Isolation of an Antibiotic for Citrus Greening Disease

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Abstract

Citrus Greening Disease is an illness caused by a bacterium that renders citrus fruit unmarketable and kills infected citrus trees. Good bacteria in the microbiota of citrus trees may produce an organic molecule with antibiotic properties. Using natural product isolation, this project aims to isolate an antibiotic for Citrus Greening Disease.

Introduction

Background

In recent years, citrus orchards have been facing a pandemic. Citrus greening disease has swept across the nation, from Florida to California, devastating citrus trees and the citrus economy. Since the disease was introduced in Florida in 2004, citrus orchards have been decimated. In 2004, the effect of the disease on crops was barely visible. Farmers in Florida produced nearly 242 million 90-pound boxes of Oranges. In 2022, the projected crop is only 20% of that.[1] Citrus Greening Disease has cost the citrus economy over $1 billion per year, with nearly 5000 jobs lost annually.[2] Today, the disease and its vector has spread west, reaching California, where the results will be the same. Finding a cure for Citrus greening disease is crucial for the citrus industry in America to survive.

Figure 1. A picture of the asian citrus psyllid on the leaf of a citrus tree. Citrus greening disease is spread through vector transmission by the asian citrus psyllid.

Figure 2. Citrus orchards have been ravaged by citrus greening disease. Citrus greening disease causes canopy thinning, twig dieback, mottled leaves, root starvation, the accumulation of starch and changes in metabolite profiles. This leads to a decline of tree health and ultimately death.

Citrus greening disease is caused by a bacteria called *Candidatus* Liberibacter asiaticus, referred to as *C*Las, and is spread through vector transmission of the Asian Citrus Psyllid. It first affected citrus crops in China, where it was named Huanglongbing, meaning yellow dragon disease.[1] Today it affects over 50 countries in the world, including 7 of the top 10 citrus producing countries in the world.[3] Trees infected by Citrus Greening produce small, asymmetrical and green oranges that are ultimately unmarketable. Their taste has been described as bitter, sour, salty and lacking in sweetness. Citrus Greening disease causes canopy thinning and twig dieback, discolored and mottled leaves, and poor or undeveloped root systems. It ultimately causes accumulation of starch and changes in the secondary metabolite profiles which results in root starvation, decline of health and death.[4]

There are a few methods to prevent the spread of the disease and mitigate symptoms, but ultimately a cure for the disease is necessary. One option is developing an antibiotic that targets the cause of the disease, *C*Las.[4] One interesting aspect of the disease is that not all trees are affected in the same way. While trees in a single orchard are clonally propagated, and therefore the genome of the plants are identical, some trees live for years with minimal symptoms of the disease while others deteriorate rapidly. A possible explanation for this can be found in the differences in the microbiomes of the healthy trees compared to the susceptible trees. Those that resist Citrus Greening Disease have different organisms living in their microbiome.[5]

The microbiome of a plant has an important role in its health. The microbiome is made up of a collection of endophytes, microorganisms that form symbiotic relationships by colonizing plants. Plants provide protection from the elements and nutrients, while the endophytes produce bioactive compounds that may help the plant by protecting it from abiotic factors like drought, or defending against microbial pathogens.[6] These bacteria may be producing a key molecule, or natural product, that has antibacterial properties against *C*Las.

These compounds are often referred to as natural products. Natural Products, also known as secondary metabolites, are organic compounds produced by a living organism. While they provide a competitive advantage to the organism, they aren't necessary for survival. Our hypothesis for why some trees resist citrus greening while others fall susceptible is that an organism in the microbiota of citrus trees is producing a natural product with antibiotic properties. Analyzing these molecules and isolating a bioactive compound could be a possible step for developing antibiotic treatment for Citrus Greening Disease.

Past Work

This project is in collaboration with Microbiologists at UC Riverside. In a previous study they described the niche diversity of endophytes living in the citrus holosystem. They collected bacteria and fungi from the roots, stem and leaves of citrus trees, creating a microbial repository of the citrus microbiome. A crude extract of citrus-derived bacterial strain CB687 (identified as *Bacillus velezensis*) displayed promising antibacterial properties when analyzed by a disc-diffusion bioassay against *Liberibacter crescens* (a culturable surrogate for *C*Las).

Methods

Extraction

Six liters of LB broth was prepared as per the manufacturing instructions and inoculated with CB687 in liquid culture. The strain was first streaked out using serial dilution technique and left overnight in a 30 ℃ incubator to grow. The next day, a single colony was used to inoculate each 6 × 15 mL of LB and shaken overnight for 20 hours at 30 °C. The mature culture was added to 6 × 1L LB in Thomson Ultra Yield 2.5L flasks, and shaken at 30 °C for 2 weeks. One 15 mL culture was added to each 1L of LB. The organic molecules were extracted from the solution by solid phase extraction with HP-20 resin. The liquid culture was stirred with resin beads for 30 minutes and separated using vacuum filtration. The resin was shaken with ethyl acetate for 30 minutes and again separated using vacuum filtration. This was repeated for a total of three extractions and the solvent was removed using a rotary evaporator, to give a crude

weight of 0.8544 g. The crude extract was tested for antibiotic activity against *L. crescens* using the disk-diffusion bioassay.

Isolation

0.8389 g of the crude extract of CB687 was fractionated using normal phase flash column chromatography on a Combiflash RF (Teledyne Isco, Inc.). The crude extract was loaded using the dry-load method, and fractionated on a 10 g RediSep RF pre-packed silica gel column, using a gradient method: 100% hexanes to 100% ethyl acetate over 20 minutes, followed by 100% dichloromethane to 20% methanol in dichloromethane over 10 minutes. Thin layer chromatography (with visualization using UV light and anisealdehyde stain) was used to guide the consolidation into seven fractions obtained from the autocolumn. Of these, fractions D, E, F and G were active in the bioassay. These fractions were again analyzed by thin layer chromatography. F and G were the most active, contained the majority of the active material, and were determined to be similar enough to combine. Fractions F and G were used in moving forward with the bioassay guided isolation.

Combined Fractions F and G were prepared for HPLC (High Pressure Liquid Chromatography) by dissolving in 200 µL of methanol, and centrifuging to remove any particulate matter. The fractionation was performed on a Shimadzu Prominence-i Liquid Chromatograph. The method for the first HPLC run increased from 1%-40% acetonitrile over 12 minutes. Eleven fractions were obtained from this method. Of these, fractions A, B and J were active. Their masses were 0.0129g, 0.0030g, and 0.0080 g, respectively. Fractions A and B were combined and subjected to a second run on the HPLC with a Shimadzu ELSD-LT II (Low Temperature Evaporative Light Scattering Detector) attached for universal visualization. The method for the second HPLC with ELSD run increased from 1%-30% acetonitrile over 16 minutes. A splitter was added for the HPLC and ELSD with a 4:1 split ratio (solvent collection: ELSD input). Four fractions were obtained from this method. Fraction beta was subjected to further analysis by NMR. It had a mass of 0.0061 g.

Bioassay

The fractions were prepared for bioassay by diluting the sample with methanol to give a concentration of 1 milligram per 15 microliters. 15 microliters of this solution were pipetted onto each of three replicate sterile filter disks for each fraction, and allowed to evaporate. The dry disks were placed in a sterile eppendorf tube, sealed with parafilm and sent to collaborators at UC Riverside, who performed the disk-diffusion bioassay against *L. cresens*.[7]

NMR Spectroscopy

Fraction beta was analyzed by ^{1}H and HSQC NMR spectroscopy using a JEOL ECS 400 MHz NMR Spectrometer at 400 MHz. The ^{1}H NMR showed peaks between 1.0-1.5 and 3.0-4.0 ppm. The carbon and proton shifts corresponding to each crosspeak in the HSQC spectrum was tabulated using MestreNova, and the resulting .csv file was used as the input for a Small Molecule Accurate Recognition Technology (SMART) NMR search (https://smart.ucsd.edu/) to identify similar natural products. SMART NMR was created using nonuniform NMR data samples and deep Convolutional Neural Networks and can be used to assist discovery efforts of natural products.

antiSMASH

The genome sequence of CB687 was uploaded to the antibiotics & Secondary Metabolite Analysis Shell (antiSMASH: https://antismash.secondarymetabolites.org) an online tool for the identification, annotation and analysis of natural product biosynthesis gene clusters within the genome of bacterial or fungal organisms.

Results & Discussion

CB687 crude extract was first separated into eleven fractions, as shown in Figure 3. Fractions D, E, F and G were determined to have antibiotic activity by bioassay, performed by collaborators at UC Riverside. Fraction G had the largest zone of inhibition, at 3.3 cm. The four active fractions were analyzed by hin layer chromatography under UV light and by anisaldehyde stain, shown in Figure 4. Fractions F and G seemed to contain similar compounds. Further, fractions D and E had two small, distinct bands. Fractions F and G seemed like a more promising option to pursue because it had a larger mass compared to Fractions D and E. In addition, it would be easier to purify since only one compound showed up in UV. These reasons made us pursue further purification with Fractions F and G.

Fractions F and G were combined, then separated into eleven fractions using the HPLC. Each fraction was tested in the bioassay against *L. crescens*, and also subjected to ^{1}H NMR. While some of the data seemed to contain interesting peaks, none of them were pure enough to elucidate the structure of a molecule. It was clear further purification was still necessary. From the bioassay data, we learned that Fractions A, B and J had antibiotic activity.

Figure 3. Isolation of Bioactive Compounds from CB 687. Starting with the crude extract of CB 687, we used flash column chromatography to yield 7 different fractions, labeled A-G. Fractions D, E, F and G showed antibiotic activity by bioassay, as displayed on the chart. Fractions from chromatography are listed as letters. Masses below each fraction denote the mass of the dried fraction. For active fractions, the zone of inhibition (diameter in cm) for the disk-diffusion assay against L. crescens is also given.

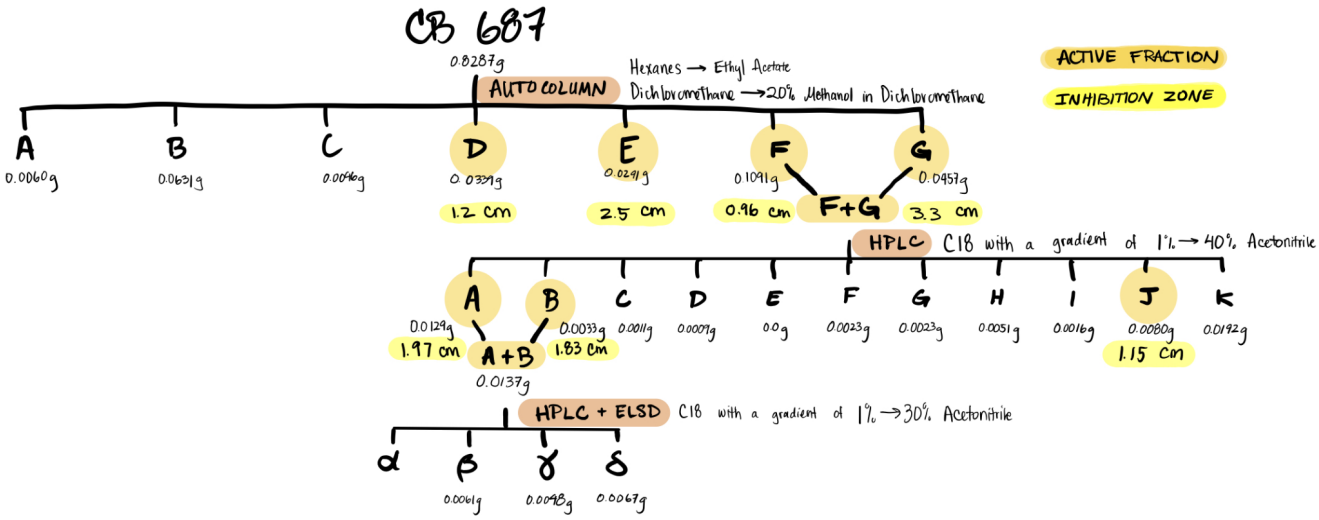
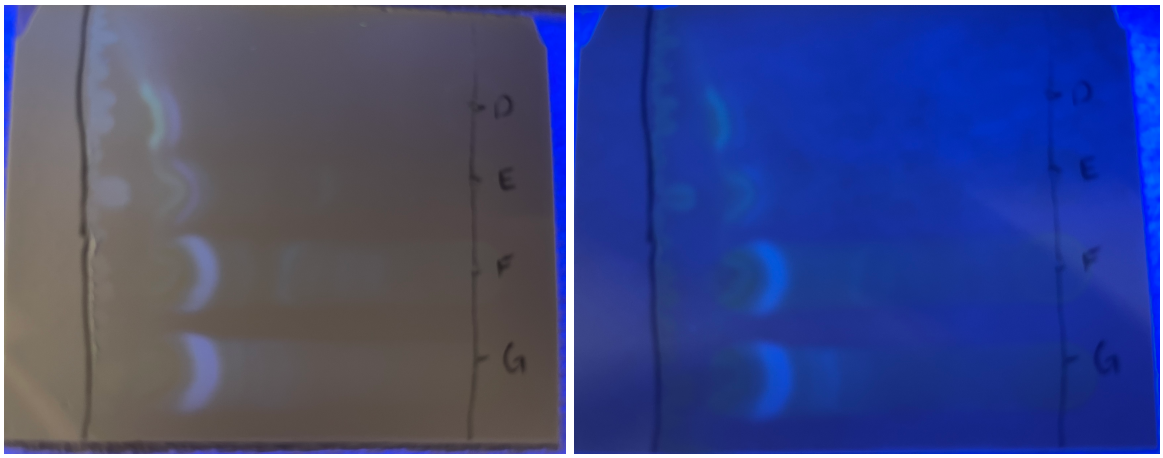


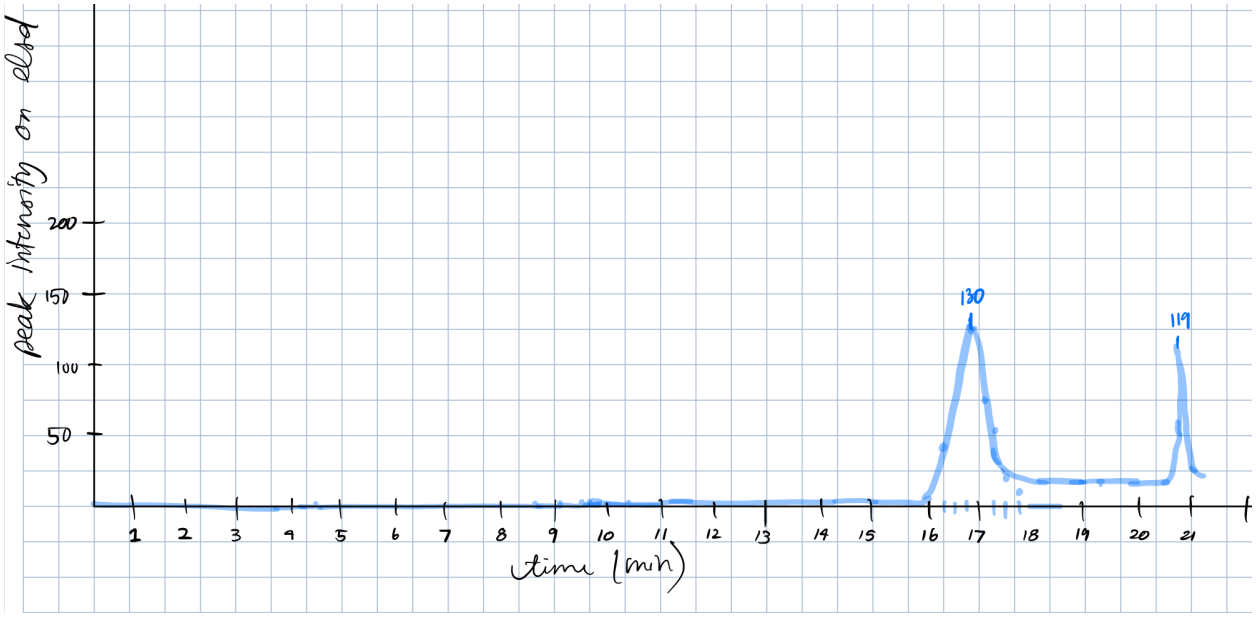
Figure 4. TLC Plates for Fractions D, E, F and G. Analysis under UV light suggests that F and G contain similar, and relatively less complex mixtures of molecules.



Surprisingly, fractions A and B, which eluted at a time in the chromatogram with no discernible UV peaks, had the largest zone of inhibition, at 1.97 cm and 1.83 cm, respectively. Moreover, when dried down, fractions A and B contained the majority of the mass of the fractions. This observation led us to conclude that the active molecule doesn't absorb UV light, and thus is invisible by the most commonly used type of HPLC detector. In order to visualize the molecules that were coming off the HPLC, we needed to use another type of detector. The ELSD, or Evaporative-Light Scattering Detector, detects non-volatile sample components in a volatile eluent and allows for visualization of individual molecules that didn't absorb UV light. It is a more universal type of detector. However, unlike the UV detector, it is destructive. As a result, we needed to install a splitter to send a fraction of our sample to the ELSD, while simultaneously collecting the majority of the sample for further study.

Fractions A and B were combined, due to similarity in content, and subjected to a second round of HPLC with the ELSD. Unfortunately, The ELSD was not communicating with the HPLC computer, making it much more difficult to visualize when a peak came off the HPLC. Fortunately, the ELSD was able to detect and display the live signal as the sample passed through it, even though a graph wasn't generated. To compensate for this, the ELSD signal was recorded using an iPhone camera and the resulting chromatogram was plotted by hand to guide future separations. Using this, I separated the remaining sample into four fractions, labeled α through δ.

Figure 5. Graph of ELSD signal as a function of time. Hand plot of ELSD chromatogram, transcribed from an iPhone video of the run. This graph was used to guide the second separation by HPLC.



^{1}H NMR spectra were obtained for all four fractions. Fraction β appears to contain mostly one compound (or a mixture of closely-related compounds), shown in Figure 6. The ^{1}H NMR spectra for this fraction had several peaks in the 3-4 pm region, characteristic of molecules with sugar moieties, along with peaks in the 1-2 ppm region, indicating the methylene envelope of a long alkyl chain. An HSQC spectrum was taken of Fraction β for further analysis, shown in Figure 7.

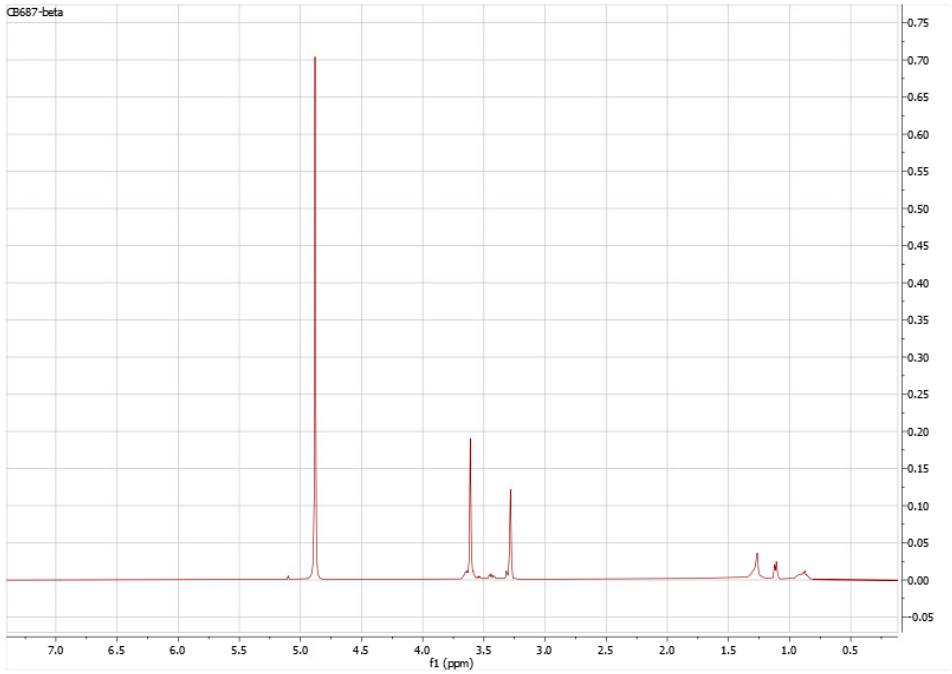
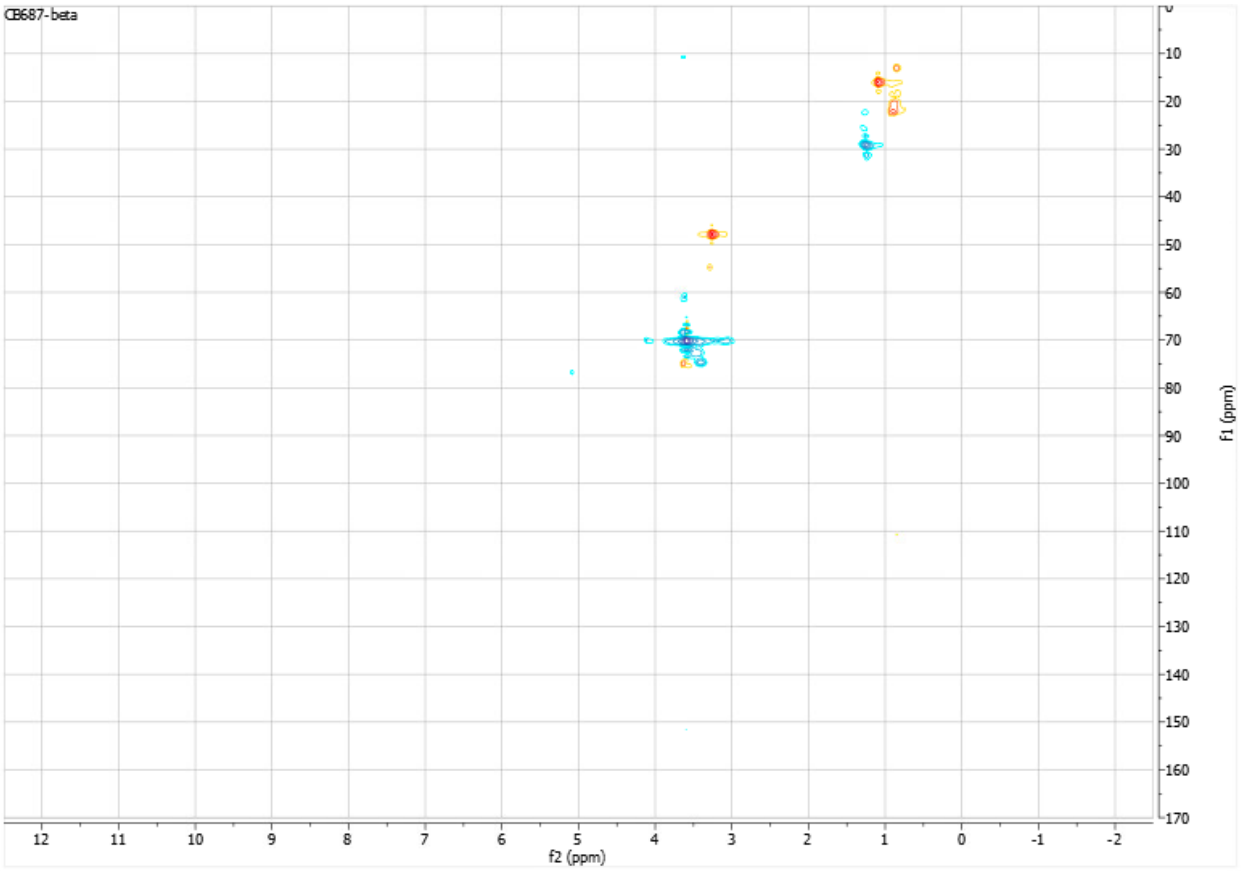


Figure 6. H-NMR of Fraction Beta. Peaks in the 1.0- 1.5 ppm region suggest that the fraction contains long carbon chains. Peaks in the 3.0 to 4.0 ppm range suggest that the fraction contains sugar-like groups.

Figure 7. HSQC for Fraction β.



The spectra from the ^{1}H NMR spectra and HSQC were converted into a .csv file on MestreNova and uploaded to SmartNMR. There were several hits, but one molecule was consistent with the ^{1}H NMR spectra and other aspects of our unknown molecule. Lysoplasmanylinositol, shown in Figure 8, has a long carbon chain and sugar group, which is consistent with the results from the ^{1}H NMR spectrum of Fraction β.

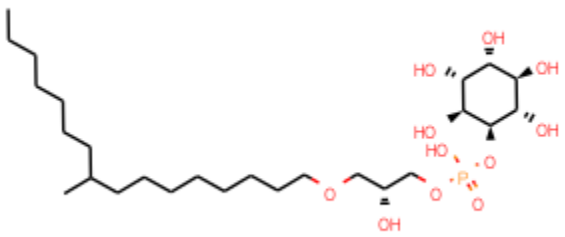
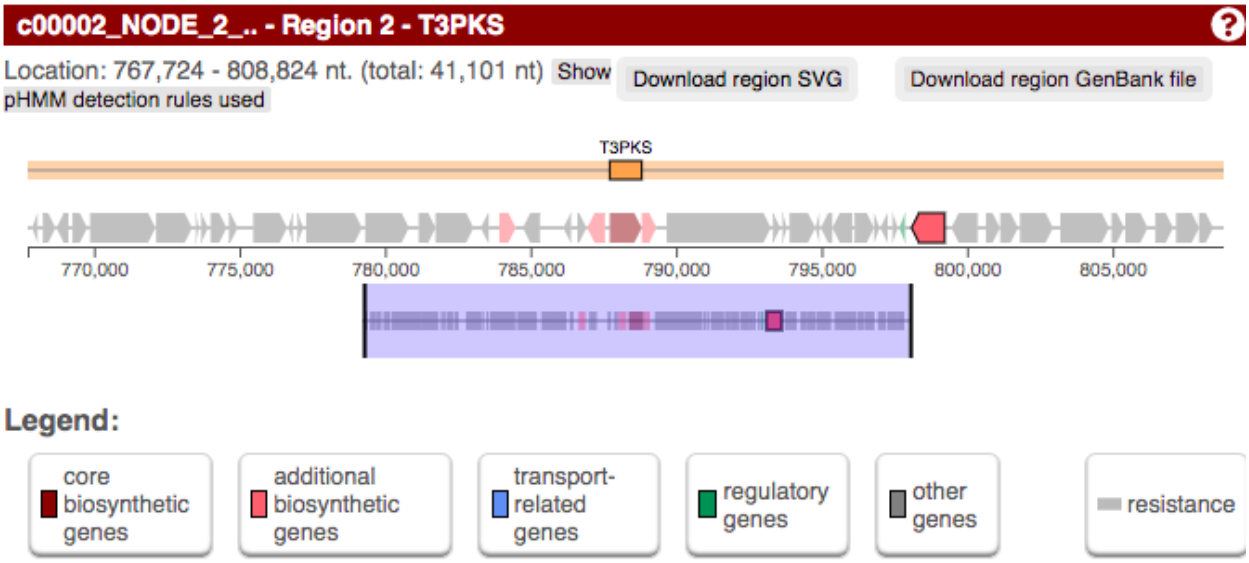


Figure 8. Lysoplasmanylinositol from SmartNMR analysis. A candidate antibiotic molecule based on the smartNMR search on data from Fraction Beta. Lysoplasmamylinositol is an antibacterial agent, known to have activity against some gram negative Bacillus species, such as *E.* coli.

After further research on this molecule, other characteristics made lysoplasmanylinositol a reasonable candidate for the bioactive molecule in fractions A and B. This molecule wouldn't absorb UV light, which could explain why it was visualized in the ELSD, but not with the UV detector of the HPLC. In addition, the phosphate group may become deprotonated if the pH were to change slightly. This could

explain why the active molecule came off in the first fractions in very polar eluent composition (~15% aqueous acetonitrile) when first separated with the HPLC, but later came off at a more nonpolar eluent composition (~50% acetonitrile) when separated with the HPLC and ELSD. The polar nature of the molecule is consistent with observations by Matsunaga, who first discovered lysoplasmanylinositol by extracting it from marine sponge *Theonella swinhoei* with 70% n-PrOH, a very polar solvent. Finally, Matsunaga identified antimicrobial activity of this molecule against *E. coli*. *C*Las is a gram negative bacterium, like *E. coli*.[8]

Figure 9. antiSMASH analysis results. c00002_NODE_2_ of Region 2 contained genes that could have a role in synthesizing the candidate molecule, lysoplasmanylinositol.



To further analyze the structure of the potential antibiotic, data was collected on the genome of CB687 using a database called antiSMASH. antiSMASH presents data regarding whether a bacterium has the gene to produce antibiotics and highlights similar gene clusters and the function of a gene, if known. The antiSMASH analysis of the CB687 genome revealed several gene clusters that make known antibiotics. None of these known antibiotics were consistent with the NMR data or the SmartNMR results for fraction beta. However, an unidentified gene cluster contained genes that could play a role in producing a compound like lysoplasmanylinositol. On c00002_NODE_2_ of Region 2, a biosynthetic gene cluster was identified containing genes ctg2_785 and ctg2_772. Based on sequence similarity to known enzymes, gene ctg2_785 is identified as a glycosyl transferase (Glycos_transf_1), which could have a role in adding a sugar group on a molecule. Gene T3PKS (type-3-PKS) is identified as a polyketide synthase, which plays a similar role to fatty-acid synthase genes. Fatty-acid synthase genes add two carbon groups on long fatty chains, meaning it could have a role in extending the carbon tail of a phospholipid like lysoplasmanylinositol. These factors make lysoplasmanylinositol an interesting compound for further consideration.

Conclusion

Fraction β may contain a structure similar to lysoplasmanylinositol. ^{1}H NMR data indicated sugar groups and a saturated hydrocarbon chain. Further, antiSMASH analysis reveals a biosynthetic gene cluster containing a few key genes that could be involved in assembling an inositol phospholipid. Finally other aspects of the molecule such as its polar characteristics and antimicrobial properties make it a good

candidate for the bioactive molecule in Fraction β. However, further analysis is needed in order to confirm the structure of the molecule with antibiotic activity. Next steps for this project would be to further purify Fraction β in order to gain clearer NMR data for structure elucidation. It also would also be beneficial to grow 12L of CB687 and following the same methods of isolating Fraction β but using the ELSD during the first round of HPLC. This could lead to isolating a pure compound with a larger mass in fewer steps. It could be used for analysis by NMR, mass spectrometry and bioassay for definitive structure elucidation.

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Appendix

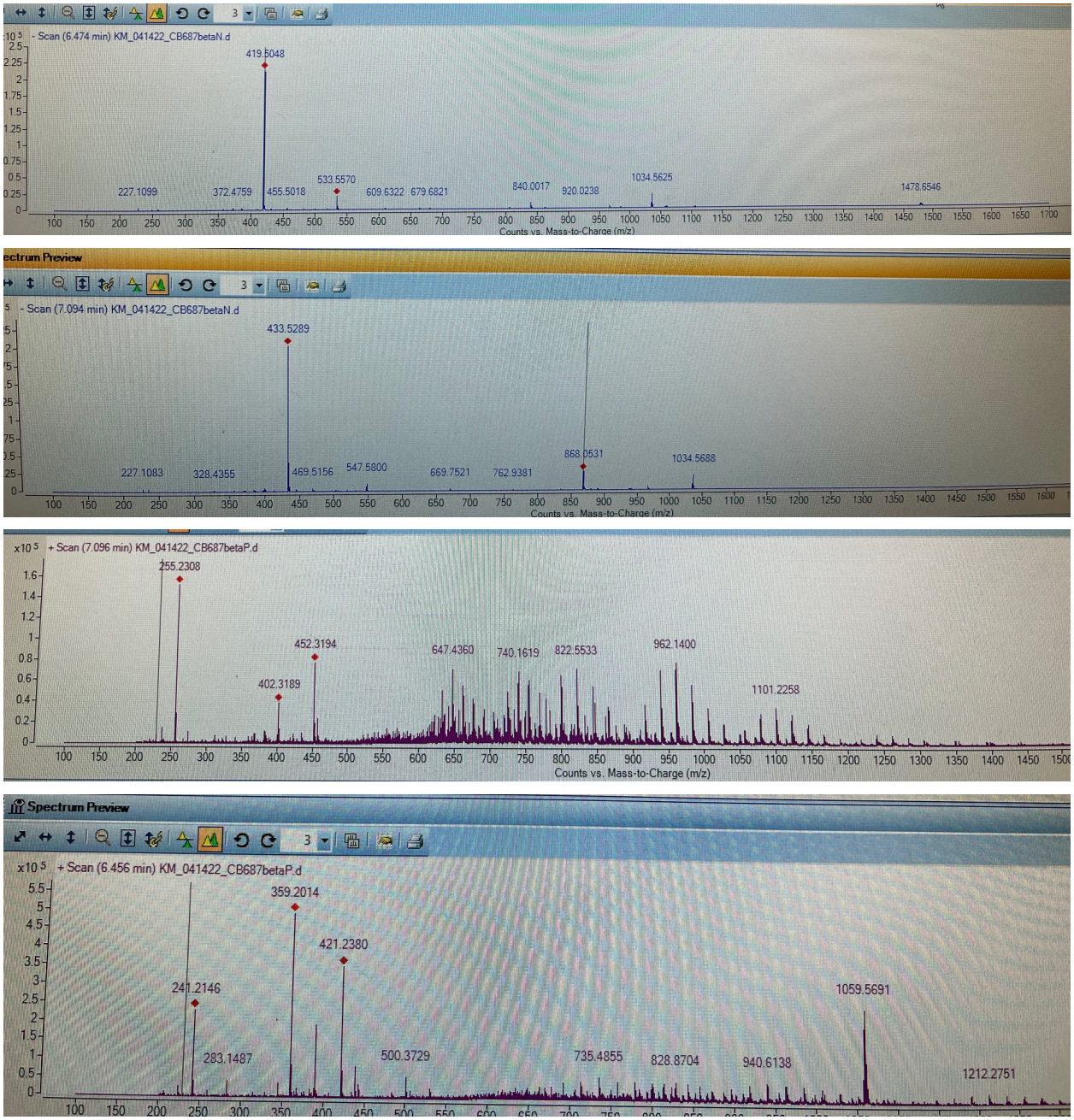
Table 1. Bioassay Results from UC Riverside for First Round of Isolation. Displays the inhibition zones of each filter disk from the fractions from the flash column chromatography. Those highlighted in orange are the fractions that were used in future separations.

Fraction	Rep 1 zone (cm)	Rep 2 zone (cm)	Rep 3 zone (cm)	**Ave zone (cm)**
CB687 (6L)- A	0	0	0	0
CB687 (6L)- B	0	0	0	0
CB687 (6L)- C	0	0	0	0
CB687 (6L)- D	1.1	1.2	1.3	1.2
CB687 (6L)- E	2.4	2.5	2.7	2.533333333
CB687 (6L)- F	0.9	1	1	0.9666666667
CB687 (6L)- G	3.2	3.3	3.4	3.3

Table 2. Bioassay Results from UC Riverside for Second Round of Isolation. Displays the inhibition zones of each filter disk from the fractions from the first separation with the HPLC. Those highlighted in orange are the fractions that were used in future separations.

Fraction	Rep 1 zone (cm)	Rep 2 zone (cm)	Rep 3 zone (cm)	**Ave zone (cm)**
CB687F+G	3.4	3.3	3.7	3.466666667
CB687F+G - A	2	2.1	1.8	1.966666667
CB687F+G - B	2.1	1.8	1.6	1.833333333
CB687F+G - C	0	0	0	0
CB687F+G - D	0	0	0	0
CB687F+G - E	0	0	0	0
CB687F+G - F	0	C	C	0
CB687F+G - G	0	0	0	0
CB687F+G - H	~	0	0	0
CB687F+G - I	0	~	0	0
CB687F+G - J	C	1.1	1.2	1.15
CB687F+G - K	C	C	C	

Figure 10. LCMS Data of fraction beta.



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