Bioassay-guided isolation of a natural product inhibitor of *Epicoccum nigrum* to combat citrus greening disease

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ABSTRACT

Huanglongbing disease (HLB), commonly known as citrus greening disease, is a bacterial infection that impacts citrus trees across the United States. Citrus greening is caused by *Candidatus* Liberibacter asiaticus (*C*Las), a bacterium that blocks sugar flow through the phloem, leading to unprofitable harvests. In this study, we used bioassay-guided isolation and organic structure elucidation to identify an inhibitor produced by *Epicoccum nigrum*, an endophytic fungus isolated from citrus trees residing in infected groves.

INTRODUCTION

Background

Citrus greening (aka Huanglongbing disease, HLB) is a bacterial infection caused by *Candidatus* Liberibacter asiaticus (*C*Las), which wreaks havoc on citrus trees. *C*Las blocks sugar flow through the phloem, leading to green and bitter citrus fruits, discolored leaves, and limp branches.[1] The bacterium is transmitted among citrus trees by the Asian citrus psyllid.[2] Citrus greening was first seen in China in the early 1900s and made its way to Florida by 2005 via shipments of citrus trees from Southeast Asia.[3] Florida is the largest citrus producer in the US, and since 2005 citrus greening has led to a devastating 75% decline in Florida's $9 billion citrus economy.[4]

All counties in the Southeastern US, as well as in Southern California, have had citrus groves quarantined for the vector of citrus greening, the Asian citrus psyllid (*Diaphorina citri* Kuwayama, Figure 1), indicating that the disease has the potential to devastate citrus economies throughout the US (Figure 2). Since 2016, citrus groves in California have also been quarantined for citrus greening, suggesting that the disease is spreading. Solutions have been proposed to help prevent the spread of citrus greening, such as tree steaming, and advances towards genetically modified citrus trees.[5] Once infected though, most farmers resort to removing the infected trees, throwing away several years invested into growing the trees.[2]

Figure 1: Adult stage of Asian citrus psyllid (*Diaphorina citri* Kuwayama): The Asian citrus psyllid serves as the vector for *C*Las, the pathogen of citrus greening. [2]

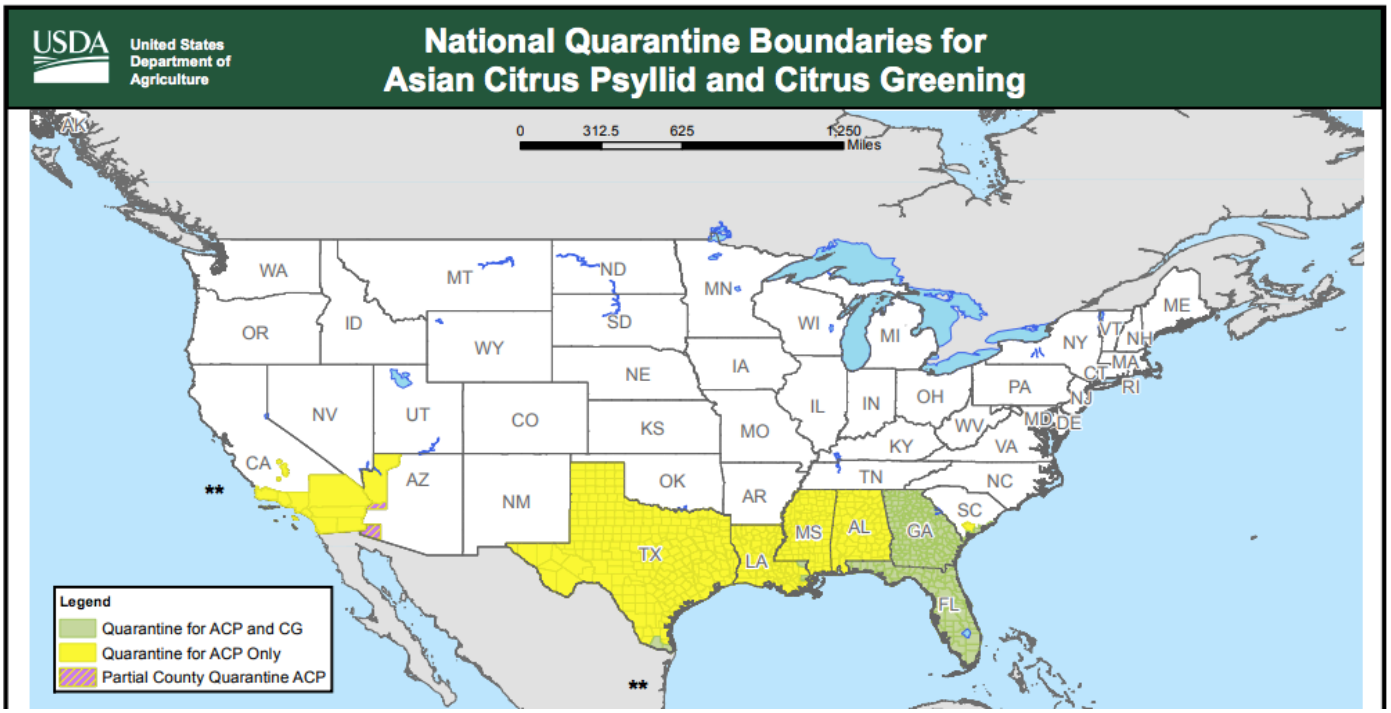


Figure 2: Quarantined counties in the US for the Asian citrus psyllid and citrus greening (2016). Since the introduction of citrus greening in Florida in 2005, it has spread to citrus groves throughout Florida and Georgia. The spread of the vector, the Asian citrus psyllid, suggests that citrus greening has the ability to spread throughout the Southeastern US, as well as Southern California. Since 2016, cases of citrus greening have appeared in California, the second largest citrus producer in the US. [7]

One interesting observation in citrus groves quarantined for citrus greening is that symptomatic and asymptomatic citrus trees coexist in the same grove (Figure 3). Since all trees in a citrus grove are clonally propagated, and therefore have identical genomes, if the citrus trees provided the only defenses against *C*Las, this would result in either all trees or no trees being infected with citrus greening, which is not consistent with the given observation.

All plants act as hosts for communities of microorganisms, and these microorganisms are called endophytes. Endophytes and the host plant live in symbiosis, with the plant providing simple sugars and protection against weather elements to the microbe, and the microorganisms providing protection from potential pathogens.[6] Endophytes sometimes produce compounds known as secondary metabolites that can protect the plant host.

Figure 3: The varying degrees of citrus greening symptoms in citrus trees. Within a given citrus grove, some trees are more susceptible to showing symptoms similar to the Stage 4 and Stage 5 trees, while other trees remain asymptomatic, such as the Stage 1 tree.

Secondary metabolites (aka natural products) are compounds produced by an organism that serve another function other than normal growth and development, such as for protection against predators.[8] Numerous natural products that exhibit activity against pathogenic bacteria have been isolated. These include antibiotics such as penicillin.[6]

Given our observation that healthy and infected citrus trees can reside in the same grove, along with the knowledge of endophytes and their natural products, we hypothesize that there is a secondary metabolite being produced by an endophyte that is protecting the escaped citrus trees from *C*Las. In this study, we cultured *Epicoccum nigrum* (*E. nigrum,* aka *Epicoccum purpurascens*), an endophytic fungus isolated from citrus trees in infected groves, and worked toward the isolation and structure elucidation of the bioactive natural product through the process of bioassay-guided isolation.

Past Work

Endophyte samples were extracted from leaf, budwood, and root systems of both asymptomatic and symptomatic citrus trees in quarantined citrus groves, and the surface sterilized tissue was grown up in lab to obtain a consortium, a collection of microorganisms. Microbes from the consortia were isolated and cross-streaked against *Liberibacter crescens* (*L. crescens*), a culturable relative to *C*Las, to determine potential inhibitors.

Through this process, we obtained several strains of bacterial and fungal endophyte inhibitors. One of the endophytes isolated through this process was *Epicoccum nigrum,* the endophyte that we focused on in this study.

METHODS

Fermentation

Six liters of potato dextrose broth (PDB) was prepared following manufacturer instructions, in 24x 250mL samples. One plug of *E. nigrum* growing on solid media was used as the inoculum in each flask and flasks were incubated with shaking at 190 RPM at room temperature. After the fermentation period, 2.5L was taken off at the 15-day mark, and another 2.5L at the 22-day mark for organic extraction. One liter of samples were contaminated and deemed unusable for the study.

Organic extraction

Broth extraction

After incubation, 250mL ethyl acetate was added to each sample and samples were shaken for 30 minutes at 190 RPM. The fungal supernatant and solid fungal matter (pellet) were separated using vacuum filtration. The pellet samples were collected in a conical vial and stored in a freezer until the pellet extraction. The supernatant was partitioned with ethyl acetate three times and the solvent was removed under vacuum.

Pellet extraction

Liquid nitrogen was used to freeze the pellet samples, and then the frozen pellet was ground up using mortar and pestle. 1:1 DCM:MeOH was poured over the ground pellet, mixed, and then vacuum filtered. The ground pellet was extracted two more times with organic solvent, and the organic solvent was removed under vacuum.

Bioassay

Extracts were dissolved in methanol to a concentration of one milligram per 15 microliters (1 mg/ 15 µL). To make a triplicate of each sample, 15µL aliquots of each solution were pipetted onto three sterile filter disks. The triplicate was placed into a sterile Eppendorf tube and then sealed with parafilm. Samples were sent to our collaborators at the University of California, Riverside for bioassay.

For the bioassay, a prepared filter disk was placed on a plate with agar mixed with *L. crescens*. After two days of incubation, the radius of the *L. crescens*-cleared zone from the disk was measured. With the radii of zones of inhibition, we determined which samples to focus on for further analysis.

Isolation

After receiving bioassay data for the crude extracts, fractionation was performed on the extracts of interest. Normal phase flash column chromatography on a CombiFlashRf, Teledyne Instruments, Inc. was used to separate the crude sample into fractions. Thin layer chromatography was used to help combine similar samples.

Structure elucidation

The Antimarin database was used to find known compounds produced by *E. nigrum.* ^{1}H NMR spectra and mass spectrometry data was collected from each active fraction. Using the collected ^{1}H NMR and LCMS data, we were able to speculate as to which potential compounds were present in the active fractions. ^{1}H NMR spectra were collected on a JEOL ECS 400 MHz NMR Spectrometer at 400 MHz, using $CDCl_3$ from Cambridge Isotope Laboratories, Inc (99.8% D) or D-MeOH from Cambridge Isotope Laboratories, Inc (99.8% D). Liquid chromatography-mass spectrometry (LCMS) and mass spectrometer data was obtained from an analytical Agilent 1260 Infinity LC system coupled with a 6530 Q-TOF mass spectrometer at the Scripps Institution of Oceanography.

RESULTS AND DISCUSSION

Crude extracts were tested for bioactivity, and the broth crude extract as well as the DCM pellet crude sample showed the most inhibition. Interestingly, a layer developed at the bottom of the 22-day 60% MeOH (aq) fraction. The crude broth extracts were combined and fractionated via silica autocolumn. In the broth fractions, Fractions 1, 2, and 8 showed the most activity against *L. crescens* (Figure 4).

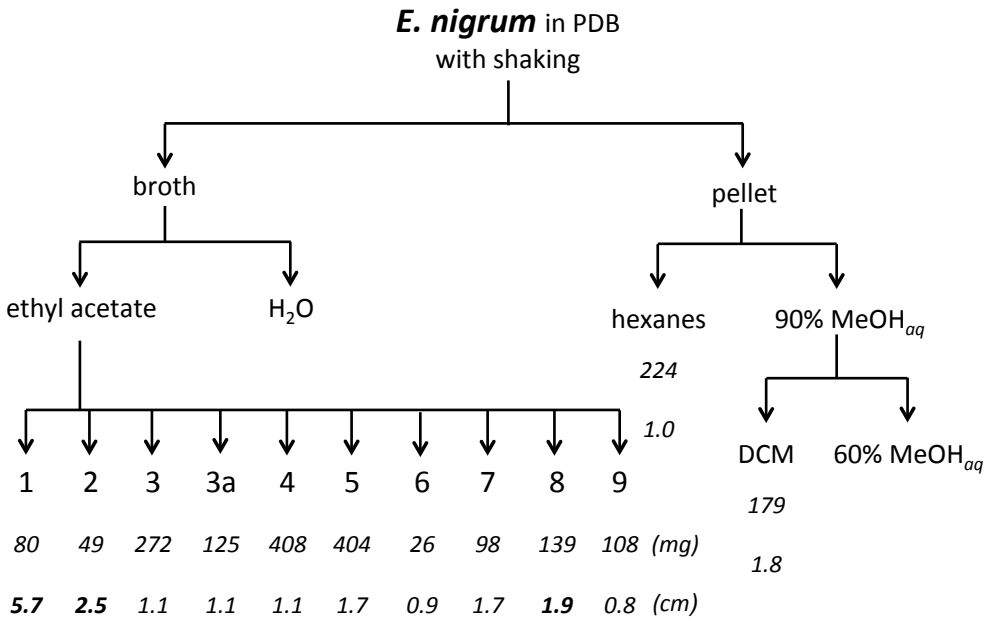
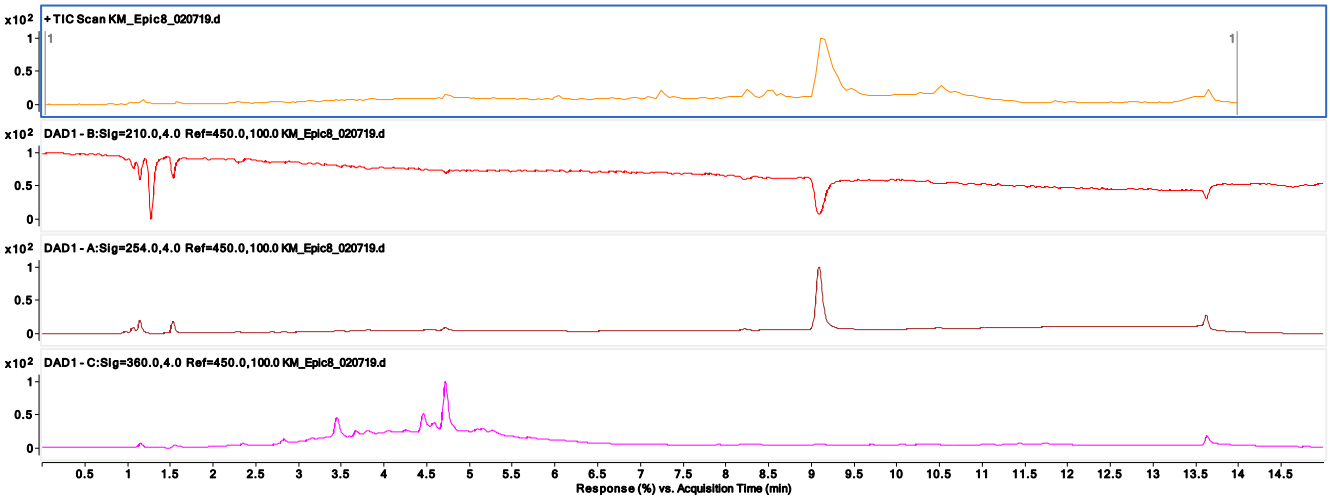


Figure 4: Bioassay-guided isolation of *E. nigrum*. Average diameters of the zones of inhibition (cm) are presented under each tested sample and its corresponding mass (mg). From the broth crude extract, Fractions 1, 2, and 8 exhibit the most bioactivity, while the DCM fraction from the pellet extract exhibits the most from the pellet.

^{1}H NMR spectra were collected for all fractions, and LCMS data was collected for the fractions showing the most activity, namely Fractions 1, 2, and 8. Unfortunately, the LCMS data and ^{1}H NMR spectra of Fractions 1 and 2 reveal these fractions contain mixtures of compounds. Further purification is necessary to identify the active molecule(s) in these fractions (Appendix, Figures 8-11).

The rest of this paper will discuss Fraction 8, since it has the cleanest ionization peak in the LCMS spectrum, which occurs at the 9-minute mark (Figure 5). This peak corresponds to *m/z* ratios at 397.31 and 580.34, which correspond to molecular weights of 396 and 579.



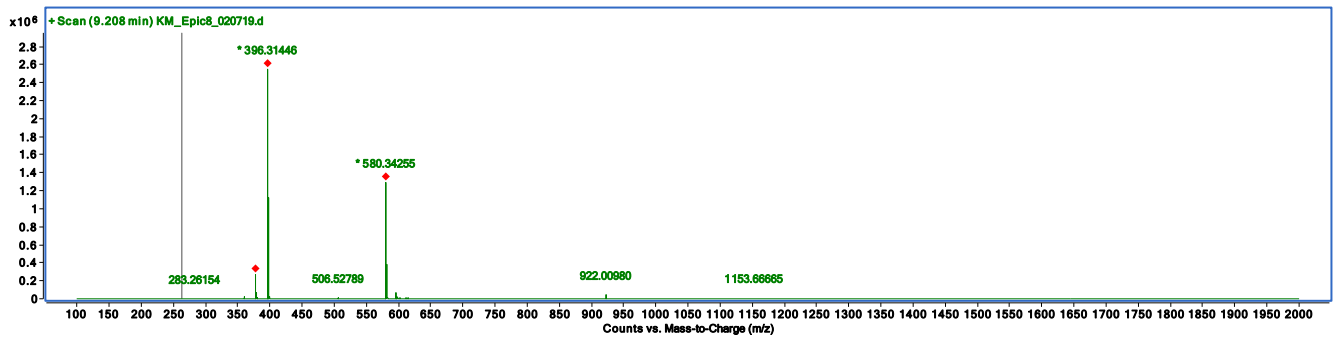


Figure 5: Liquid chromatography-mass spectrometry (LCMS) data for *E. nigrum* Fraction 8. Fraction 8 has a prominent ionization peak, which occurs around 9 minutes (top three chromatograms). The mass spectrum for this peak (bottom table) displays mass-to-charge (*m/z*) peaks at 397.314 and 580.342. These *m/z* peaks correspond to potential masses of 396 or 579 of the most prominent compound in the sample.

The ^{1}H NMR spectrum of Fraction 8 features several signals from 3.1 to 3.9 ppm, which could correspond to a series of protons next to oxygen atoms. A class of molecules that exhibit a similar motif to the multiplet between 3.1 and 4.9 ppm are sugars.

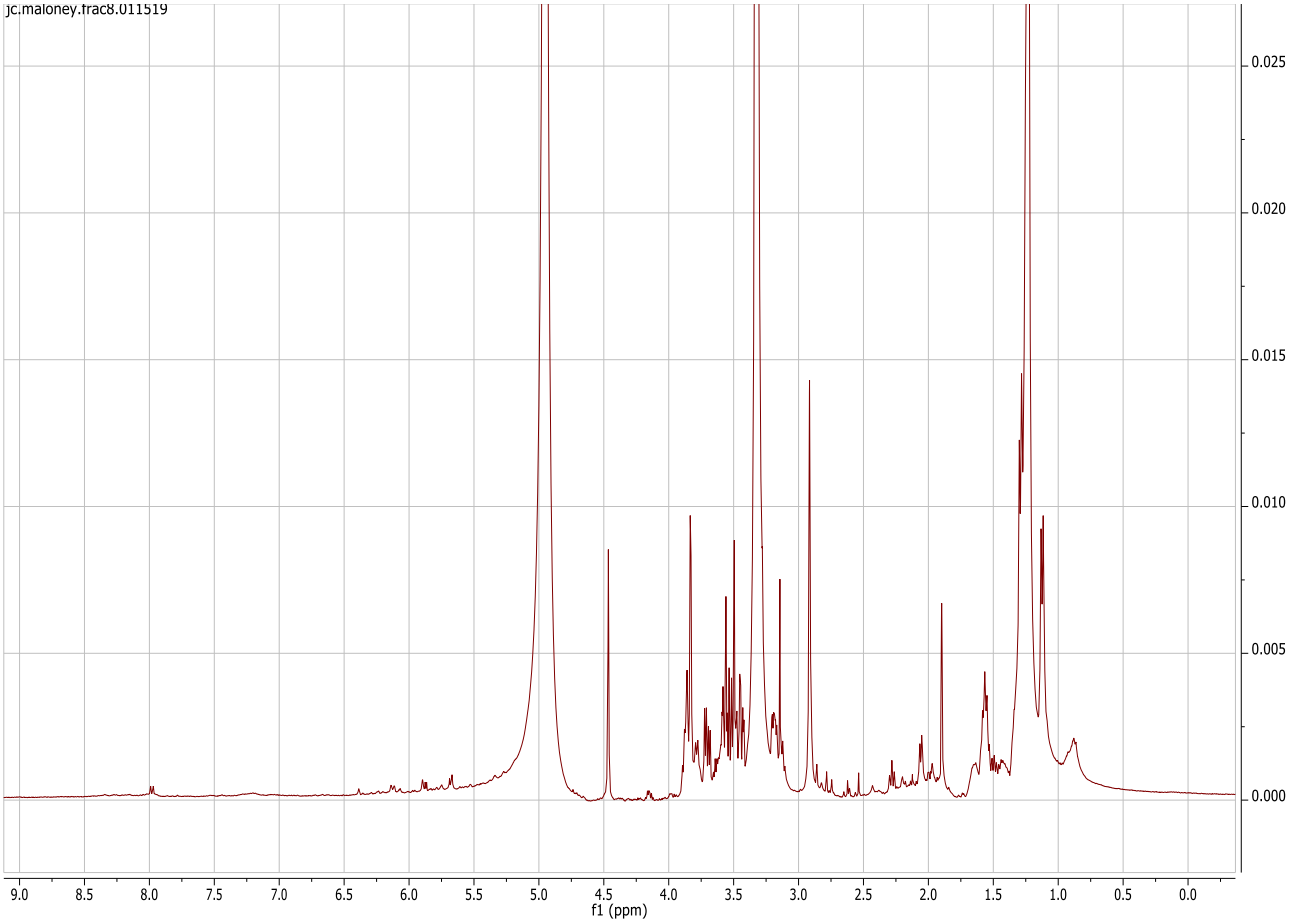


Figure 6: ^{1}H NMR spectrum for *E. nigrum* Fraction 8 in the solvent deuterated methanol (D-MeOH). Aside from a solvent peak from methanol at 3.25 ppm, and a peak at 4.9 ppm from water, peaks corresponding to a sugar moiety (3.1 to 3.9 ppm) and an alkyl chain (1.2 ppm) can be seen.

Using the Antimarin database, we found one molecule that could have similar structures to the molecule that gives off some of the signals in the ^{1}H NMR spectrum of Fraction 8, while also matching one of the *m/z* peaks exhibited from the LCMS data. Ustilipid F3, isolated from *Ustilago maydis* and *Geotrichum candidum*, contains the sugar motif with a predicted chemical shift from 3.25 to 4.0 ppm (Figure 7). The predicted sugar motif could account for the pattern of signals seen in the ^{1}H NMR for Fraction 8 from 3.1 to 3.9 ppm. The predicted ^{1}H NMR also contains an overlap of several proton signals from an alkane chain at a chemical shift of 1.15 ppm. The ^{1}H NMR of Fraction 8 (Figure 6) shows similar alkyl chain at 1.15 ppm in Fraction 8.

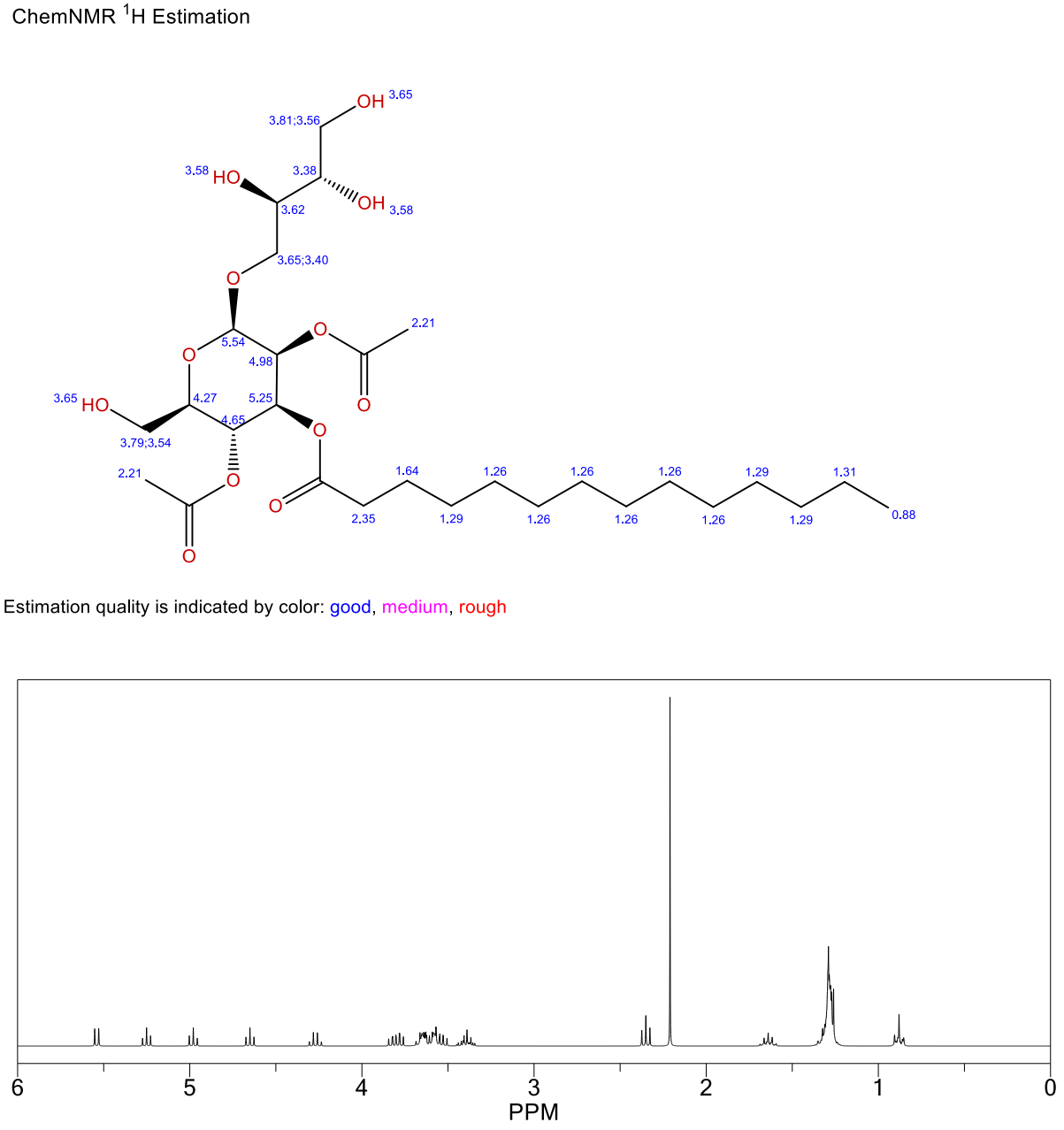


Figure 7: Structure and predicted ^{1}H NMR of ustilipid F3.

CONCLUSION

Fraction 8 could have a structure similar to ustilipid F3, in the sense that it has a sugar group and possibly even an alkyl chain. The next step for this project would be to run HPLC fractionation on the bioactive samples to obtain cleaner NMR spectra. For Fraction 8, we will begin running 2D NMR to finish the structure elucidation of the bioactive compound. We will also continue the bioassay-guided isolations on Fractions 1 and 2, and begin the structure elucidation on those fractions. Once the bioactive compounds have been identified, we will test the purified compounds against *L. crescens* and begin *in planta* testing to begin developing a pesticide against the *C*Las bacterium.

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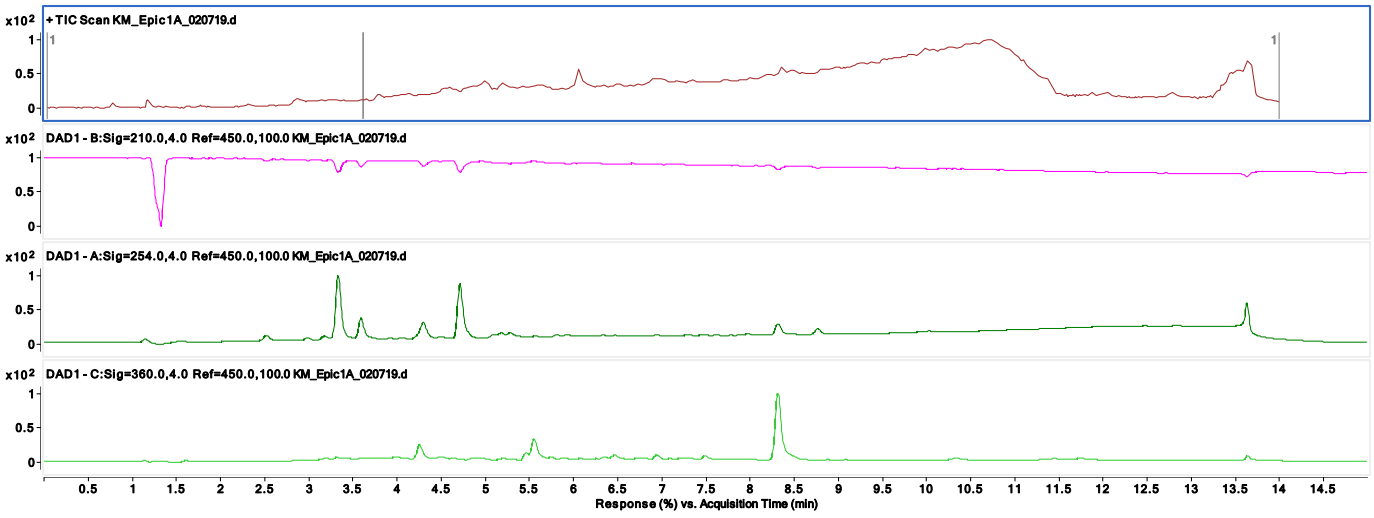
APPENDIX



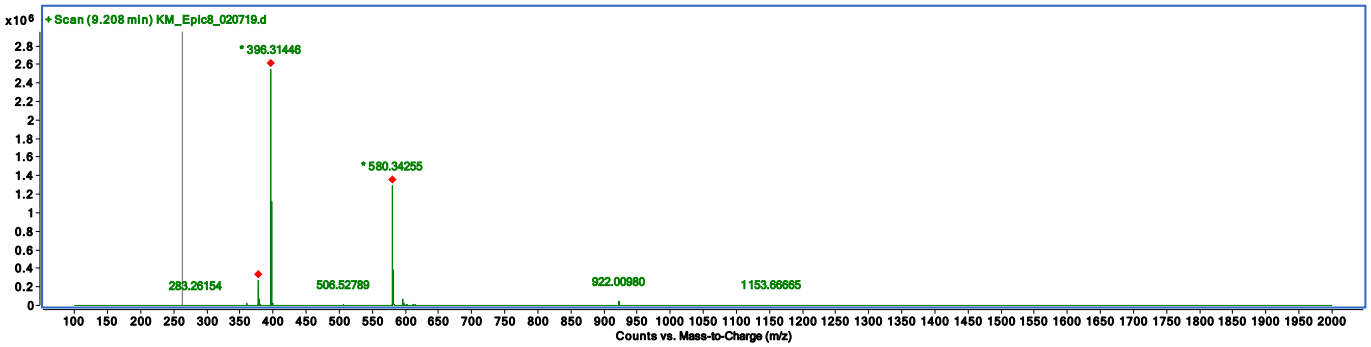


Figure 8: Liquid-chromatography mass spectroscopy data for *E. nigrum* Fraction 1. *M/z* peak at 297.08 at 8.5 minutes.

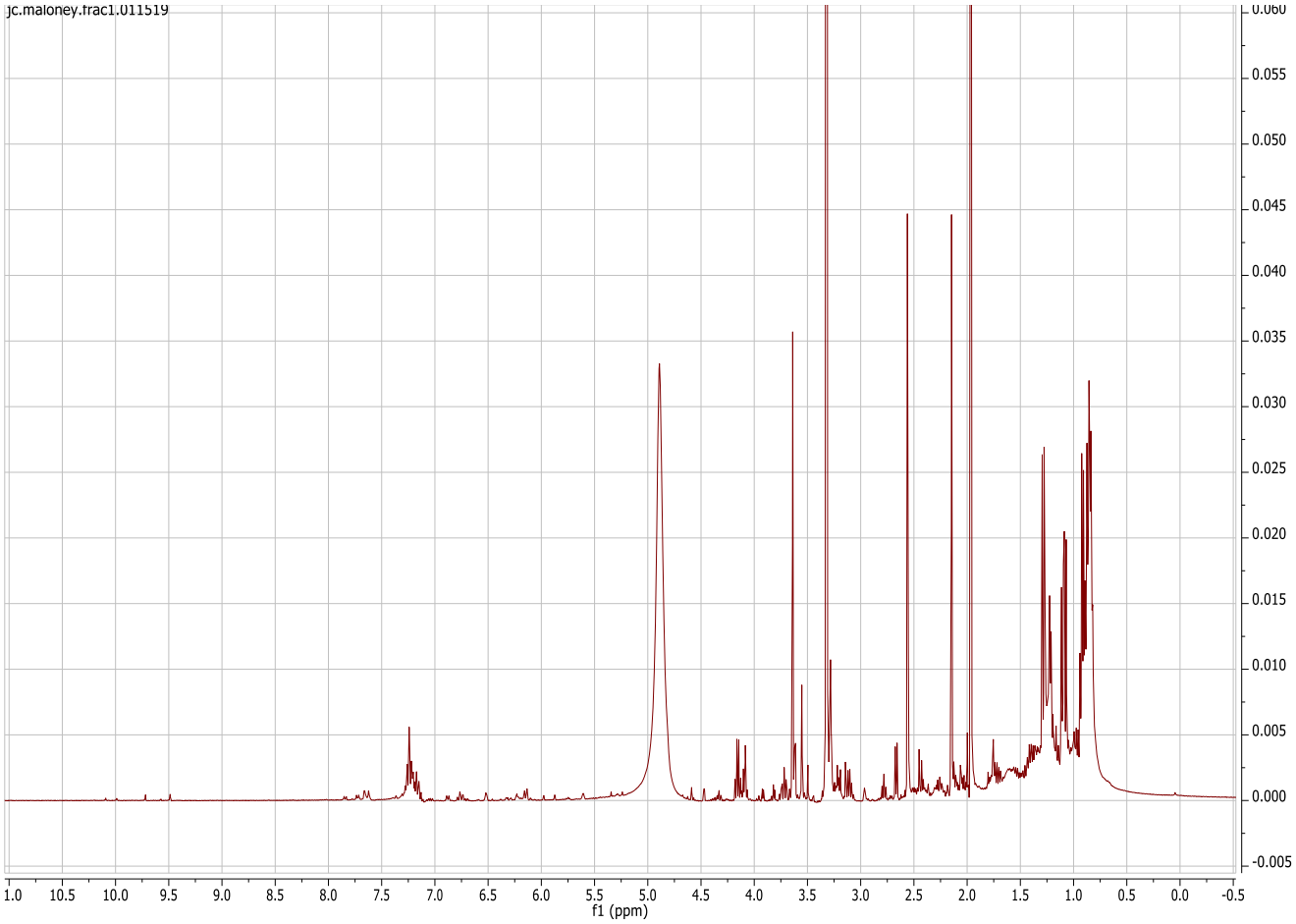


Figure 9: *E. nigrum* Fraction 1 ^{1}H NMR spectra. Potential aromatic ring at 7.2 ppm.

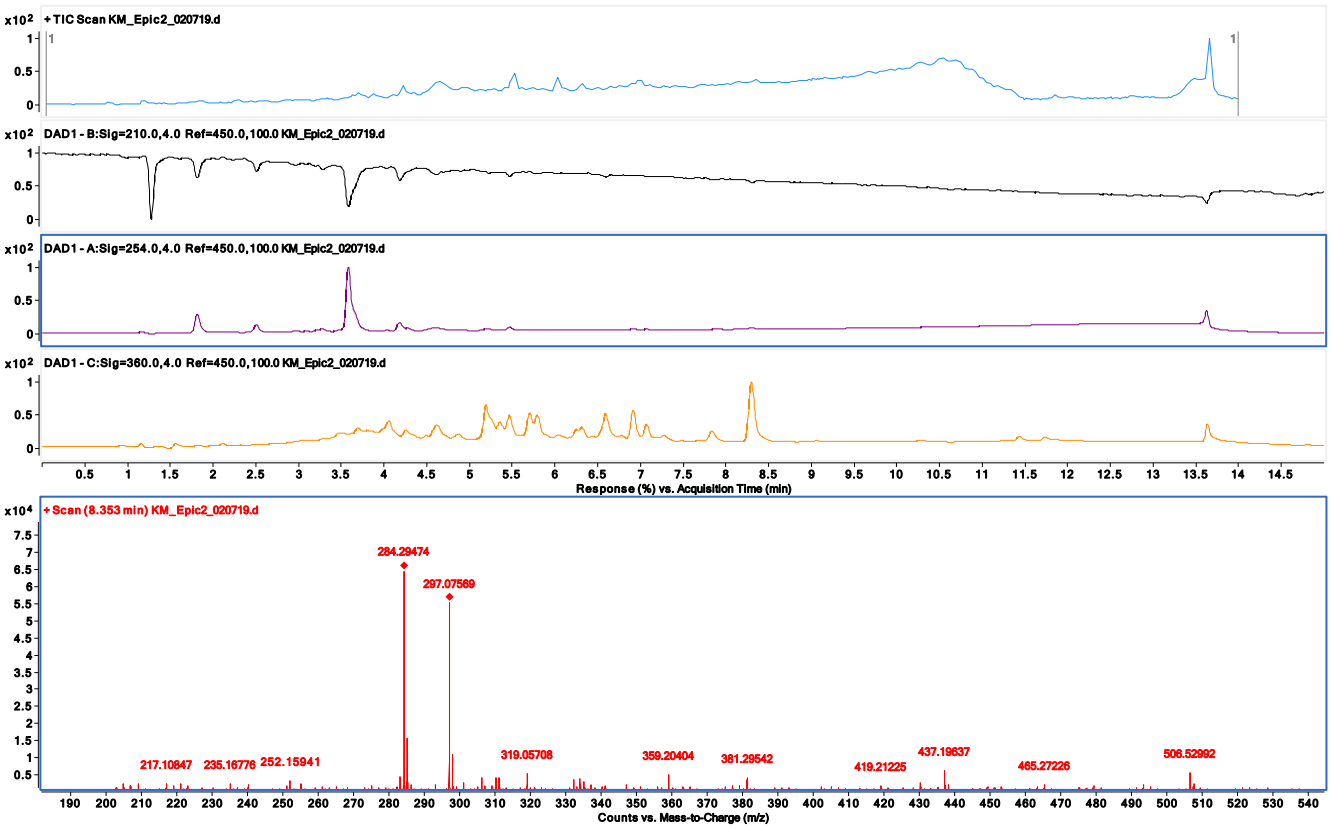


Figure 10: *E. nigrum* LCMS data for Fraction 2. Ionization peak at 8.2 minutes with m/z ratios of 284.30 and 297.08 ppm.

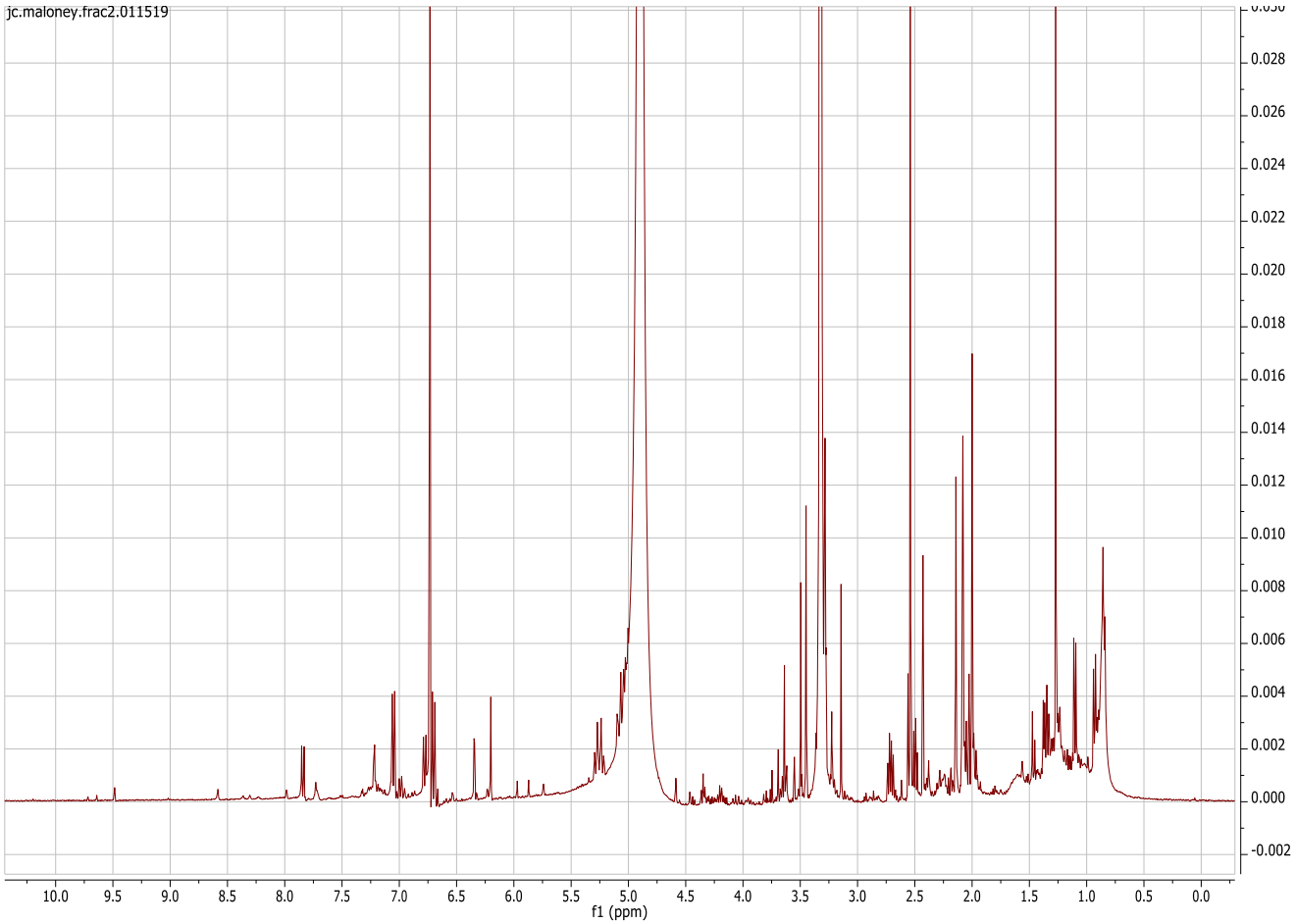


Figure 11: ^{1}H NMR spectrum for *E. nigrum* Fraction 2.

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