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Extraction, Isolation, and Characterization of a Novel Compound Presenting with Antibiotic Properties from *Herbaspirillum* sp.

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Abstract

Due to the lack of novel antibiotic discovery since the 1980's, the rapidly increasing number of antibiotic resistant bacteria has become a global health concern. Correlating with the misuse of antibiotics and the lack of funding for research, once treatable bacterial infections are now presenting with multi-drug resistance leading to more than 35,000 deaths a year in the United States alone. To slow the progression of multi-drug resistant bacteria, research in this field is being refunded and is focusing on developing new methods for antibiotic discoveries. Mining for natural products produced by microbes has regained popularity and advances in technology are proving to drastically reduce the rediscovery rate that once halted antibiotic research. Almost all antibiotics currently in use were mined from natural products, proving this method is extremely successful and has more to offer. Here an overview of the history of antibiotic resistance, priority pathogens, streamlined and new research techniques, and research of extracting a novel antibiotic from a *Herbaspirillum* species of bacteria that is capable of inhibiting both *Staphylococcus aureus* and *Escherichia coli* is discussed.

1. Introduction

As there are many serious bacterial infections now presenting with multi (MDR) and extensive (XDR) drug resistance that are effectively untreatable, the World Health Organization (WHO) recognizes the antimicrobial resistance (AMR) crisis as the largest global health concern because it affects the healthcare, veterinary, and agricultural industries.[1]

As reported by the Center for Disease Control (CDC) in 2019, 2.8 million people in the United States will be diagnosed with an antibiotic-resistant infection and at least 35,000 people will die from its effects. If these trends continue, we could see up to 10 million deaths worldwide per year due to these infections (Figure 1).

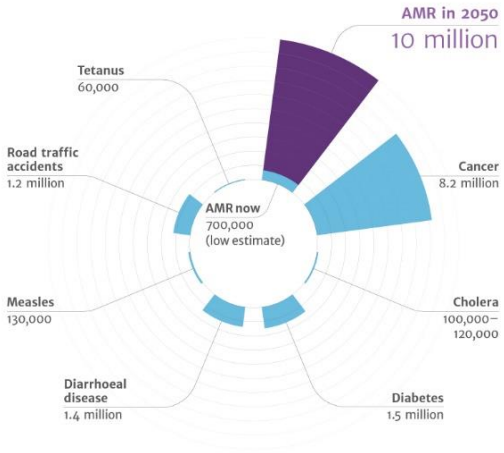

Figure 1. Current and predicted deaths due to antibiotic resistant infections in comparison with common causes of death.[2]

Further explained by the CDC, the veterinary community will experience a similar increase in AMR and XDR cases. The agricultural community is also hit hard from this health crisis and is viewed as a major contributor to the crisis itself as 80% of antibiotics produced in the US are sold to farmers, 75% to livestock and 5% to crops.[3] Due to these statistics, the CDC recommended that these industries take precaution in prescribing antibiotics.

The WHO recently released a list of the most prevalent pathogens labeling *Mycobacterium tuberculosis* "priority one" as it alone is responsible for 250,000 deaths a year.[1] The WHO continues to list these pathogens as "critical priority": *Acinetobacter baumannii*, *Pseudomonas aeruginosa* (PA), *Enterobacteriaceae*, and these under "high priority": *Enterococcus faecium*, *Helicobacter pylori*, *Campylobacter* species, *Staphylococcus aureus* (SA), *Salmonella* species, *Neisseria gonorrhoeae.* [1] All these pathogens currently present with MDR or XDR in addition to having insufficient antibiotic-discovery pipelines, which is what puts them at the top of the WHO prevalent pathogen list. The goal of this list is to encourage researchers to aim their research towards finding novel antibiotics to combat these pathogens. The pathogens of particular importance to most researchers are the "ESKAPE" pathogens (*Enterococcus faecium*, SA, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, PA, and *Enterobacter* species) as they are the leading causes of hospital infections globally since 2015.[4,5]

The resistance expressed by pathogens that are determined to be MDR and XDR can be acquired or inherited. Typically, resistance can begin to occur when antibiotics are overprescribed, the wrong antibiotics are used to treat an infection, a course of antibiotics goes unfinished, the antibiotics do not kill the entirety of the pathogen population, or when random mutations occur allowing the pathogen population to persist.[4] These pathogens then can reproduce and perform horizontal gene transfer to survive.[6] To circumvent further antimicrobial resistant bacteria, there must be a steady influx of novel antibiotics in the pipeline until there are new treatment alternatives for bacterial infections.[5]

Referred to as the golden era of antibiotic discovery, between 1920-1960 there were 20 novel classes of antibiotics approved by the FDA. However, after this golden era there were only 2 novel antibiotics and only one novel class approved from 2000-2014 despite enhancements in technology and understanding of antibiotic mechanisms of action (Figure 2).[7]

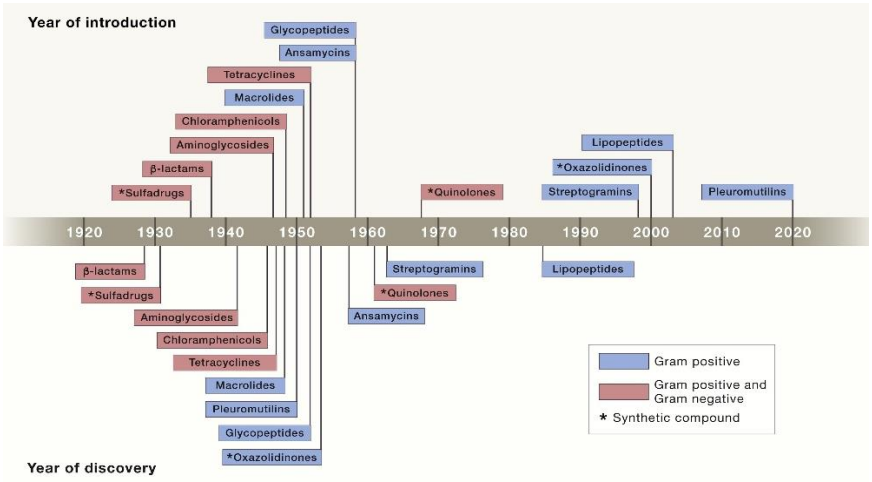

Figure 2. Discovery timeline of antibiotic classes.[8]

This lack of continued discovery is largely attributed to a high re-discovery rate of antibiotics which caused researchers to lose interest and funding to be cut. In turn, lack of funding and participation caused the antibiotic discovery and clinical pipeline to dry up just as the AMR crises started emerging, furthering its effects.[1] After being recognized as the leading global health issue by the CDC and WHO, antibiotic discovery programs have started to be put into place in both big and small pharmaceutical companies as well as academic laboratories world-wide.

Since the golden era, the current focus of antibiotic discovery is on mining for natural products (NP) which are classified as any secondary metabolite produced by an organism. NP's have continued to be a significant source of novel antibiotics and account for more than 73% of antibiotic classes approved by the FDA, more half of which were harvested from soil microbes.[6,7] The most accepted hypothesis as to why there are so many bioactive compounds produced by these microbes is because they produce multi-functional compounds that act as a chemical defense during competition and stress. However, the rediscovery of antibiotics in the golden era was caused by natural product research as there was a lack of bacterial strains able to be cultured in a laboratory. That is because during that time, only easy to cultivate bacteria was used with little methodology variation. Therefore, the same compounds were being rediscovered causing research labs and pharmaceutical companies to discontinue most of its antibiotic discovery research.[8]

Now that research has been resumed, researchers are utilizing many different techniques to combat the rediscovery rate of antibiotics such as the use of more versatile NP sources like filamentous actinomycetes, bacteria, and fungi. Additionally, researchers have been able to get the bacteria producing more NP's by subjecting them to stressful growing conditions.[8] Due to the abundance of abiotic factors that could influence the production of new NP's, this field of discovery has untapped potential. However, a popular method to increase the versatility of NP output is by growing the bacteria in monoculture, co-culture, and multi-culture growing conditions. This technique widens the range of potential NP's one bacterium can produce by exposing them to direct competition from other bacteria. Many other environment alterations to factors such as temperature, light, nutrient, and pH, are also being conducted in these types of growing conditions to increase possible NP production.[8]

To further combat re-discovery many novel techniques are being practiced that are aimed to increase bacterial sources. To aid the influx of bacteria for screening the sampling underexplored environments, such as the seafloor, arid deserts, and cave systems, has become very popular among research groups. In addition to this sampling of multi-organismal microbiomes, such as insect, plant endophytes and epiphytes, coral, fish, and mammal, is being surveyed for potential antibiotic producing bacteria or fungi. Researchers have also been able to widen the range of potential bacteria to survey by using the iChip which allows researchers to cultivate hard to grow bacteria.[9] Synthesis of novel and derivative antibiotics also plays a major role in this new wave of antibiotic discovery.

Despite these new programs, sources of funding, and scientific advancements, there have only been 11 antibiotics approved since 2014, most of which were derivatives from previously discovered antibiotics.[1,10] Additionally, the pipelines outlook is improved, but not sufficient as the AMR crisis continues to escalate despite researchers' efforts.

In the Wolfe Laboratory at the University of North Carolina at Asheville the use of mono-, co-, and multi-culture screening to mine for antibiotics, in addition to adjuvant and synthesis work, is a large focus. With a library of over

400 isolated bacterial strains that were harvested from pitcher plant water and rhizosphere soil collected on the UNCA's campus and another location in Southwestern United States (undisclosed) all the bacteria were processed

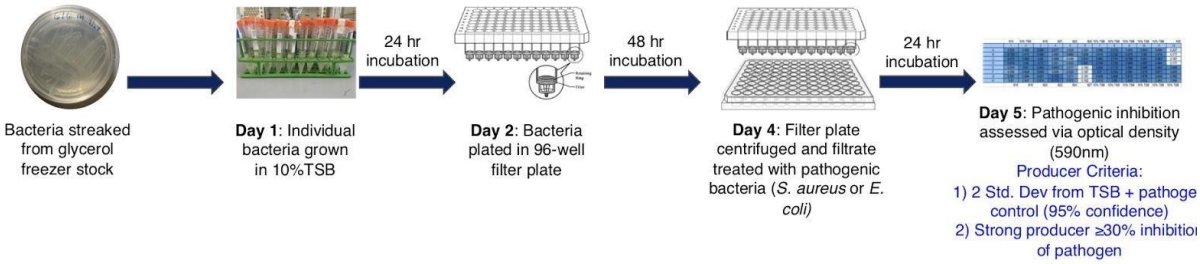

Figure 3. High-throughput screening method developed in Wolfe lab to analyze cultured bacteria's natural products for antibiotic properties in mono and co-culture environments.[12]

using high throughput screening in mono and co-culture settings against SA and EC (Figure 3).[11,12] Of the bacteria screened, 115 have been identified as antibiotic producers in monoculture against SA and 90 against EC, while in co-culture settings where 303 pairings were analyzed, 14% produced moderate activity against SA and 3% produced strong activity, additionally in trials for EC where 278 pairs were analyzed with 30% producing moderate activity.[12] Current research on the Wolfe lab is being conducted to test the bacterial stains for antibiotic activity in multi-culture trials.

During the mono-culture screening process, few novel antibiotic producers were discovered. Of importance to this research, the bacteria culture 614 was identified as an antibiotic producer strong enough for further testing. In this paper, methods for extracting, isolating, and characterizing a novel antibiotic compound from bacteria 614 from the Wolfe Lab library is discussed. The bacterium producing this compound was characterized to be *Herbaspirillum* sp. by previous researchers in Wolfe laboratory. However, due to the complexity or impurity of the compound, characterization was impossible. In addition to the compound being hard to extract in large quantities, further characterization efforts were not feasible at that time. With an improvement in isolation techniques, extraction of this monoculture is revisited. It is important to note that this bacterium has not yet been reported to produce antibiotics in any published research not produced by Wolfe Lab. However, in research by Batista et al. (2013), a gene encoded in this bacteria's DNA was found that does present with antibiotic activity by acting to inhibit respiration of certain pathogens.[13]

2. Methodology

2.1. Aseptic Technique

All procedures involving bacteria were done under sterile conditions. All working surfaces were cleaned with 10% Bleach prior to conducting experiments. All bacteria used were worked with under sterile flame conditions using gloves cleaned with ethanol. Pipet tips used to inoculate bacteria were sterilized in an autoclave (121 ºC) (Tuttnauer 5075EL). Media was sterilized either by autoclaving (121 ºC) or filtered through a 0.2µM Polyethersulfone (PES) filter. The pathogenic bacterial strains, *Staphylococcus aureus* (SA, ATCC 29213) and *Escherichia coli* (ATCC 25922). All bacteria were stored in 50% glycerol stock at -80 ºC and grown onto 10% Tryptic Soy Agar (TSA) (20 g Agar and 3 g Tryptic Soy Broth (TSB) per 1 L DI H_2O) plates. Once grown, the streaked bacteria were kept at (2 ºC) to prevent further growth and mutation.

2.2. Media and Scale-Up Process

A minimal media solution of 12.5 mM Citrate, 10x PPM, and 1M $MgSO_4$ was used for this experiment. 3 L of the media was prepared for each bacterial scale up and 10% (300mL) of each 3 L solution was separated for an intermediate volume in the scale up process. A singular bacterial colony of 614, from the soil isolate library at UNCA,

was inoculated into 30 mL of 10% TSB and shaken (Excella E25 Incubator shaker) at 130 RPM and 26 ºC for 24 hours. The caps were left loose to increase the oxygen exchange rate. The samples were transferred to 300 mL of 12.5 mM citrate and shaken for 24 hours under the same conditions. The samples were then transferred to the 3 L solution of 12.5 mM citrate and shaken for 120 hours. This was done with two colonies at once for a final total volume of 6L.

2.3. Extraction Process

To remove cellular components post incubation, the cultures were centrifuged (Thermo Scientific Legend XTR centrifuge) at 4,000 RPM for 20 minutes at 26 ºC. The supernatant was collected and 40 g per 3 L culture of Diaion HP20 resin beads (2% w/v) that had been presoaked in methanol for 15 minutes were added to the centrifuged supernatant and shaken for 24 hours at 26 ºC and 130 RPM. The beads were loaded into a column and six fractions were eluted off the beads using a water to methanol gradient of 1 L solutions: DI H_2O, 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, 100% MeOH. Fractions were dried via assisted air drying or rotovap (Buchi R-100 complex) and crude product was weighted. Active fractions were identified using cell death assay. TLC was used to follow the compounds through the rest of the experimentation process. Conditions for TLC were 100% EA and 3 drops of trimethylamine.

2.4. Cell Death Assay

Crude product from each fraction was diluted with 10 µL of Dimethyl sulfoxide (DMSO) per 1 mg of crude. In a 96-well plate with 89 µL of full-strength TSB, and 10 µL of overnight cultures of pathogen (EC or SA) 1 µL of dissolved crude was added. Chloramphenicol and DMSO only columns were used as positive and negative controls respectively. The plate was incubated (Fisher Scientific Isotemp Incubator) and shaken for 24 hours at 37 ºC. Using a Biotek plate reader (OD_{590}) the antibiotic activity for each extract was determined by analyzing absorbance values. Lower absorbance values correlated with a relatively lower pathogen cell count which suggests antibiotic activity.

2.5. Antibiotic Isolation

Active fractions from the extraction process were subjected to further purification to isolate the compounds by preparative high performance liquid chromatography (HPLC) (Shimadzu LC 20AR). The compounds were diluted with 50% MeOH in water solution then sonicated (Fisher Scientific Ultrasonic Bath). The HPLC was run using a 20% - 80% MeOH gradient that spanned for 30 minutes. Compounds isolated from this step were subjected to the cell death assay.

2.6. Characterization

Once enough antibiotic extract (>2mg) was obtained, LC/MS (Shimadzu LCMS-2020), ^{1}H-NMR, ^{13}C-NMR, single quad mass spectrometer, and triple quad mass spectrometer experiments were conducted. The results of these experiments were used in determining the structure of the antibiotic compound.

3. Results and Discussion

3.1. SS 614, *Herbaspirillum* monoculture

After the initial extraction procedures with resin beads was completed, the cell death assay indicated that the monoculture had antibiotic activity in the 40%, 60%, 80%,100% MeOH fractions against EC (40,60,80,100) and SA (40,60,80) (Figure 4). There was also some activity seen in the 0% and 20% fraction however, it was not deemed significant based on standards set by Murray et al. (2019). These fractions were visualized using Thin Layer Chromatography (TLC) with UV light. In initial TLC testing the compounds would not move up the plate under any concentration of ethyl acetate (EA) and hexane (Hx) mixture suggesting that the compounds produced by this bacterium are very polar. Due to the high polarity of the compounds, the TLC was run in 100% EA and with 3-4 drops of triethylamine, for de-salting purposes, which resulted in compound movement up the TLC plate. From the TLC,

the 40%, 60%, and 80% all presented with long and short-wave marks at both R_f values of 0.54 and 0.00 while the 100% presented with a short wave hit at an R_f of 0.84 and a long wave hit at an R_f of 0.00. This led to the combination of the 40-80% extracts. Further isolation was done with the 40-80% and 100% extracts at first using a normal phase silica gel column chromatography. After the silica column fractions were put through a cell death assay it was found that one compound, Compound A, presented with antibiotic properties against EC and SA within the 40%, 60%, and 80% fractions (Figure 5). All other compounds were inactive. Compound A presented an R_f value of 0.48 (in 100% EA, 2-3 drops of triethylamine) and a long wave light hit. The activity of Compound A in this cell death assay was lower than predicted for reasons unknown at the time**.** The compounds eluted from the 100% extract silica column have yet to be analyzed by cell death assay.

Collection of compound A was variable as the 2nd, 3rd, and 4th extractions had a collection range of 1.0 – 1.6 mg, which was assumed to be caused by poor collection timing. As an effort to improve the collection of compound A, a reverse phase HPLC was used to purify the compounds instead of the silica column after the 4th scale up. Using the HPLC, it was found that compound A had a retention time of 13.5000-15.206 minutes: however, there was significantly less (0.2-1.1 mg) product produced using this method. This decrease may be attributed to the compound collected from the HPLC being purer while the compound from the silica column may have had additional compounds in it, causing a weight increase. Additionally, the collection of the compound was still variable suggesting that the variability came from unintentional changes in the scale up process.

Before additional scale up was completed, Compound A was analyzed using LCMS with a 20-80% methanol procedure that ran for 8 minutes (Table 1). The major MS positive and negative masses were not consistent with any known antibiotic or any byproduct from *Herbaspirillum* sp, suggesting that Compound A may be a novel discovery (Table 2). The data bases used to analyze if the LC/MS peaks were novel are AntibioticDB and The Natural Products Atlas.[14, 15]

As Compound A collection and purification continued, its properties began to change. It went from a light orange to neon yellow in color and became relatively polar. This polarity made the compound insoluble causing proton and carbon NMR's challenging or impossible to read. Compound A was attempted to solubilize in DMSO, compound would not fully dissolve, and deuterated acetone, compound would crash out of solution after ~1 minute. This made the ^{1}H NMR results unreadable and unreliable. The compounds inability to dissolve in the DMSO suggests the cell death assays from the silica extracts may be inaccurate as the compound might not have been fully dissolved in the DMSO (Figure 5). This suggests that the compound may cause a greater percent of inhibition, as predicted, than the results indicate. Direct correlation between insolubility in DMSO and cell death assay inaccuracies were directly seen as the pure Compound A lost all inhibition against EC and SA. A reading of an ^{1}H NMR was attempted again with deuterated MeOH, which fully solubilized the compound. The ^{1}H NMR was run for 63,000 scans (Figure 6). From this NMR it is concluded there is an aromatic group and there appears to be no Cl. A ^{13}C-NMR was also run in deuterated MeOH. The data was inconclusive as no peaks were seen even with a run of 3,000 scans. This left ^{13}C-NMR data unobtainable. To progress in characterization of compound A, from the results of the single quad the peak 225 (+) was selected to be run in the triple quad mass spectrometer (Table 1). The masses of the compound as it broke follow: 255, 123, 72 (Figure 7). Using results from the triple-quad paired MS and the ^{1}H NMR, the compound with antibiotic properties extracted and isolated from this research was characterized to be a possibility of 19 different compounds with various splitting possibilities (Table 3).

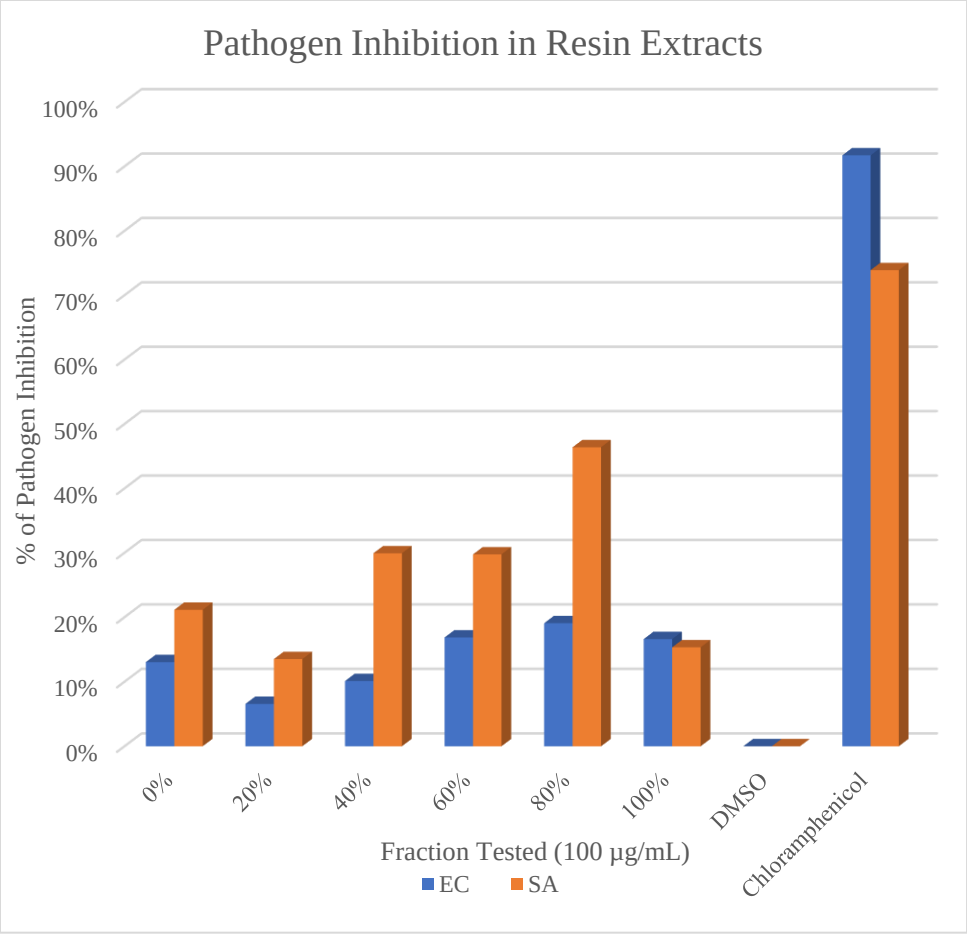

Figure 4. Pathogen Inhibition in Resin Extracts

Figure 4. Pathogen inhibition results from the cell death assay of the 0-100% extracts collected from the resin bead column. All tested fractions had a concentration of 100 µg/mL. n=2.

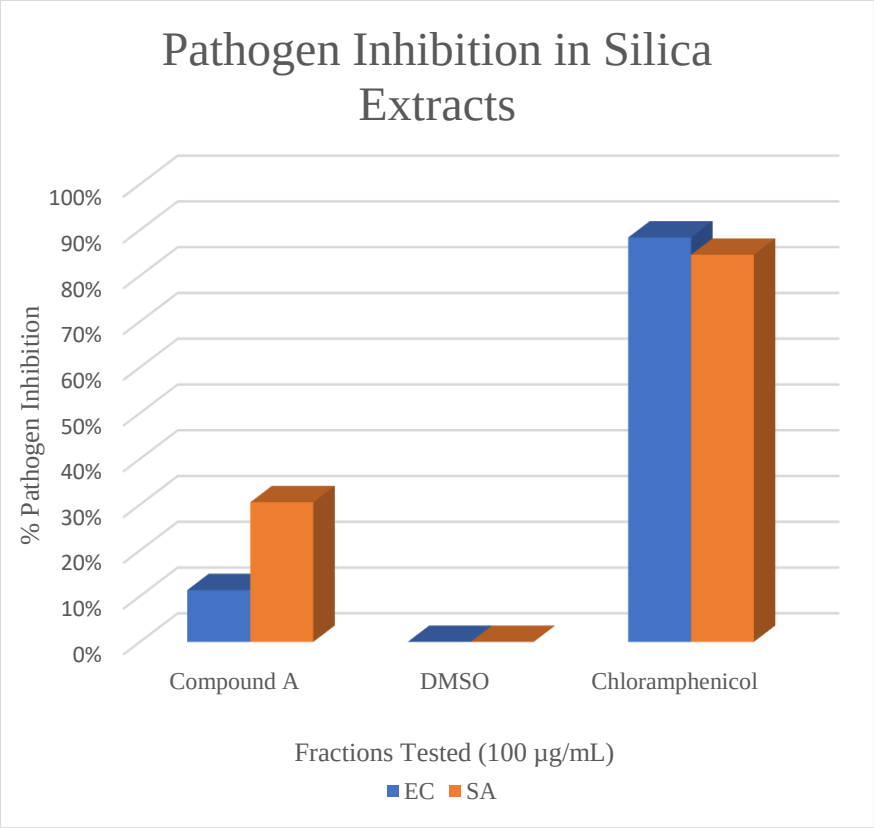

Figure 5. Pathogen Inhibition in Silica Extracts

Figure 5. Pathogen inhibition results from the cell death assay of the active compound eluted off the 40-80% extracts silica gel column. All tested fractions had a concentration of 100 µg/mL. n=2. In this step over 40 compounds were tested. Represented in this figure is the only active compound.

Table 1. MS data from pure Compound A. Method: 20-80% MeOH gradient over 8 minutes. The masses are listed by peak height, the tallest being first and the shortest being last. Peaks under **0.5 were not considered.**

	Retention Time (minutes)	[M+1] m/z	[M-1] m/z
Compound A	**0.0 – 0.25**	255, 479, 370, 537	487, 713, 421, 265

Table 2. Known natural products harvested from *Herbaspirillum* sp. Data sourced from AntibioticBD.[14]

Compound Name	Molecular Weight
Serobactin A	821.835
Serobactin B	849.889
Serobactin C	877.943

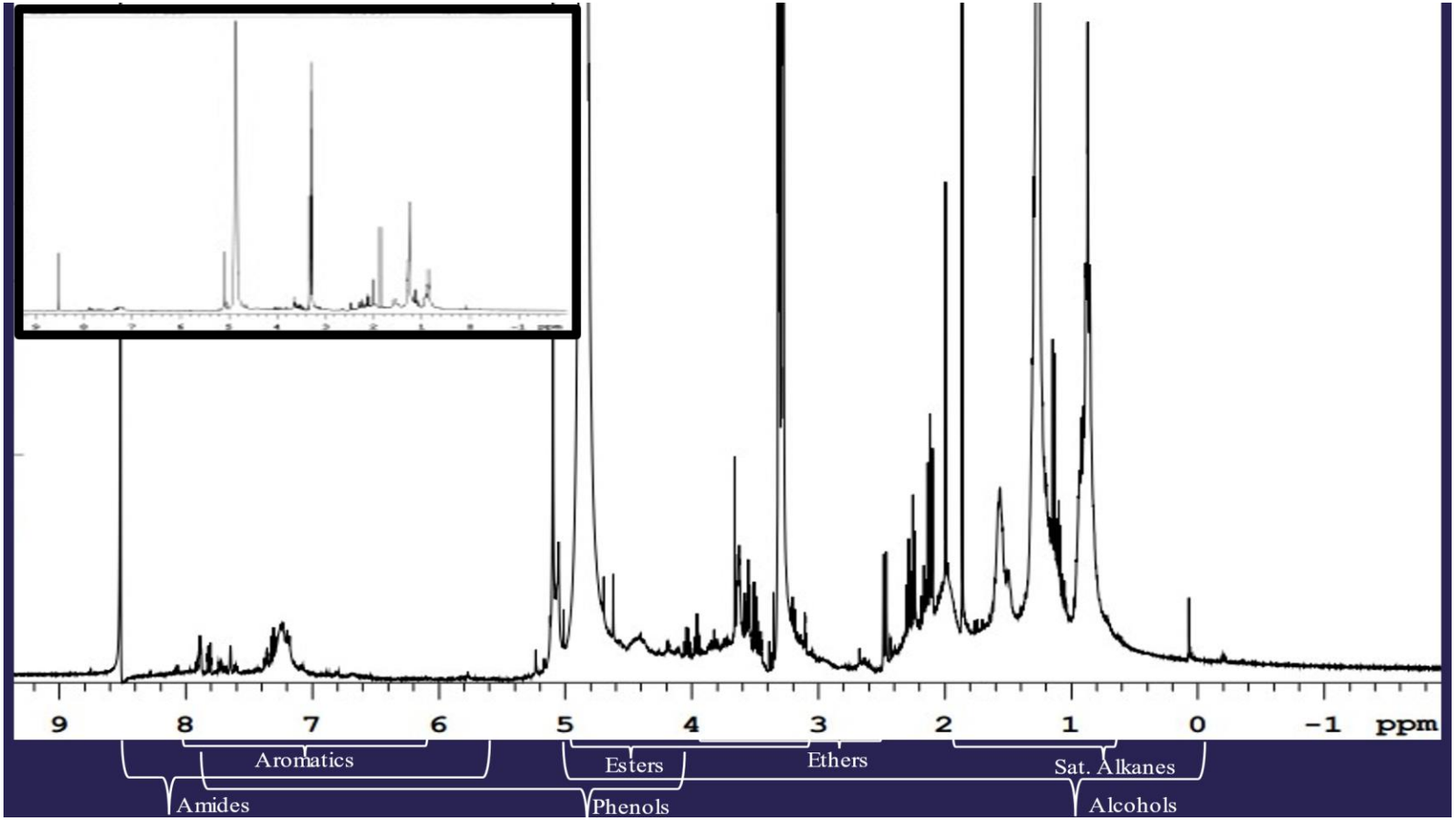

Figure 6. ^{1}H NMR of Compound A

Figure 6. NMR data from Compound A with chemical shifts labeled. Solvent: Deuterated Methanol. 63,000 scans. Aromatic groups present.

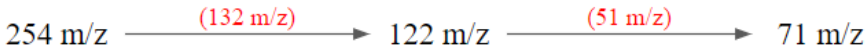

Figure 7. Triple-quad mass spectrometer analysis of compound breakdown at peak 255 m+1.

Table 3. Possible final compound and its splitting possibilities.

254 m/z	132 m/z	122 m/z	51 m/z	71 m/z
C6H10N10O2	C2H2N3O4	C5H2N2O2	C3HN	C2HNO2
C7H10N8O3	C4H4O5	C4H2N4O	C4H3	CHN3O
C8H10N6O4	C3H2NO5	C6H4NO2		C3H3O2
C9H10N4O5	C7H2NO2	C5H4N3O		C2H3N2O
C9H14N6O3	CH2N5O3	C7H6O2		CH3N4
C10H10N2O6	C3H4N2O4	C4H4N5		C3H5NO
C10H14N4O4	C2N2O5	C6H6N2O		C2H5N3
C11H10O7	C6H2N3O	C5H6N4		C4H7O
C11H14N2O5	C8H4O2	C7H8NO		C3H7N2
C11H10N8	C2H4N4O3	C6H8N3		C4H9N
C12H14O6	C4H6NO4	C8H10O		C5H11
C12H10N6O	C5H2N5	C7H10N2		
C13H10N4O2	C7H4N2O	C8H12N		
C13H18O5	CH4N6O2	C9H14		
C14H10N2O3	C3H6N3O3			
C14H14N4O	C5H8O4			
C15H10O4	C6H4N4			
C15H14N2O2	C8H6NO			
C16H14O3	C2H6N5O2			
	C4H8N2O3			
	C7H6N3			
	C9H8O			
	CH6N7O			
	C3H8N4O2			
	C5H10NO3			
	C8H8N2			
	C2H8N6O			
	C4H10N3O2			
	C6H12O3			
	C9H10N			
	CH8N8			
	C3H10N5O			
	C5H12N2O2			
	C10H12			
	C4H12N4O			
	C5H14N3O			
	C7H16O2			
	C4H14N5			
	C6H16N2O			
	C5H16N4			

4. Conclusion

The novel compound extracted from this *Herbaspirillum* sp. presents with a mass of 254 m/z however, due to the inability to produce a readable ^{13}C-NMR the Compound A was only able to be deduced to 19 possible compounds with the information provided from the ^{1}H NMR and triple-quad MS. Currently due to the insolubility, polarity, and limited quantity of compound A, further characterization is impossible. Future steps in this research would include either collecting additional compound A and reattempting a ^{13}C-NMR or sending compound A to another research facility for further analysis with instrumentation that UNCA does not have access to. Additionally, due to the limited

quantity of compound A the mechanism of action of this antibiotic still needs to be assessed. The ability for compound A to inhibit the growth of both gram-negative and gram-positive bacteria make it of high importance for characterization efforts. Once characterized, this compound has the potential advance towards assessment and qualification as an antibiotic or adjuvant in the medical, veterinary, and agricultural industries.

5. Acknowledgements

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