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Antifungal defense molecules from bacterial symbionts of North American Trachymyrmex Ants

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Antifungal defense molecules from bacterial symbionts of North American *Trachymyrmex* Ants

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Abstract

Defensive symbioses, in which microbes provide molecular defenses for an animal host, hold great potential as untapped sources of therapeutically useful antibiotics. Fungus-growing ants use antifungal defenses from bacterial symbionts to suppress pathogenic fungi in their nests. Preliminary chemical investigations of symbiotic bacteria from this large family of ants have uncovered novel antifungal molecules with therapeutic potential, such as dentigerumycin and selvamicin.

In this study, the bacterial symbionts of North American *Trachymyrmex* fungus-growing ants are investigated for antifungal molecules. Plate-based bioassays using ecologically-relevant fungal pathogens confirmed that these bacteria have antifungal activity. In order to purify and identify the antifungal molecules produced by a single strain we are using reversed-phase liquid chromatography for activity-guided fractionation. Preliminary mass spectrometry data suggests this is a novel compound. Identification of the antifungal molecules will allow us to assess their structural novelty, therapeutic potential, and to contextualize antifungal defense in nature.

Introduction

From plants to microbes to humans, fungi can pose a threat to many forms of life. Fungal infections are surprisingly common and life-threatening, especially to those already in the hospital for other reasons.[1] Specifically, invasive candidiasis is a prevalent mycoses, or fungal infection, that accounts for 8-10% of bloodstream infections originating in the hospital in the United States. *Candida* bloodstream infections have an astounding 40% mortality rate.[1] These fungal infections disproportionately affect immunocompromised people such as those with immune deficiencies such as HIV/AIDS or those undergoing immune-affecting therapies such as cancer chemotherapy.[2] Unfortunately, clinically available antifungal compounds are not nearly as abundant as clinically available antibacterial compounds, and it is only becoming harder to identify more. Only three major structural classes of antifungal molecules have been identified: polyenes, echinocandins, and azoles.[3] Polyenes are characterized by a series of at least three conjugated double bonds, echinocandins are lipopeptides that inhibit a specific enzyme in the fungal cell wall, and azoles are five-membered heterocycles containing a nitrogen atom and at least one other non-carbon atom. Additionally, compounds that have been identified are not always clinically applicable due to low potency or toxicity, creating a need for broadly effective antifungal molecules with low toxicity.

Furthermore, fungi are constantly evolving and developing resistance to these treatments, creating an ongoing need for new antifungal molecules. This necessity is exacerbated due to overuse of existing antifungals, low financial incentive to discover new antifungals, and other factors.[4] Historically, many therapeutically relevant antibiotic molecules have been

secondary metabolites produced by microbes themselves.[5] Fungal secondary metabolites are especially interesting for their therapeutic relevance due to their ability to affect eukaryotic cells, such as other, possibly competing, fungi.[6] Also having eukaryotic cells, humans have taken advantage of these metabolites in remarkable ways. However, fungal cells have similarities to human cells that make it hard to derive effective and specific antifungal molecules from fungi that are not detrimental to human cells. While some antifungal molecules derived from fungi can be toxic to human cells, they can still serve as inspiration for antifungal drug development, or make good cancer therapeutics due to their ability to kill eukaryotic cells.[6]

Bacteria have been a remarkable resource for antifungal discovery in the past due to their production of small, targeted defense molecules. Antibiotics have been produced by bacteria for about 1 billion years,[7] allowing for plenty of genetic development and diversity through natural selection and random mutations. Therefore, a diverse assortment of antibiotic molecules are produced by bacteria and they are a great place to look for novel antibiotics. Not only do these compounds themselves have therapeutic promise, but they can also be used as jumping-off points for drug-development techniques.[5]

Insect-microbe defensive symbioses provide an interesting system in which to not only look for antibiotic molecules but also analyze interesting ecological systems.[10,11] A defensive symbiosis is a symbiosis in which the participants form a symbiosis in defense of a threat or invasion. A wide range of mutualisms exist, allowing the success of both the microbe and insect. Some insects protect their defenseless eggs or larvae by distributing antibiotic-producing bacteria over the eggs, fending off microbial pathogens. Other insects

host gut bacteria that protect them from parasites or unwanted microbes, such as mosquitoes, who protect themselves from the malaria-causing parasite through mutualism with bacteria.[10] Pine beetles, termites and ants have mutualisms with fungi, and protect that fungus from other pathogens with antibiotics produced by an additional bacterial symbiont. These ecological systems present some fascinating questions about horizontal gene transfer, and the existence of bacteria exist across different ecological niches.

Here, we look at the 4 part symbiosis between North American *Trachymyrmex* leaf-cutter ants, their cultivar fungus, the fungal competitors of the cultivar fungus and Actinobacteria that grows and lives on the underside of these ants (Figure 1).[12,13]

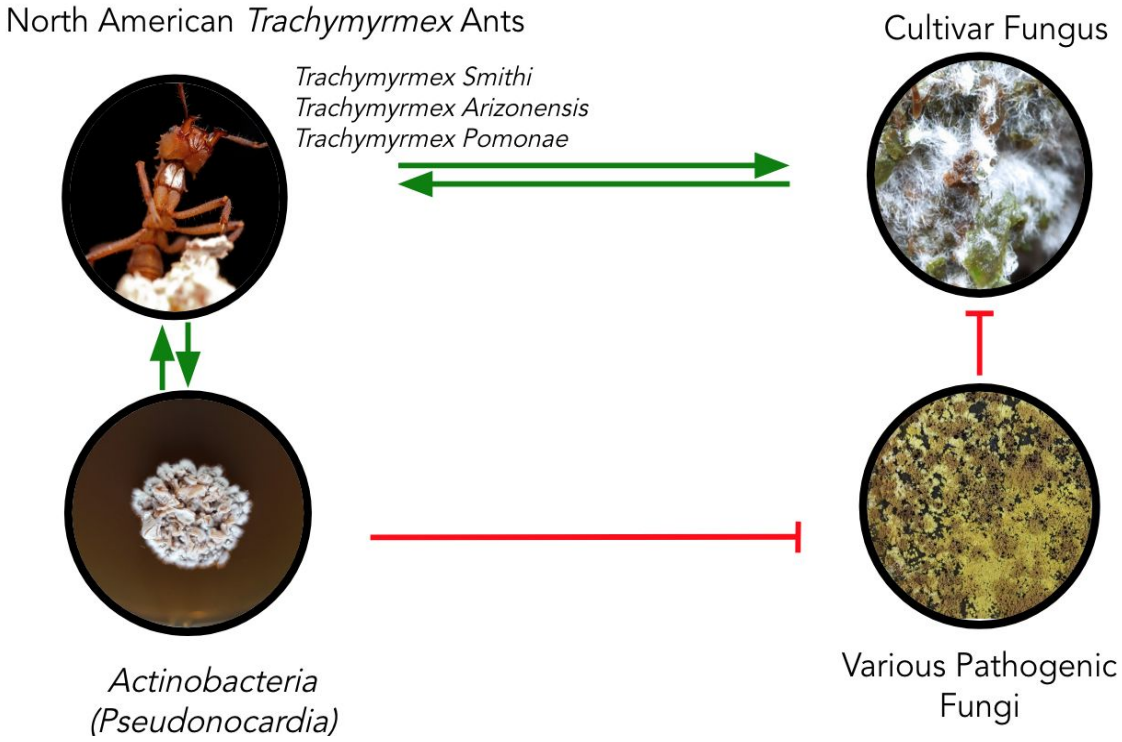

Figure 1. Visual representation of the symbiotic relationship between North American *Trachymyrmex* ants and their microbial symbionts, as well as the pathogenic fungi that may threaten the livelihood of the cultivar fungus. Green arrows indicate mutualistic relationships, and red bars indicate antagonistic relationships.

It has been shown that leaf-cutter ants have two seperate symbioses with microbial organisms, but are not limited to these two symbioses. Interestingly, the primary food source of these ants is a fungus that the ants grow in their nests. In order to complete this mutualism, the ants bring organic material to the fungus which allows it to grow.[13] It is thought that ant agriculture began about 50 million years ago[14]. Another symbiosis in which the ants participate is with an Actinobacteria that lives and grows on the nutrient-rich underside of the ant itself. In return for a place to grow, the Actinobacteria produces antifungal molecules. These antifungal molecules serve to protect the monoculture cultivar fungus from potentially pathogenic fungi in the soil surrounding the nest.[15] *Trachymyrmex* ants keep their nests deep in the soil where many other microbes live, including other fungi. Due to the slow growing nature of the cultivar fungus, it runs the risk of being overtaken by a more aggressive soil fungus. Furthermore, as mentioned above, some fungi produce antifungal molecules and may chemically kill or inhibit the cultivar fungus.[6] These potentially pathogenic fungi are therefore the primary targets of the antifungal molecules produced by the Actinobacteria, due to their ecological relevance.

The Actinobacteria that lives on the *Trachymyrmex* ants has been genetically associated with soil bacteria living in the same habitat as the ants. A study on different genuses of ants found that ants in the same physical location had more genetically similar bacterial symbionts to each other and to nearby soil than those farther apart. This indicates horizontal transfer of bacteria between soil bacteria and that growing on the ant.[16] While the ants studied in this case were lower attine ants and therefore not *Trachymyrmex* ants, there is also indication of horizontal gene transfer in higher attine ants. In further support of horizontal

transfer of antibiotic gene clusters, it has been found that in *Pseudonocardia* bacteria growing on *Apterostigma* ants, the gene cluster for the antifungal molecule is found both as a plasmid and in the chromosomes of the bacteria.[17] More broadly, this indicates that the antifungal molecules found in ant-associated bacteria may have developed non-specifically to the ecological context in which these bacteria are found. However, slight variations in these molecules may be found due to niche adaptation. It would not therefore be surprising if these antifungal molecules, or their analogues were previously discovered in ecological contexts outside the ant system.

Multiple distinct antifungal molecules have been discovered as a part of this complex symbiotic system. Fungus-growing *Apterostigma dentigerum* ants from Panama have a symbiosis with a *Pseudonocardia* bacteria. This bacteria presents a novel antibiotic molecule, dentigerumycin, which effectively inhibited the growth of the fungal pathogen *Escovopsis*. Importantly, this compound was selectively inhibiting and did not inhibit the growth of the food source of the ant, the cultivar fungus.[18] Selvamicin, an antifungal polyene, was extracted bacteria living on fungus-growing ants in Costa Rica. Selvamicin showed inhibitory activity against not only fungal pathogens but also the human pathogen *C. albicans*.[17] This encouraging result indicates that antifungal molecules extracted from bacteria may be therapeutically relevant for treating fungal infections in humans. Moreover, both studies show promise for identifying novel antifungal molecules from this mutualistic scaffold.

While previous work has identified antifungal molecules, there are many other genera of fungus-growing ants which have not yet been studied in depth, including North American

Trachymyrmex ants. We suspect that the symbiotic bacteria living on *Trachymyrmex* ants may produce different antifungal molecules than previously studied fungus-growing ants of the genus *Apterostigma* in Central America due to residing in a drier, geographically distinct environments on different species of ants, and foraging on different plant material. Furthermore, the cultivar fungi of these ants may be threatened by different fungal pathogens. The genus of *Escovopsis* fungi has been shown to be pathogenic to the cultivar fungus of *Apterostigma* ants. However, the fungal pathogen or pathogens for North American *Trachymyrmex* ants have yet to be identified. Here, we look at fungi collected from near the cultivar fungus, as those fungi in the same physical vicinity as the cultivar fungus are most likely to be it's pathogens. While *Escovopsis* can be useful as a test strain, it has not been found in our collections of fungi from North American *Trachymyrmex* ant nests.

There are also many different species of North American *Trachymyrmex* ants including, but not limited to, *T. arizonensis, T. pomonae, and T. smithi*, adding to ecological diversity. There is yet further variability in the species of filamentous actinomycete bacteria that live on these ants including *Pseudonocardia* and *Amycolatopsis* bacteria.[10,19] This diversity in bacteria which likely utilize endogenously created antifungal molecules leads to a high likelihood of discovering a novel antifungal molecule. As stated above, novel antifungal molecules are in high demand due to both antibiotic resistance and difficulty discovering new antifungal molecules. A novel antifungal molecule could help those suffering from *Candida* infections, and help save lives. In this study, we look deeper at an *Amycolatopsis* bacterial strain, 17SM-2A collected from the cuticle of a *T. smithi* ant in New Mexico, an especially hot and dry location. Notably, most *Trachymyrmex* associated bacteria are of the genus

Pseudonocardia, making this strain especially intriguing. So far, the antifungal molecule of interest appears to be a novel compound.

Methods

Resident-Intruder Assay

In order to assess the antifungal activity of ant-associated bacteria, a resident-intruder assay was performed. In this plate-based assay, 5 μL of 1:4 diluted bacterial spore stock with potential antifungal activity was plated 1 cm from the edge of a YPD plate and allowed to grow at 30ºC for two weeks. After this point, the bacteria has established residency on the plate, allowing it to begin producing any antifungal molecules it may produce. Therefore, a plug of fungus was introduced using a sterile straw and plunger 1 cm from the edge of the plate opposite the bacteria. Fungus was also plated onto a control plate with no bacteria in order to see uninhibited growth of the fungus, and replicates of each plate were produced in order to control for inconsistencies in growth. Once the fungus on the control plate had grown to 1 cm from the edge of the plate, the radius of the fungal growth was measured on each plate. Inhibition of fungal growth by the bacterial resident was determined by subtracting the average radius of the treatment groups from that of the control.

Growth and Extraction Optimization

150 μL sterile water and 15 μL of the spore stock of the bacteria of interest was pipetted and spread onto 4 100 mm diameter plates. The plates were allowed to grow for two weeks and then cut up and soaked in 80 mL of tetrahydrofuran. The extract was then filtered from the agar with a coffee filter and the solvent was evaporated off using a SpeedVac. The dried extracts were then redissolved in 200 μL MeOH or THF and then tested in a Spot-on-Lawn assay.

Growth and Extraction of 17SM-2A

Once the bacterial strain of interest for growth and extraction had been selected, it was plated on a large scale. 150 µL sterile water and 10 µL spore stock were spread onto 20 150 mm diameter YMEA plates. These plates were then allowed to grow for two weeks, allowing the bacteria to cover the plate and produce antifungal molecules. The plates were then all cut up and put in a large beaker. Ethyl acetate was used as an extraction solvent and the chunks of agar and bacteria were submerged in ethyl acetate overnight. The following day, the agar was filtered out using coffee filters and disposed of, resulting in an ethyl acetate extraction of the bacteria and agar.

In order to concentrate the active molecules in this extract, the extract was dried down and redissolved. It was dried dowth through rotary evaporation, followed by redissolving in about 5mL of methanol. In order to confirm that the antifungal compound was in fact present in this extract, a spot-on-lawn assay was performed.

Spot-on-Lawn Assay

In a spot-on-lawn assay, 2-3 plugs of fungal mycelia are mixed with 1.5mL of sterile water in a test tube and vortexed in order to get the mycelia loose from the agar. Then, 1mL of the water containing mycelia is mixed with 15 mL of 0.75% agar YMEA media, and spread across a 150 mm 2% agar YMEA plate. Then, 5 µL of each compound of interest is spotted on the plate. Once the fungus begins to grow, antifungal activity can be visualized by

a zone of inhibition created by antifungal compounds. The diameter of the zone of inhibition was measured, difference in antifungal activity of different compounds..

Purification

Bioassay-guided fractionation was performed in order to purify the compound using a 5g reverse phase C18 Sep-Pak cartridge from Waters and High Performance Liquid Chromatography (HPLC). First, the Sep-Pak column was used to fractionate the compounds in the extract into 10 fractions based on polarity. Before running the sample on the Sep-Pak column, celite was added to the extract solution and dried down using the rotary evaporator. The column was equilibrated by drawing through 3 column volumes of acetonitrile with 0.1% formic acid, 3 column volumes of water with 0.1% formic acid and 3 column volumes of 20% acetonitrile in water with 0.1% formic acid with vacuum. Then, the dried, celite-adsorbed extract was transferred to the column and acetonitrile was added up to approximately 0.5 cm. Once the extract was loaded onto the column, a solvent gradient was used to draw increasingly less polar molecules through the column. In this case, a 20-40-60-80-100% acetonitrile in water gradient was used, running approximately 2 column volumes of mobile phase per step while collecting each column in one vial. Upon completion, Sep-Pak fractions were dried down using a SpeedVac vacuum concentrator, and redissolved in methanol.

Redissolved Sep-Pak fractions were tested for activity by performing a spot-on lawn assay as described above, spotting 5 µL of each fraction onto a lawn of fungus. Fractions with activity were further purified using HPLC (Phenomenex Kinetex 5 µm Biphenyl 100 Å

250 mm x 10 mm column, 2.5 mL/min). The sample was run through a biphenyl column with a biphenyl guard. Acetonitrile and millipore water with 0.1% formic acid each were used in a gradient to fractionate the sample based on polarity. The starting mobile phase was 35% acetonitrile which was increased to 70% over the course of 20 minutes at a 2.5 mL/min flow rate.

UV

The major peaks in the active Sep-Pak fractions were collected and a spot-on-lawn assay was performed to identify which peak contained the compound exhibiting antifungal activity. The HPLC collected the UV absorbance of the compounds eluting off the column, so once the compound of interest was identified through the bioassay, the compound's UV absorbance spectrum was found. The peak itself, eluting at approximately 17 minutes along with neighboring fractions were collected using the HPLC software. The purity of the compound was confirmed through liquid chromatography-mass spectrometry (LC-MS).

Mass Spectrometry

A few mass spectrometry experiments were performed on the purified and unpurified compounds. The most reliable mass spectrum was a result of LC-MS performed by Waters on a purified fraction of the antifungal compound. The mass spectrum was performed by Waters Corporation. The sample was dissolved in 200 µL of methanol and then diluted 10X, before being injected onto the ACQUITY HSS T3, 1.8 µm; 2.1 mm x 100mm column. A gradient of water with 0.1% formic acid and Acetonitrile with 0.1% formic acid was used.

Waters also provided a UV spectrum of the compound of interest, giving limited but valuable structural information on the antifungal compound.

Nuclear Magnetic Resonance

NMR was then performed to show the structural relationship of atoms to each-other in the molecule. To minimize a signal from water in the NMR, the dried compound was subjected to vacuum, and the NMR tube was dried in a 125ºC oven. Deuterated dimethyl sulfoxide (DMSO) was used as a solvent. A proton NMR was performed.

Results

Screening and Assay Development

A comprehensive resident-intruder screening of bacterial symbionts and potential fungal competitors was performed. These assays were used to test the selectivity and potency of antifungal activity. Due to *Trachymyrmex* fungus-farming ants relying on their cultivar fungus for food, it would not be to their evolutionary advantage to have antifungal molecules that restricted the growth of the cultivar fungus. Therefore, these resident-intruder assays served to determine if there was indeed more antifungal activity against fungal pathogens as compared to the cultivar fungus. All bacterial symbiont strains of interest were tested against a full panel of fungal test strains collected from the nests of different ant species (Supplementary Table 1, 2).

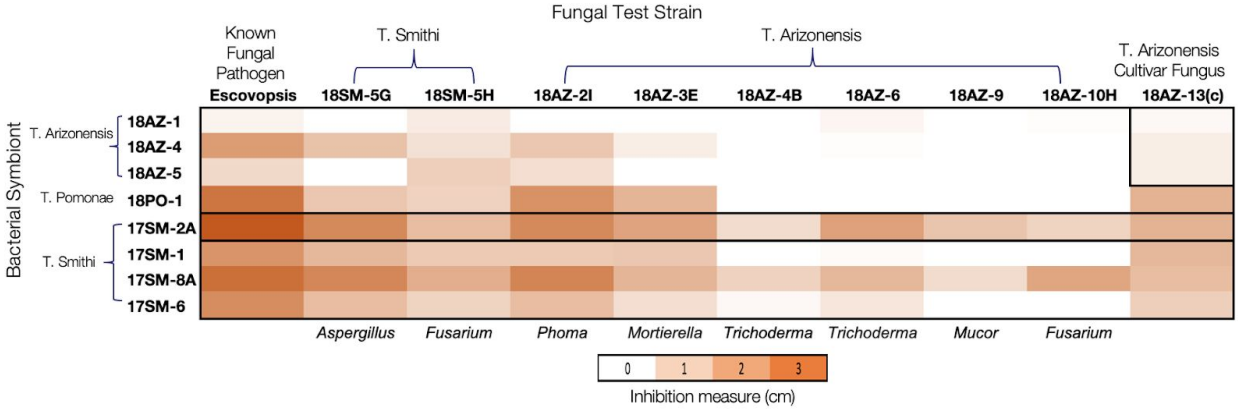

Figure 2. Compiled results of resident-intruder assay cross between fungi and bacteria from *T. arizonensis*, *T. pomona*e and *T. smithi* ants and nests. The ant species from which the bacterial symbiont was isolated is indicated down the left side. The ant species' nest from which each fungus was taken is shown across the top with their respective genus as determined from ITS DNA sequencing across the bottom. A cultivar fungus from a *T. arizonensis* ant is shown in the far right column. Black boxes indicate pairings of interest.

This heat-map shows important information about both fungi found in ant nests and the bacterial symbionts. Firstly, the far right column displays inhibition or lack thereof of the cultivar fungus. In this case, the cultivar fungus was extracted from a *T. arizonensis* ant nest. Some inhibition is shown by bacterial symbionts of ants from other species against the cultivar fungus of a *T. arizonensis* ant. However, *T. arizonensis* bacterial symbionts did not show a strong inhibition of the cultivar fungus. A two sample t-test was performed and the difference between the inhibition of cultivar fungal growth by *T. arizonensis* bacterial symbionts was statistically significantly lower than inhibition of cultivar fungal growth by *T. smithi* or *T. pomonae* bacterial symbionts ($t=7.2470$, 6 d.f., $P=0.0004$).

Rows with darker orange shading across many different fungal strains show the bacterial strains with more antifungal activity, indicating that 17SM-2A and 17SM-8A have noticeably more antifungal activity. While both 17SM-2A and 17SM-8A are of interest for future study, here we look more deeply into the strain 17SM-2A.

Additionally, the data shown in Figure 1 provides us with information about which fungal strains are best to use in future assays. We want to focus on the fungal strains that these bacteria would have the most pronounced effect on, including *Escovopsis*, 18SM-5G and 18AZ-2I. 18AZ-2I and *Escovopsis* specifically were used in Spot-on-Lawn assays with the bacteria of interest.

Bacterial extracts of strains of interest were also produced and tested in spot-on-lawn assays against potential fungal pathogens. The crude extracts, of four bacterial strains are shown below: 17SM-1 and 17SM-2A which are shown in Figure 1 and GA6-1 and 17SE-9 which are not shown in Figure 1. All the crude extracts shown were extracted using tetrahydrofuran (THF) and redissolved in THF with the exception of 17SM-2A which was extracted with THF and redissolved in methanol. Note that the extraction solvents used in this figure are not the same as those used through the fractionation steps following.

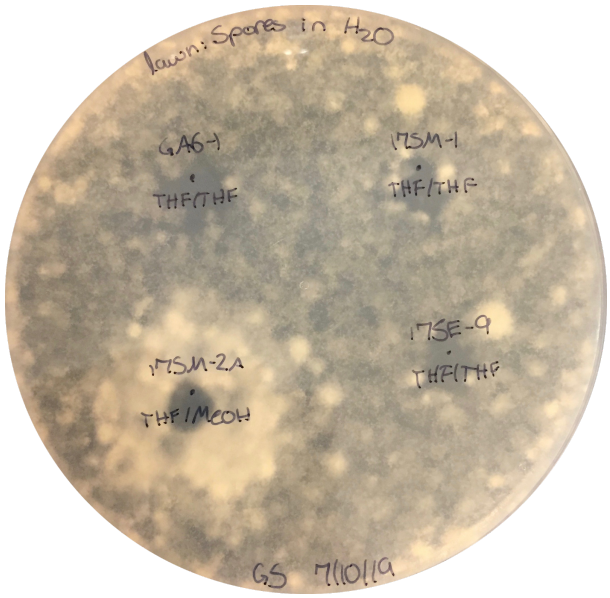

Figure 3. Spot-on-lawn assay of crude, unfractionated extracts of ant-associated bacteria. 5µL of each compound was spotted onto a fungal lawn of *Escovopsis*.

While all 4 crude extracts show a moderate zone of inhibition, it is notable that our compound of interest 17SM-2A has a zone of inhibition, preventing fungal growth near where the compound was spotted. This confirmed the antifungal activity of the compound and further indicated that the compound was extractable through solvent extraction of solid cultures of the bacteria. Primarily, this assay shows that 17SM-2A has antifungal activity against a known antagonist to the cultivar fungus, *Escovopsis*.

Activity Guided Fractionation

In order to determine the active compound in 17SM-2A, activity guided fractionation was performed. After a Sep-Pak fractionation with a C18 column, 5µL of each fraction was spotted against a lawn of *Phoma sp.* fungus of the strain 18AZ-2I.

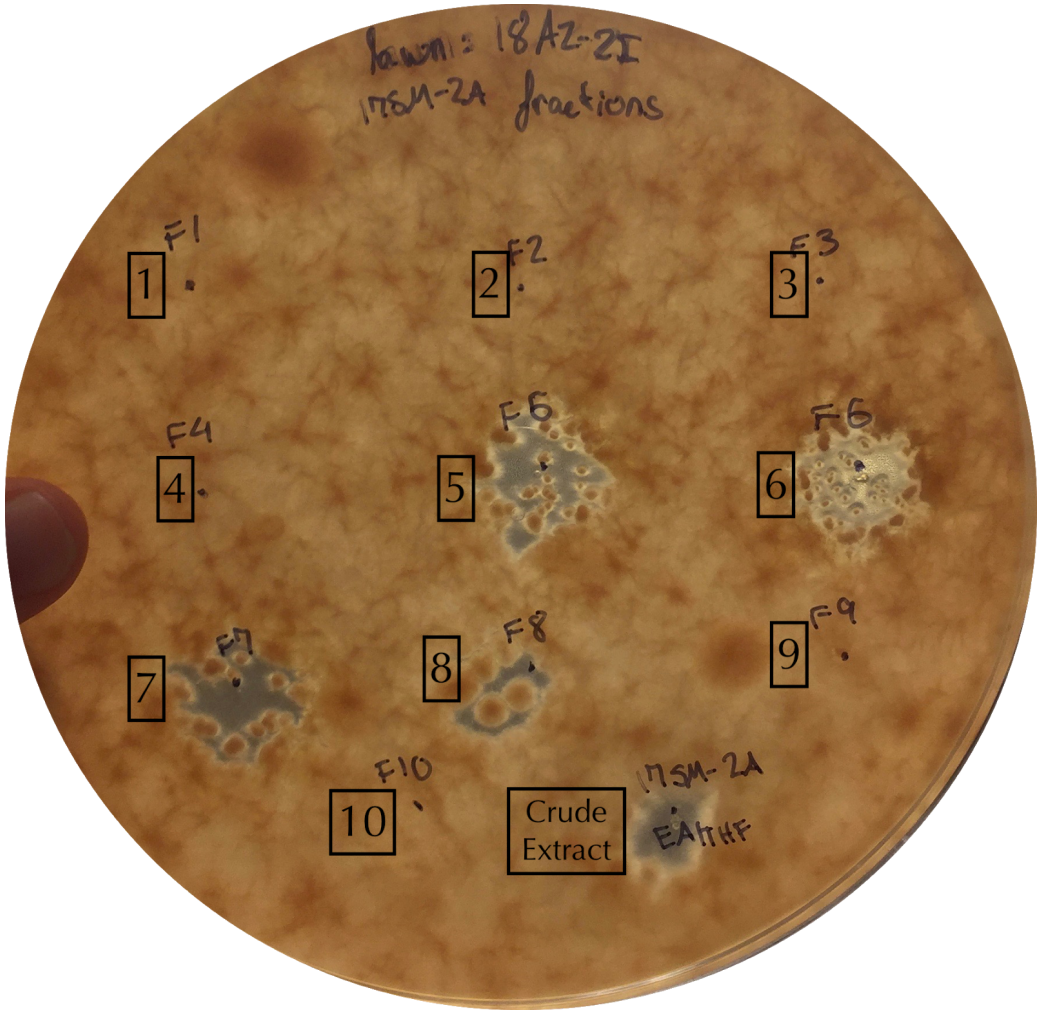

Figure 4. Spot-on-lawn assay of Sep-Pak fractions of 17SM-2A against a lawn of 18AZ-2I. 5µL of each fraction was tested alongside 5µL of a crude extract as a control.

Strong activity was shown in fractions 5 and 6 with less activity in fractions 7 and 8. The crude extract spot was a comparable size to that of spots F7 or F8 indicating that the fractions have strong antifungal activity.

Upon HPLC fractionation of the Sep-Pak fraction 6, three major peaks were seen. All 3 were collected and activity tested yet again against 18AZ-2I for antifungal activity.

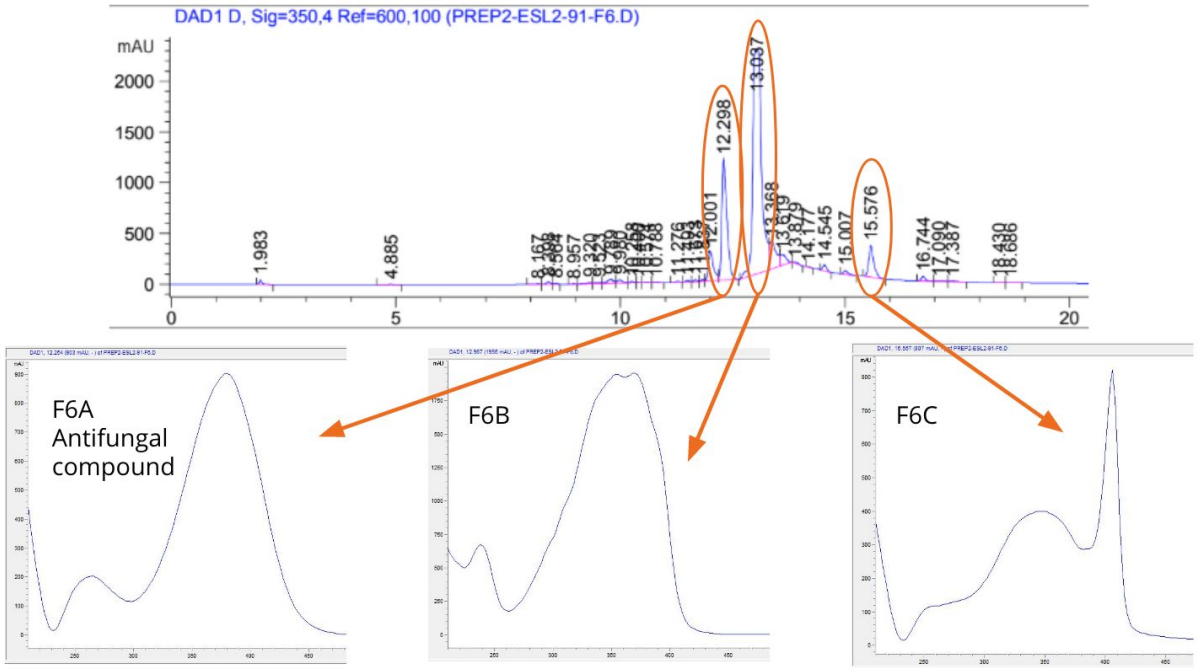

Figure 5. HPLC chromatogram (above) and UV absorbance of collected peaks (panels below) of the Sep-Pak Fraction 6. The three major peaks, here A, B and C were collected.

A spot-on-lawn assay of three major peaks in Sep-Pak fraction 6 was performed to see which one had antifungal activity.

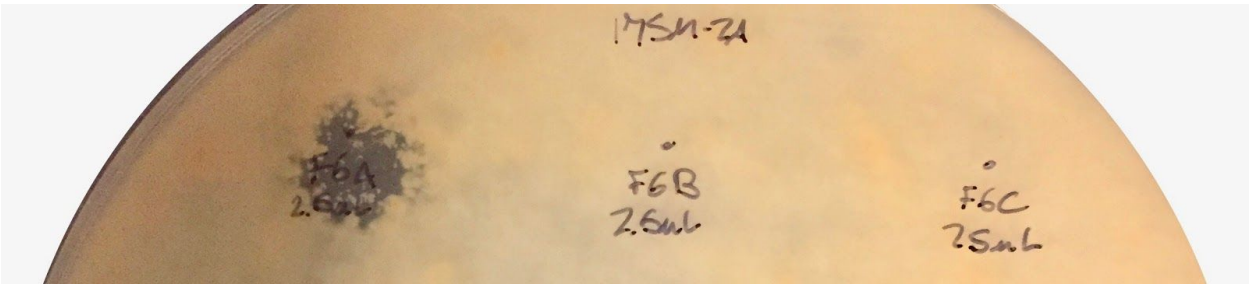

Figure 6. Spot-on-lawn assay of the three major peaks in Sep-Pak fraction 6 of 17SM-2A against a lawn of 18AZ-2I.

This assay allowed the identification of the peak at 12.298 minutes on the HPLC as that with the antifungal compound. Interestingly, the fraction F6B, which showed no antifungal

activity, shows strong antibacterial activity (results not shown). After confirmation of the antifungal activity of this particular compound, the UV spectrum for that peak on the HPLC chromatogram was obtained, allowing differentiation of this compound from others in the extract.

Structure Elucidation

Liquid chromatography-mass spectrometry was performed on a purified sample of the antifungal compound produced by 17SM-2A.

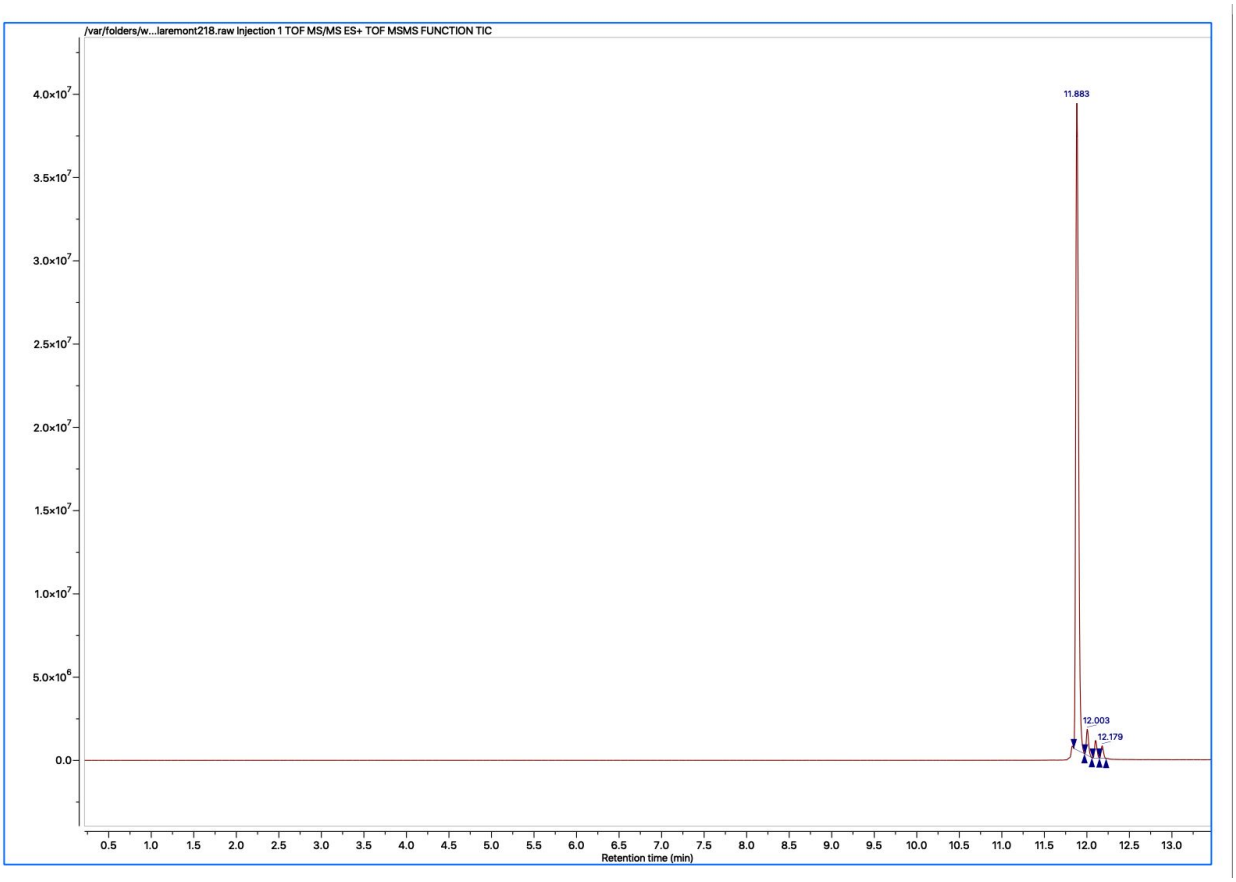

Figure 7. High resolution liquid chromatography-Mass spectrometry chromatogram showing total ion count over time from an LCMS of a purified fraction of the antifungal compound. On an ACQUITY HSS T3 column at Waters, the retention time for 0.2 µL of the compound was 11.883 minutes, and had very few nearby peaks, indicating a pure sample, and therefore a reliable mass spectrum.

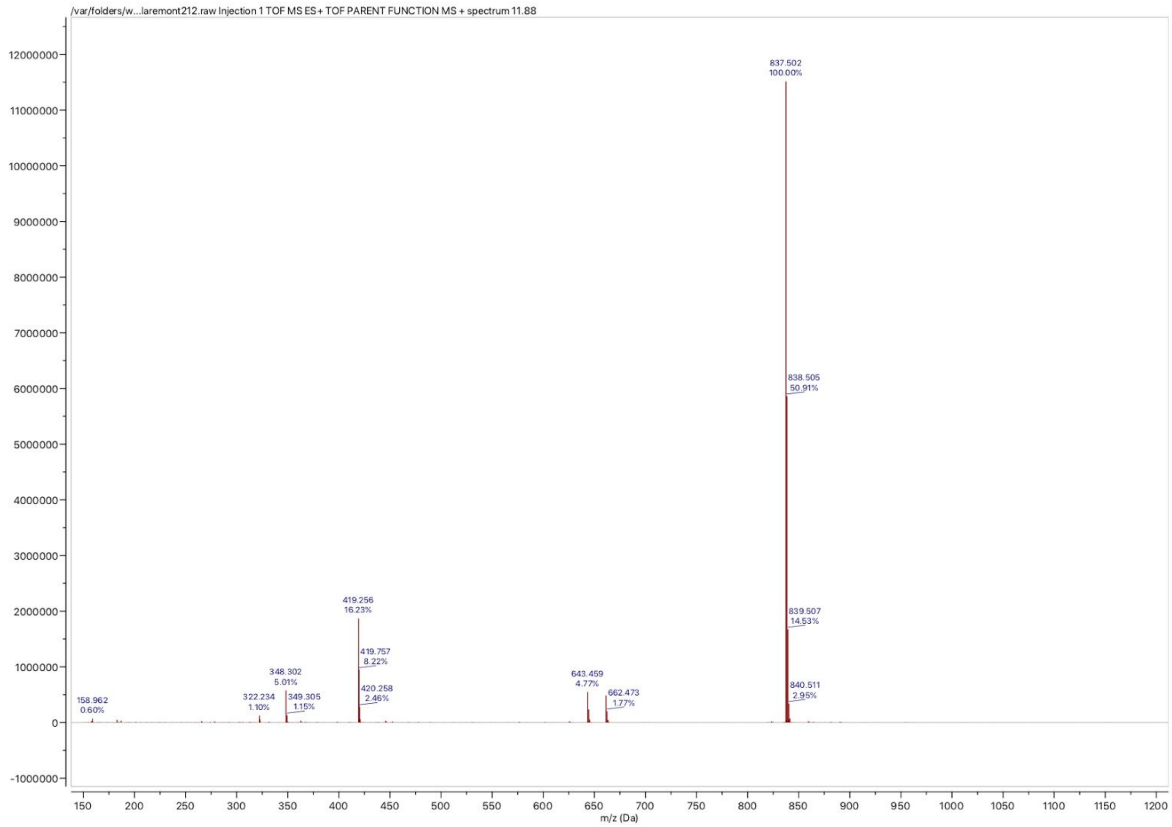

Figure 8. High resolution mass spectrum of the purified antifungal compound of interest.

This chromatogram indicates that the [M+H]+ peak lies at 837.502 m/z, suggesting an exact mass of 836.494 atomic mass units after subtracting the monoisotopic mass of a proton, 1.007825. Within the Dictionary of Natural Products, there is not an exact match for this mass, indicating that this molecule may be a novel antifungal.[20]

After identifying the exact mass of this antifungal compound, LCMS data from other *Trachymyrmex smithi*-associated bacteria was scanned for the presence of this particular molecular defense molecule.

Table 1. The occurrence across populations of the antifungal compound m/z value of 837.502 found in 17SM-2A. Black lines separate different ant nests, with each line representing bacteria from a different ant in the nest (Supplementary Table 3). The presence of an antibacterial compound also found in 17SM-2A is listed on the right with an m/z value of 519.2. LCMS data was produced by Elizabeth Lawton.

Strain Code	Bacterial Genus	Presence of 837.502 (M+H)	Presence of 519.2 (M+H)
17SM-2*	Amycolatopsis	Yes	Yes
17SM-2**	Amycolatopsis	No	No
17SM-8*	Amycolatopsis	No	No
17SM-8**	Amycolatopsis	No	No
18SM-1	Amycolatopsis	No	No
18SM-2A	Amycolatopsis	No	Yes
18SM-2B	Amycolatopsis	No	No
18SM-2C	Amycolatopsis	No	No
18SM-3A	Amycolatopsis	Yes	Yes
18SM-3B	Amycolatopsis	Yes	Yes
18SM-3C	Amycolatopsis	Yes	Yes
18SM-4	Amycolatopsis	No	No
18SM-5A	Pseudonocardia	No	No
18SM-5B	Pseudonocardia	No	No
18SM-6	Pseudonocardia	No	No
18SM-7A	Pseudonocardia	No	No
18SM-7B	Pseudonocardia	No	No
18SM-7C	Pseudonocardia	No	No

Four of the *T. Smithi* associated bacterial strains tested, all of which were *Amycolatopsis* appeared to contain the compound. Three of these strains were isolated from ants in the same colony, whereas the other was isolated off an ant from a different colony at a different time point. The mass spectra of these compounds was also analyzed for the antibacterial compound also produced by 17SM-2A, and the compound was found in the same bacterial strains, however the antibacterial compound was found in one more strain.

UV absorbance data extracted from an LCMS chromatogram also gave us some insight into the molecule of interest (Figure 5).

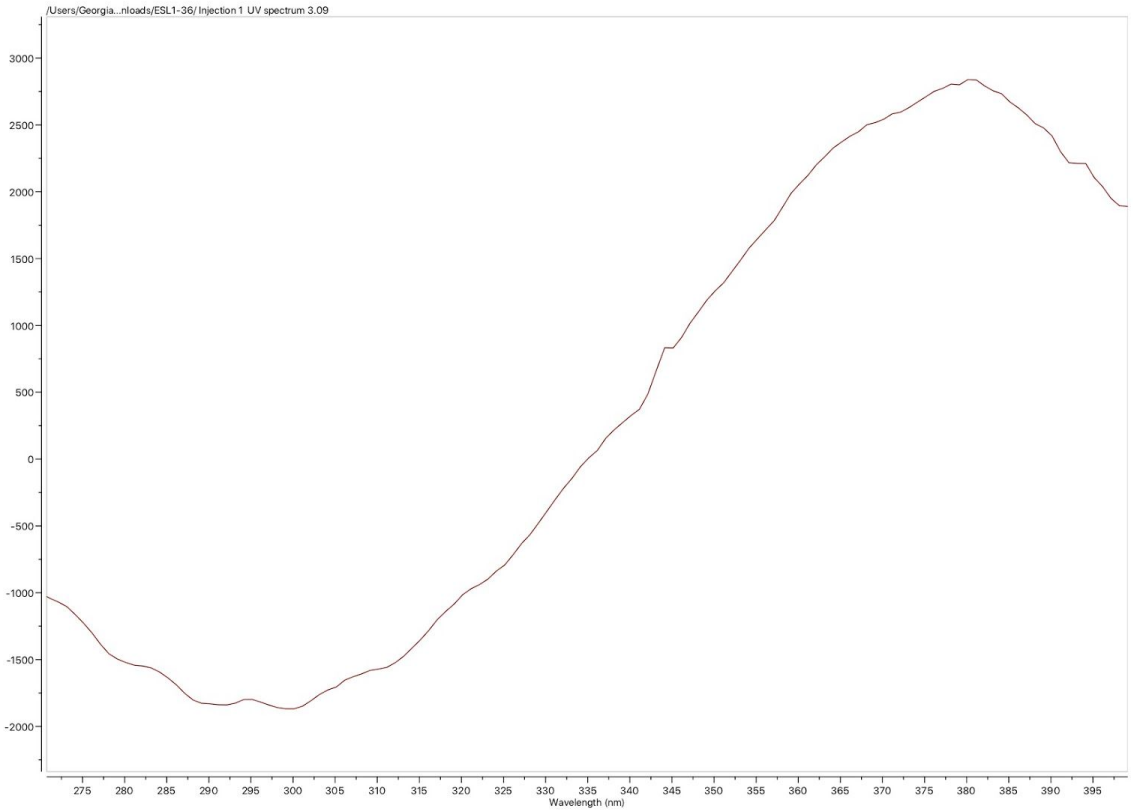

Figure 9. UV Spectrum of the antifungal compound of interest.

Peak absorbance was found to be about 380nm, hinting at some conjugation or aromaticity of the compound. Furthermore, the shape of the UV spectrum rules out the possibility of being a pentaene, a common antifungal structure.[20]

Lastly, NMR was performed in order to get a better sense of the chemical structure. The PROTON NMR did not contain enough pure compound to obtain definitive structural information.

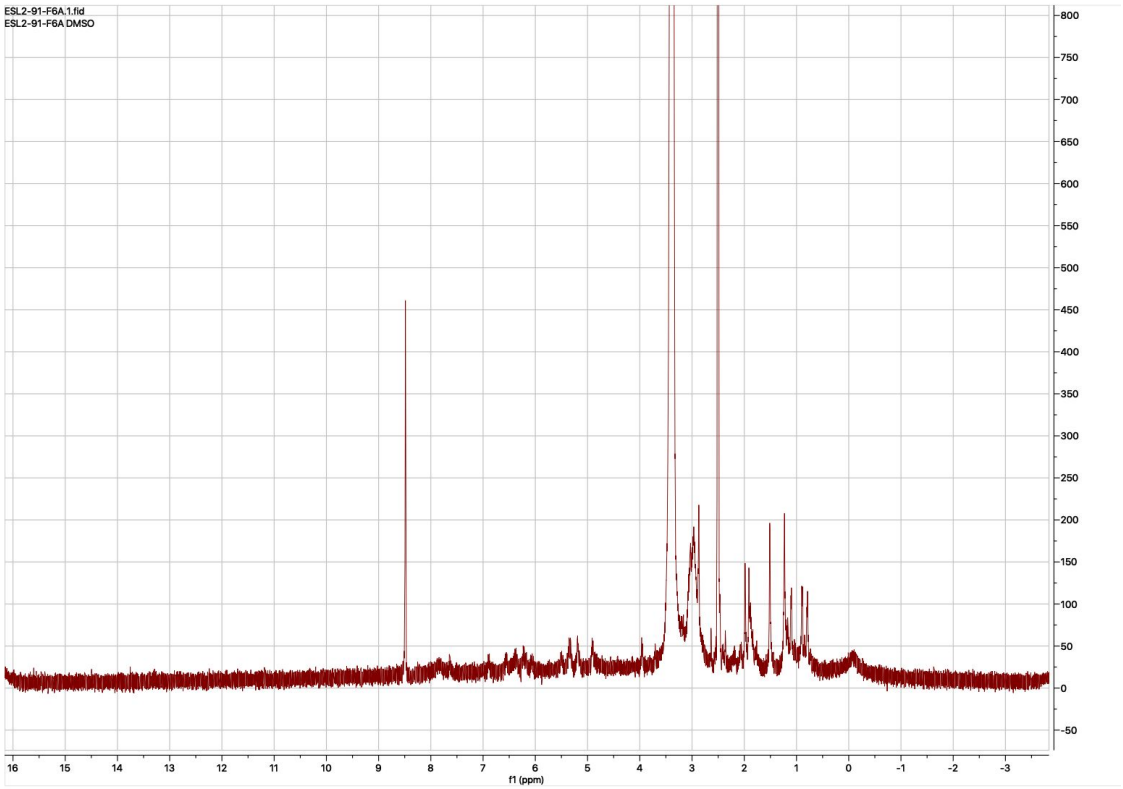

Figure 10. 1D NMR of the antifungal compound.

Discussion

Antifungal Screening

In this study, bacterial symbionts of North American *Trachymyrmex* ants were investigated for their antifungal properties. Plate-based activity testing using ecologically relevant fungal pathogens confirmed that these bacteria have antifungal activity (Figure 2).

This screening also revealed an interesting selective inhibition pattern with the cultivar fungus. Minimal antifungal inhibition was seen when the cultivar fungus from a *T. arizonensis* nest was plated against bacteria derived from *T. arizonensis* ants. Intriguingly, statistically significantly more inhibition of the cultivar fungus was seen by bacteria extracted off different species of ants, *T. pomonae* and *T. smithi*. This points towards a selective inhibition mechanism of the symbiotic bacteria. It is evolutionarily favorable for the ants to have a bacterial symbiont that produces defense molecules against a potentially pathogenic fungus. However, it is evolutionarily unfavorable for this bacterial symbiont to produce antifungal molecules that effectively inhibit their primary food source, or cultivar fungus. Future investigation of other cultivar fungi from different ant species would further support this idea. For example, the antifungal dentigerumycin, which was extracted off ants in Panama, was found to be selectively inhibiting of fungal parasites, but not of it's own cultivar fungus.[18] The specificity this data implies would support highly specialized and co-evolved relationships between the bacterial symbiont, the ant and the cultivar fungus.

Fungal putative competitors used in Figure 2 were collected from the nests of *Trachymyrmex* ants as indicated on the figure, and each column has some dark orange boxes indicating that each strain was inhibited to some degree by some bacteria. The purpose of

performing activity testing with these putative competitors was to evaluate the degree to which these fungi may be antagonistic in the nest of the ant. This would draw in a fourth character into the symbiosis, since *Escovopsis* has not yet been found and successfully cultured from the habitats of *Trachymyrmex* ants. However, we cannot confirm an antagonistic relationship between the fungal test strains and the cultivar fungus in that nest, since we do not have enough ecological data to show that any one of these fungi consistently threatens cultivar fungi growth in ant nests. DNA sequencing can be used to identify the relative abundance of different possible fungal pathogens in the immediate environment of the cultivar fungus.[16] While the Actinobacteria in this system often shows effective inhibition of the putative competitors, we cannot be sure that these fungi have significant ecological impact on the nest of *Trachymyrmex* ants. However, due to the antifungal inhibition shown by 17SM-2A, against this panel of fungi we moved forward with fractionation of the extract. Furthermore, significant inhibition was seen of the known fungal pathogen *Escovopsis* by the *Amycolatopsis* strain in this screening assay. This implies that the antifungal compound is important for inhibiting fungal pathogens that may threaten the cultivar fungus.

From the bioassays in Figure 2, *Amycolatopsis sp.* 17SM-2A was selected due to exceptionally high antifungal activity. In order to purify and identify more information about the molecule responsible for the antifungal activity, we have used HPLC, mass spectrometry, UV spectroscopy and NMR.

From the resident-intruder assay screening (Figure X), 17SM-2A and 17SM-8A arose as strains with especially strong antifungal activity. 17SM-2A has been investigated more deeply in this thesis however, 17SM-8A is of great interest for future research.

Activity-Guided Fractionation

Activity guided fractionation provided a method by which to determine the active antifungal compound produced by 17SM-2A. This allowed us to confirm our active compound and obtain it's UV absorbance spectrum. The only successful Spot-on-Lawn assay for the initial activity testing with the Sep-Pak fractionation was on a lawn of *Phoma sp*. fungus 18AZ-2I, which, as stated above, is not a known fungal pathogen of the cultivar fungus. However, we were able to show that the purified active compound inhibited both the *Phoma sp.* fungus and a known fungal pathogen, *Escovopsis*.

Remarkably, the Sep-Pak fractions that showed antifungal activity showed antibacterial activity. We have confirmed through this spot-on-lawn assay and further purification with HPLC that there are two separate compounds that contribute antifungal and antibacterial activity to the bacteria despite them eluting in similar fractions during the Sep-Pak fractionation process. The antibacterial compound has been researched and does not, in fact, have antifungal activity against two different strains of fungus tested, and is therefore a different compound. Analysis on this compound has indicated it is a nocamycin derivative. Therefore, activity guided fractionation allowed us to differentiate these two compounds which eluted in similar fractions during fractionation, likely due to similar interaction with a C18 column (Figures 5 and 6). This has also allowed us to determine that this extract has strong antibacterial and antifungal activity, each of which is caused by a distinct defense molecule.

Spectrometry and Spectroscopy

After confirming the identity of the compound through activity-guided fractionation we were able to view the UV spectrum of this compound obtained by the HPLC. The UV absorbance of 381nm for the antifungal compound served as a useful guide for finding the compound on other HPLC conditions then used for further purification of the compound.

The high resolution mass spectrum obtained from the purified antifungal compound gives significant structural information about the compound. First of all, it shows an [M+H]+ peak of 837.502, indicating an exact mass of 836.494. With this and the isotopic ratio, a number of molecular formulas can be proposed for the molecule. $C_{38}H_{72}N_6O_{14}$ (ppm 0.417)and $C_{37}H_{66}N_{13}O_9$ (ppm 0.423)and $C_{53}H_{66}N_5O_4$ (ppm 0.575) have exact masses that match that indicated by the high resolution mass spectrum. UV absorbance increases as conjugation increases and the three proposed formulas have degrees of unsaturation of 6, 11.5 and 23.5 respectively, consistent with the compound having a large UV absorbance peaking at 381nm. Furthermore, the third formula has an isotopic ratio most similar to that seen in experimental results. However, due to the high molecular weight of the molecule, it is not possible to establish an exact molecular formula from the high-resolution monoisotopic mass alone.

Analysis of the fragmentation patterns of the mass spectral data can be used to determine molecular substructures and build from those substructures to the full antifungal molecule. MSMS data, in which each fragments are separated by weight by the first mass spectrometer and then each of those fragments is fragmented again to show the composition of that particular fragment, can also be analyzed in tandem with NMR in order to shed light on structural information.

Moving forward, more NMR on pure antifungal compound would be helpful for further insight on structural elucidation. Proton NMR spectra show the spatial relationships between protons on adjacent carbons. NMR experiments to be performed include COSY, HSQC, HMBC, ROESY, TOCSY and carbon-13. These complementary NMR experiments provide relationships between protons and carbons in a variety of ways. For example, a COSY shows which protons are coupled to each other on a proton NMR spectrum.

Dereplication

While the antifungal compound being isolated may be a novel compound, there is a significant likelihood that the compound may have been previously extracted and identified from a different bacterium in a different ecological context. Bacteria of significantly different species can produce the same molecules due to horizontal gene transfer.[19] Therefore, ruling out known antifungal compounds, a process known as dereplication, is essential to efficiently identify antibiotics that may have already been characterized. In 1965, a paper was published detailing the discovery of a new antifungal molecule, pentafungin which had a proposed molecular formula of $C_{41}H_{74}O_{16}N$, and a proposed exact mass of 836.501, within a reasonable range of the exact mass of the compound of interest at 836.511. However, due to pentafungin being a polyene, it's UV spectrum had a distinct pattern that was not seen in the UV spectrum for our antifungal compound of interest.We can conclude that this compound is not pentafungin, and no other reasonable matches were found in the dictionary of natural products that have both a similar exact mass and potential or known antifungal activity.[20]

Population-Level Diversity

Analysis across *T. Smithi* associated bacterial strains allowed insight into questions on evolution of these antibiotic-producing bacteria. Interestingly, most of the strains that produced the antifungal also produced an antibacterial molecule with an m/z of 519.2. This evidence suggests that the biosynthetic gene clusters of these two molecules are close together or the bacterial strains that produce these molecules are closely related. Interestingly, the two colonies from which the bacteria was isolated were obtained over a year apart in Las Cruces, New Mexico. However, there was one bacterial strain that did produce the antibacterial compound but did not produce the antifungal compound, which may indicate horizontal gene transfer, allowing otherwise genetically dissimilar strains of bacteria to endogenously create the same antifungal molecule.

Therapeutic Implications and Broader Relevance

The potency of this antifungal compound in comparison with other antifungals is unknown at this point, but a minimum inhibitory concentration could be determined through plate-based assays of the compound at different concentrations. This would allow the antifungal activity of this compound to be assessed compared to other, known, antifungal compounds.

The possibility that this antifungal compound could be a potent, novel antifungal compound is exciting. This compound could be useful for a diverse set of purposes. For example, it could be used in agriculture, or for treating possibly deadly *Candida* infections in humans.[2] Furthermore, as antifungal resistance becomes an increasingly prevalent issue, a

novel antifungal, possibly with a different structural class, could be important for treating antifungal-resistant strains of fungus. Due to this ecological niche being largely unstudied, and we have already shown that this compound is not a pentaene, one of the three classes, we may find a novel structural class of antifungal compounds from this unique context.

It is unlikely that this compound is perfectly potent and nontoxic for use in treating human infections because it is very likely that if something is toxic to a eukaryotic fungal cell, it may also be toxic to a eukaryotic human cells.[6] Furthermore, the compound may only be potent enough to treat human infections in high doses which may cause the compound to become toxic as well, if it is not easily broken down by the human body. While it is improbable that this compound can be used, unmodified as a human therapeutic, the structure could still serve as the starting point of investigation into other therapeutically relevant compounds. Moreover, it could provide us with insight on the fascinating tripartite symbiosis in which North American *Trachymyrmex* ants partake. Data indicates that *Trachymyrmex*-associated bacteria produce antifungals which are selectively inhibitory of all other fungi except their cultivar fungus, suggesting a tight coevolution between the ant-associated bacteria and the cultivar fungus of that ant. Furthermore, evidence points to evolutionarily related bacterial strains in different parts of the world. While the structure of the molecule would aid in establishing ecological and evolutionary relationships, data presented here gives us a window into how these relationships play out in *Trachymyrmex* ants.

I have shown here an Actinobacteria strain of the genus *Amycolatopsis*, which was isolated from the cuticle of a *T. smithi* ant in New Mexico produces an antifungal defense

molecule. This defense molecule inhibits potentially pathogenic fungi from the same environment as this compound, and a known fungal pathogen, *Escovopsis*.

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Appendix

Supplementary Table 1. Fungal test strains used in Figure 2. The ant nest from which the fungi were extracted are noted as well as the genus of each fungus from a DNA BLAST.

Fungal Strain	*Escovopsis*	18SM-5G	18SM-5H	18AZ-2I	18AZ-3E	18AZ-4B	18AZ-6	18AZ-9	18AZ-10H	18AZ-13(c)
Ant Nest Origin	Fungal Pathogen	*T. Smithi*	*T. Smithi*	*T. Arizonensis*	*T. Arizonensis*	*T. Arizonensis*	*T. Arizonensis*	*T. Arizonensis*	*T. Arizonensis*	*T. Arizonensis*
Fungus Genus	*Escovopsis*	*Aspergillus*	*Fusarium*	*Phoma*	*Mortierella*	*Trichoderma*	*Trichoderma*	*Mucor*	*Fusarium*	

Supplementary Table 2. Bacterial test strains used in Figure 2. The ant from which each bacteria was extracted is noted as well as the genus of each bacteria from DNA sequencing.

Bacterial Strain	*18AZ-1*	18AZ-4	18AZ-5	18PO-1	17SM-2A	17SM-1	17SM-8A	17SM-6
Ant Species of Origin	*T. Arizonensis*	*T. Arizonensis*	*T. Arizonensis*	*T. Pomonae*	*T. Smithi*	*T. Smithi*	*T. Smithi*	*T. Smithi*
Bacterial Genus	*Pseudonocardia*	*Pseudonocardia*	*Pseudonocardia*	*Pseudonocardia*	*Amycolatopsis*	*Pseudonocardia*	*Amycolatopsis*	*Pseudonocardia*

Supplementary Table 3. Bacterial strains of mass spectrometry data analyzed in Table 1. The ant from which each bacteria was extracted is noted as well as the genus of each bacteria from DNA sequencing and the year collected.

Bacterial Strain	*17SM-2**	17SM-8	18SM-1	18SM-2	18SM-3	18SM-4	18SM-5	18SM-6	18SM-7
Ant Species of Origin	*T. Smithi*	*T. Smithi*	*T. Smithi*	*T. Smithi*	*T. Smithi*	*T. Smithi*	*T. Smithi*	*T. Smithi*	*T. Smithi*
Bacterial Genus	*Amycolatopsis*	*Amycolatopsis*	*Amycolatopsis*	*Amycolatopsis*	*Amycolatopsis*	*Amycolatopsis*	*Pseudonocardia*	*Pseudonocardia*	*Pseudonocardia*
Year Collected	*2017*	*2017*	*2018*	*2018*	*2018*	*2018*	*2018*	*2018*	*2018*

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