 and not with the 1x, indicating a lack of stimulation. The 5x aspartic acid actually did not overlap with the lower error bar of PM7, indicating an inhibitory effect at this high concentration, with zone sizes around 10% smaller than PM7. The combination of aspartic acid and KCl was not stimulatory. The trend toward inhibition with higher levels of aspartic acid was more

pronounced with the added KCl; the 3x combination had zone sizes 10% smaller than PM7 and the 5x combination was around 15% smaller (Figure 25).

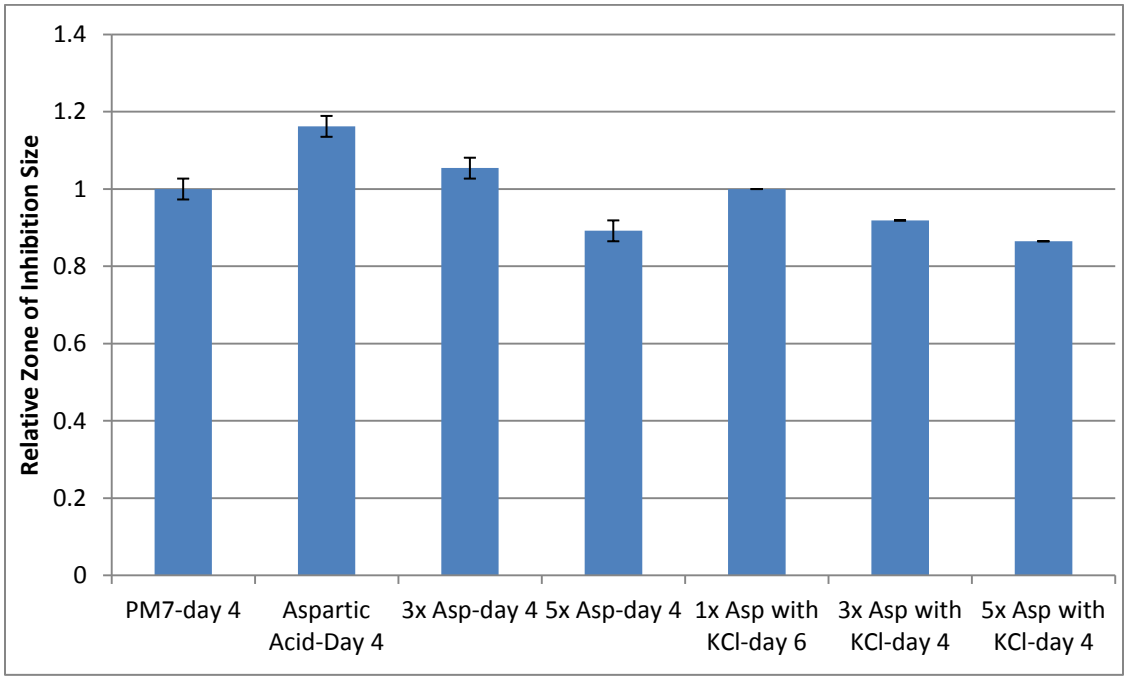

Figure 25: KCl and Aspartic Acid Combinations. Evaluation of the effect of increased concentration of aspartic acid with and without stimulatory KCl by comparing the relative largest zone of inhibition sizes using the size of PM7 as standard of comparison. Error bars represent range of standardized values between flasks of the same experimental condition; lack of error bars indicates identical values between flasks. 1x concentration is 1 mg/mL, 3x concentration is 3 mg/mL, and 5x concentration is 5 mg/mL. KCl and aspartic acid combination does not stimulate production; increased aspartic acid trends toward inhibitory.

As higher concentrations of aspartic acid were not stimulatory, lower concentrations, including 0.5 mg/mL (0.5x) and 0.25 mg/mL (0.25x), were explored. Both lower concentrations seemed stimulatory (Figure 26); the 0.5x was around 9% higher and the 0.25x was around 3% higher than the PM7 control. The 0.5x also did not

have overlapping error bars with the 1x, indicating further stimulation (Figure 26). The lower concentrations will need to be retested to confirm the stimulation.

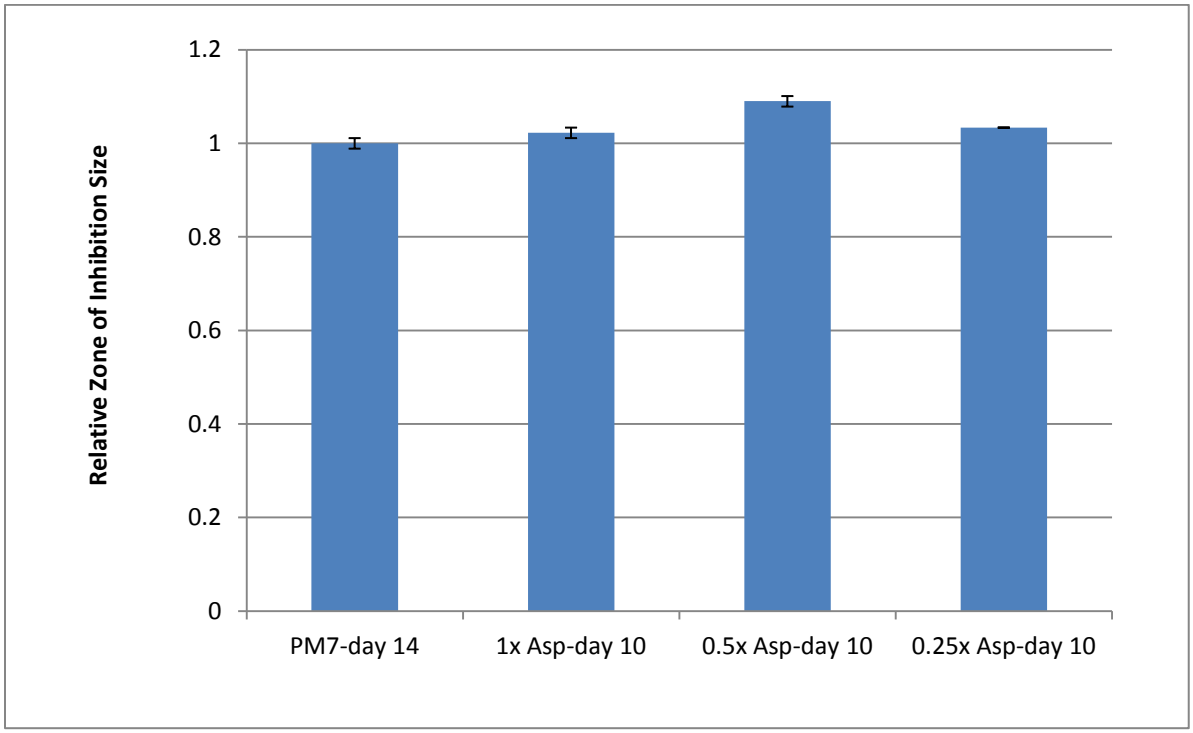

Figure 26: Decreased Concentration of Aspartic Acid. Evaluation of lower concentrations of aspartic acid comparing the relative largest zone of inhibition size using the size of PM7 as standard of comparison, error bars represent range of standardized values between flasks of the same experimental condition, lack of error bars indicates identical values between flasks. 1x concentration is 1 mg/mL, 0.5x concentration is 0.5 mg/mL µg/mL, and 0.25x concentration is 0.25 mg/mL. 0.5x was most stimulatory.

Discussion and Conclusions

The antibiotics platensimycin and platencin, discovered by Merck and Co. in the mid-to late 2000's, are produced by the actinomycete *S. platensis*. These compounds are significant because of their unique antibiotic target of bacterial fatty acid synthesis and

their efficacy against resistant, problematic pathogens (Singh *et al*. 2006). They came at a time where new antibiotic drugs were greatly needed due to a vast increase in resistance, and a lack of commercial development (Demain and Spizek 2012). Unfortunately, these compounds have poor pharmacokinetics and are inactive in feasible *in vivo* models, rendering them unusable as antibiotics (Singh *et al*. 2006). Despite the poor pharmacokinetics, the compounds are still important to antibiotic development and to medicine in general. Numerous projects are ongoing to attempt to synthesize analogs with enhanced pharmacokinetics that maintain antibiotic activity (Jang *et al*. 2013). Recently, platensimycin has shown activity as a diabetes drug in mouse models and continued research is in progress (Wu *et al*. 2011).

Because of the importance of these compounds, gaining knowledge about how they are produced and how to maximize their production is beneficial. When Merck and Co. discovered the compounds, they developed both a production medium and a seed medium. These media are both complex, which made them difficult to work with, especially for observing the effects of compounds added to the medium (Jose *et al*. 2013). Therefore, our first goal was to formulate a chemically-defined production medium and to reduce the complex nutrient carryover from the seed medium upon inoculation. Both goals were accomplished with the establishment of PM7 and the washing procedure for the seed culture (Figures 14 and 15). Unfortunately, there is some variability in the amount of antibiotic production supported by PM7. Improvement of production is a continual goal. Mass spectrometric data, shown in Figures 16 and 17, confirm that platensimycin is produced in PM7. These data also show that platencin was undetectable

in the PM7 fermentation, which was expected because of the biosynthetic pathway branching (Figure 9 and 10). At that point, the project focused on platensimycin (Smanski *et al*. 2011). No conclusions could be drawn about the effects of the additives on production of platencin due to its lack of detection.

Many of the compounds that go into the synthesis of platensimycin and platencin stem from the citric acid cycle and glycolysis (Figures 8-10) (Herath *et al*. 2007; Smanski *et al*. 2011). For example, pyruvate is involved in several reactions of the biosynthetic pathway, i.e., in the production of both moieties of the compound. Glyceraldehyde-3-phosphate and DHAP, two intermediates of glycolysis, are utilized as starting material for different reactions of the biosynthetic pathway. Moreover, the tail to tail incorporation of acetate molecules observed in the labeled feeding studies indicates the involvement of the citric acid cycle (Figure 7). This extensive linkage between these metabolic pathways and the biosynthetic pathway suggests that the biosynthesis may be influenced by these pathways. A potential influence may serve as an explanation of the necessity for glucose in the production medium. All tested production media have glucose in some form (i.e., yellow corn meal or dextrin), and the removal of the glucose resulted in a complete lack of antibiotic production, despite an additional carbon source (lactose) in the medium.

The establishment of the chemically-defined medium and the elimination of the complex nutrient carryover allowed for the evaluation of the effect of additives, specifically vitamins and amino acids, on antibiotic production. Riboflavin and d-biotin stimulated antibiotic production (Figure 18). The electron carrier flavin adenine

dinucleotide (FAD) is derived from riboflavin (Berg *et al*. 2012). It is involved in the citric acid cycle and serves as a cofactor for the pyruvate dehydrogenate complex, both of which are directly involved in the biosynthesis of platensimycin. Having additional FAD available to the cells could allow for increased citric acid cycle activity and pyruvate dehydrogenase activity, which could lead to increased production of the ADHBA moiety of platensimycin, explaining the stimulation. d-Biotin is an important cofactor in bacterial fatty acid synthesis, specifically involved with the acetyl CoA carboxylase complex (Magnusen *et al.* 1993). Additional cofactor for fatty acid synthesis could increase the overall health of the organisms, which would allow for higher levels of antibiotic production. Figure 18 also shows that B_{12} was inhibitory at the initial concentration tested, 10 µg/mL. However, at this concentration it inhibited growth of the culture. Interestingly, when the concentration was reduced to 10%, the inhibitory effects on growth and platensimycin production were diminished. This result has lead us to conclude that B_{12} is inactive.

Figure 19 shows the effects of ascorbic acid, folic acid, thiamine, and inositol. Unfortunately in this experiment, there was high variation between the two PM7 control flasks used, generating high error bars on the PM7 data points. Figure 19B shows that, as hypothesized, thiamine has a 35% larger zone size than PM7. However, despite the larger zone size, the error bars overlap with PM7 because of the high variation. The high variation in PM7 is an indicator that the experiment should be repeated, especially for thiamine. Thiamine plays a role in the synthesis of the tetracyclic enone portion of the platensimycin structure (Herath *et al*. 2007). It also serves as a cofactor of the pyruvate

dehydrogenase complex which plays a role in the synthesis of the ADHBA moiety by converting pyryuvate to acetyl CoA and stimulating the citric acid cycle, enhancing its potential to stimulate production (Berg *et al*. 2012). If a repeated experiment confirms that is not stimulatory, it could be many reasons, including a possible inability of the compound to enter the cell. In *Streptomyces cinnamonensis*, addition of thiamine to production medium increased production of the natural product antibiotics monensins, indicating that thiamine is able to enter the cells (Matějů *et al*. 1990). A remaining possibility is the idea that the thiamine utilizing step is not rate-limiting to production. It is possible that the cells are naturally producing enough thiamine. Ascorbic acid should also be retested because its largest zone size was 20% higher than PM7, but was not considered stimulatory because of the large error bars on PM7 (Figure 19).

Based on knowledge of the biosynthetic pathway, it was hypothesized that aspartic acid would stimulate antibiotic production. Aspartic acid serves as a precursor for the starting material in the pathway responsible for the ADHBA potion of the chemical structure of platensimycin and platencin (Smanski *et al*. 2011) (Figure 6). As shown in Figures 23 and 25, aspartic acid was repeatedly stimulatory to antibiotic production at 1 mg/mL and at lower concentrations, consistent with its role in the pathway. Interestingly, its effect approached inhibitory as the concentration was increased to 3 mg/mL and 5 mg/mL (Figure 25), which is close to its solubility limit in water. It is possible that there is a limit to the amount of aspartic acid that can enter the cell and the additional aspartic acid in the medium may be somehow interfering with growth of the cell.

Histidine stimulated antibiotic production (Figure 20). The stimulatory effect of histidine could possibly be explained by the necessity of key histidine residues in a number of enzymes that play roles in the biosynthesis of platensimycin. A good example of such an enzyme is DXS in the MEP pathway (Querol *et al*. 2001). Additional histidine could ensure that the cells have enough material to assemble the key enzymes necessary for the synthesis of platensimycin. In addition, the histidine biosynthesic pathway shares a common intermediate with purine synthesis, phosphoribosyl pyrophosphate, PRPP (Berg *et al*. 2012). Additional histidine could decrease the need for histidine synthesis and allow more PRPP to be used for nucleotide synthesis. Having additional available nucleotides could facilitate an increase in gene expression of the genes in the platensimycin and platencin gene clusters, increasing antibiotic production.

Alanine also stimulated antibiotic production (Figure 22). Alanine is one of the amino acids synthesized from pyruvate (Berg *et al*. 2012). Additional alanine in the cell would decrease the need for alanine synthesis and could increase the amount of pyruvate available to go into platensimycin and platencin production.

Leucine also stimulated antibiotic production (Figure 22). There is no clear reason to explain why leucine would inhibit antibiotic production. The same is true for the inhibitory effect observed with arginine and serine (Figure 20 and 21). A potential explanation is that the concentration at which they were tested i.e., 1 mg/mL, was too high for the cells. If this were the case, the inhibitory effect might not be observed at

lower concentrations, as was the case with B_{12}. Therefore, these amino acids should be rested at a lower concentration.

Despite the confirmed stimulation of KCl (Figure 24), it does not have a straightforward connection to the biosynthetic pathway. It could be possible that the KCl contributed to better growth of the cells, generating more cells, which would increase production without affecting the pathway. Two ingredients that contributed to good production in PM4 (see Table 1), i.e., yeast carbon base and vitamin-free yeast base, contain potassium and chloride ions.

It was interesting to observe that when two stimulatory additives were combined (aspartic acid and KCl) instead of the expected increased stimulation, the effects were canceled resulting in a complete lack of stimulation (Figure 25). The lack of stimulation indicates that the additives must have some interaction with each other, which should be further explored. It was later determined that the optimal concentration of aspartic acid was really below 1x concentration (Figure 26), and the combination experiment was not carried out using this concentration. The concentrations used may have affected the results and the combination experiment should be tested again using the optimal aspartic acid concentration.

In addition to the compounds already described, Drew University undergraduate students Sana Siddiqui, Carrie Perkins, and Drew Stenger, tested the effects of glutamine and asparagine, and observed that these amino acids appear inhibitory as their zone of inhibition were below that of the PM7 control (Personal communication 12/15/2013).

They also tested lysine, tryptophan, proline, glutamic acid, and determined them to be inactive. As with the other inhibitory compounds, these compounds should be tested at lower concentrations at which the inhibitory effect may be diminished.

Many of the effects of the amino acids and vitamins need to be confirmed, and additional experiments are neded. All of the inhibitory amino acids should be tested at lower concentrations. In addition, nucleosides and additional inorganic salts are of interest and should be tested. It is hypothesized that addition of nucleosides could provide increased starting material for transcription and thus potentially increase expression of the genes responsible for the synthesis of platensimycin. It is also possible that the enzymes involved in biosynthehsis could require the presence of inorganic salts, such as magnesium. If true, adding the salts could stimulate production. Upon confirming the effects of each compound, experiments altering the concentrations can be carried out to determine optimal concentration, as was done with aspartic acid. Finally, combination experiments including different stimulatory and inhibitory additives should be carried out.

Because the agar diffusion assay does not involve purified compounds, mass spectrometric assays should be conducted on all fermentations with stimulatory additives, to confirm that the increase in zone of inhibition size is specifically related to an increase in platensimycin. Improvements to the mass spectrometric assay could include identifying the impurity on the mass spectrum at 463 M/Z and separating it from the platensimycin absorbance peak, allowing for definitive quantification of platensimycin.

Alternatively, the mass spectroscopy peak for platensimycin can be separated from the impurity enough to calculate the individual area, which could allow for quantification if a dose curve using the mass spectrum curve was generated. However, the platensimycin is present in numerous different forms, including plus one isotopes and sodium adducts, complicating this method of quantification (data not shown).

The eventual goal is to piece together all the different active additives and propose an idea of how they play into or affect the biosynthetic pathway, and to develop a more effective chemically-defined production medium. Having more knowledge about the biosynthesis pathway and its regulation could potentially provide information about how to manipulate the structure of the final product, which could generate a compound with better pharmacokinetics. Optimizing a production medium could allow for increased production of platensimycin that can be used in chemical modification experiments or studies evaluating platensimycin as a diabetes drug. It is our hope that this information can contribute to making platensimycin a usable antibiotic or diabetes drug, or that the information can be used to better explore other potential antibiotic compounds.

To the overall project I have contributed to the development of the chemically-defined PM7 and the removal of the crude nutrient carryover from the seed medium. Through a mass spectrometric assay the presence of platensimycin in PM7 was definitively confirmed. Several PM7 additives, including vitamins and amino acids, have been evaluated for stimulatory or inhibitory effects on the production of platensimycin.

Of these, aspartic acid and KCl have shown stimulatory activity. These effects can provide information about the regulation of the biosynthetic pathway of platensimycin in *S. platensis*.

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