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Extraction and partial purification of an antibiotic-like compound from the soil bacterium Rhodococcus strain KCHXC3

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Extraction and partial purification of an antibiotic-like compound from the soil bacterium *Rhodococcus* strain KCHXC3

by

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East Tennessee State University

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ABSTRACT

Rhodococcus bacteria have many secondary metabolic pathways that may produce novel natural products. The bacterium *Rhodococcus* strain KCHXC3 was isolated from a soil sample collected near Kingsport, Tennessee and was found to produce an inhibitory compound active against a broad array of bacterial species, including the Gram negative pathogen *Shigella sonnei*. The aim of this research is to extract and purify the compound for future structure elucidation. A mixture of compounds from 3 month old agar plates inoculated with strain KCHXC3 was extracted using ethyl acetate. The crude extract was then partially purified utilizing a Sephadex LH-20 column, followed by an analytical NH_2 HPLC column. This purification resulted in a dried crystalline-like active compound that is white in color and needle-like in shape. Structural studies such as NMR and GC-MS revealed the presence of aromatic rings in the active compound that appears to be built with amino acids.

ACKNOWLEDGEMENTS

I would like to extend my thanks to Dr. Abbas Shilabin, my research mentor, for his kindness and guidance throughout this research project. I would also like to thank Dr. Bert Lampson for this collaboration. Thank you for your patience and allowing me to perform microbiology experiments in your lab. Thank you both for comments and instruction throughout experiments and in preparation for presenting this work.

Many thanks to the ETSU Department of Chemistry for their guidance throughout this work, and to Andy Agbakpo for helping me with NMR and GC-MS for this study. I also owe thanks to Megan Carr, who completed much of the preliminary microbiology work for this project. I would also like to extend my thanks to the ETSU Honors College and Office of Research for funding to be able to purchase the amino HPLC column along with other necessary laboratory supplies.

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INTRODUCTION

Antibiotic Use and Resistance

Antibiotic resistance among nearly all bacterial pathogens is increasing with alarming frequency. This makes treatment of even minor infections a growing problem. Bacterial pathogens can easily share their resistance genes in the form of plasmids. Research into the discovery of new antibiotics is time-consuming and not as lucrative as other medicines, which has contributed to the decreasing number of antibiotics available[1]. Another challenge to antibiotic development is that by the time a new antibiotic has made it from discovery to prescription medicine, bacteria have already developed resistance. If these trends continue, soon bacterial infections will again become a major global cause of death with the possibility of making even simple surgeries life-threatening[1].

Natural Products

Natural products are increasingly important with the increase in antibiotic resistance and decrease in number of novel antibiotics. The study of natural products focuses on compounds produced by organisms. Humans have been utilizing natural products for thousands of years[2]. Current antibiotics are an example of this. For example, Penicillin is a natural product that is part of a group of molecules called secondary metabolites. Secondary metabolites are compounds, mainly produced by bacteria, that are unnecessary for the bacteria's essential functions of life. Essential functions of life include nutrient processing, energy production, and growth and development[3]. Valuable secondary metabolites are often provided by bacteria in both tiny quantities and as a mixture of compounds that are not optimal for practical use; therefore, it is commonplace to develop these compounds synthetically[4]. In order to do this, the compound must

be extracted from its source, purified, structurally analyzed, and a reaction synthesis produced for it to move to industrial applications.

Actinomycetes Genus Rhodococcus

Actinomycetes is a phylum consisting of diverse Gram-positive and filamentous bacteria[6]. This incredibly diverse group of bacteria are accountable for 45% of the 22,500 biologically active natural products produced by microorganisms[7]. Additional novel antibiotics may still be discovered from soil, as a large percentage of soil has not been screened for antibiotic production. The genus *Rhodococcus* are Gram-positive Actinomycetes that are found primarily in the soil. Study of this genus has led to over 100 patents by the late 90s, and recent greater understanding of its genome has opened the possibility of finding previously unknown bioactive compounds[6]. The genus *Rhodococcus* is known to produce a wide variety of secondary metabolites including antibiotics, biosurfactants, and pigments (**Figure 1**)[8] indicative of their high biosynthetic capacities[9].

Figure 1. Various pigments produced by different *Rhodococcus* species[8]. Diversity in pigments show the high biosynthetic capacity of *Rhodococcus*.

KCHXC3 Isolation and Growth

Rhodococcus strain KCHXC3 was isolated from a soil sample collected in Kingsport, TN using an enrichment procedure by a previous graduate student at ETSU[10]. 16S rRNA gene analysis confirmed that KCHXC3 was a part of the phylum Actinobacteria, family Nocardiaceae, and genus *Rhodococcus*. There were 6 *R. erythropolis* species found to be most similar to the sequence of KCHXC3[5]. KCHXC3 is a rod shaped, Gram positive bacterium that produces mucoid light pink colonies (**Figure 2**).

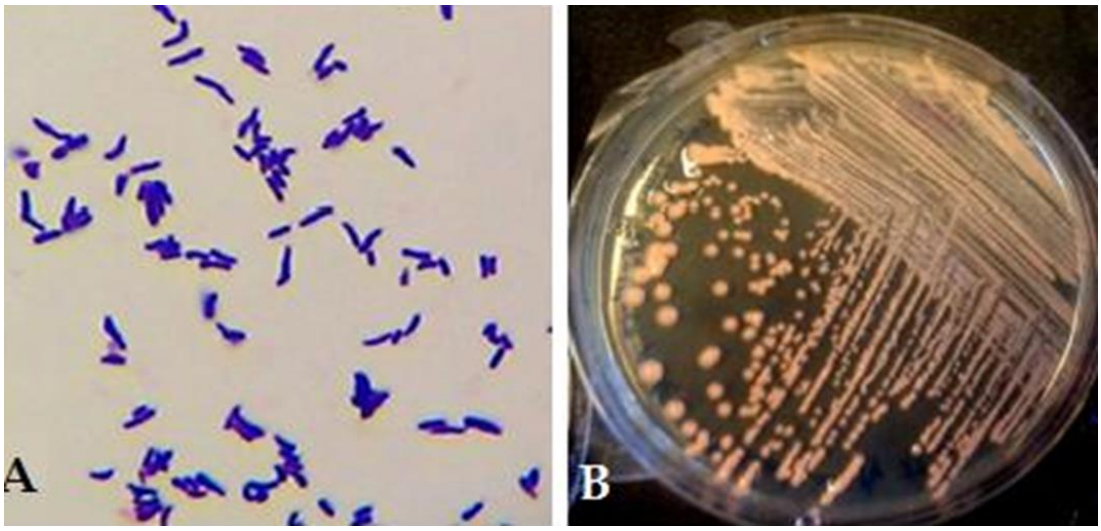

Figure 2. KCHXC3 Gram stain (A) and mucoid colonies showing light pink pigmentation (B)[5]. Gram stain indicates that KCHXC3 is a Gram-positive rod.

KCHXC3 extract partially purified by size-exclusion LH-20 column chromatography was used to test inhibitory activity against a variety of bacterial pathogens (**Table 1**). Results show that KCHXC3 produces an antibiotic-like compound that exhibits a broad spectrum of activity.

Table 1. Inhibitory compound produced by KCHXC3 against various bacterial pathogens[5].

Indicator strain	Zone of inhibition (mm) - diameter	Image	Gram (+) or Gram (-)
Escherichia coli	24		(-)
Bacillus subtilis	20		(+)
Enterobacter aerogenes	20		(-)
Klebsiella pneumonia	21		(-)
Citrobacter freundii	24		(-)
Salmonella arizonae	22		(-)

Indicator strain	Zone of inhibition (mm) - diameter	Image	Gram (+) or Gram (-)
Salmonella typhi	21		(-)
Pseudomonas aeruginosa	13		(-)
Staphylococcus aureus	19		(+)
Shigella sonnei	23		(-)
Shigella dysenteriae	30		(-)
Staphylococcus saprophyticus	16		(+)

In the table above, a control disk (right) soaked in ethyl acetate was used for all trials. Pathogenic indicator strains are categorized as Gram positive or negative. Positive activity is indicated by a large zone of inhibition. This broad spectrum of activity (**Table 1**), especially against Gram negative pathogens, is uncommon compared to previously known compounds produced by *Rhodococcus*[12].

The compound extracted was not sensitive to proteinase K and had no hemolytic activity on blood agar, suggesting that the compound is not a ribosomally produced protein[5]. Purification

of the compound also determined that the active compound is colorless in solution. Previous purification attempts of the compound produced by KCHXC3 indicate that the compound may be fairly large, over 1800 Daltons, and hydrophilic with an overall positive charge[5].

Research Objectives

The goal of this project is to purify and determine the structure of the unknown novel antibiotic-like compound produced by *Rhodococcus* strain KCHXC3. The purification of the compound is the primary focus of this project, although this is likely to give insights to the structure of the compound. With proposed methodological methods and current instruments at ETSU, it is possible to extract and purify the antibiotic-like compound produced by KCHXC3.

EXPERIMENTAL METHODS AND MATERIALS

Bacterial strains

All bacteria strains were obtained from Dr. Bert C. Lampson's research lab, Department of Health Sciences, East Tennessee State University. *Rhodococcus* strain KCHXC3 is a soil bacterium isolated near Kingsport, TN that produces the inhibitory compound of interest against a broad array of bacteria species, one of which is the Gram negative pathogen *Shigella sonnei*. This pathogen was used as an indicator of biological activity to confirm retention of the inhibitory compound throughout the extraction and purification process.

Culture Media

Rich Medium (RM)

Rich medium agar and broth was used to grow KCHXC3. The medium had the following components: 500 mL dH_2O, 5 g dextrose, 4 g nutrient broth, 0.25 g yeast extract, and 7.5 g of Bacto Agar. In order to make broth for the seed cultures of KCHXC3, the agar was left out.

Mueller-Hinton Medium (MH)

Mueller-Hinton media was used in order to perform an assay with *Shigella sonnei* in order to test compound retention. MH plates were made by dissolving 19.5 g of Difco™ Mueller-Hinton agar in 500 mL of dH_2O. The medium was autoclaved for 20 min and then transferred to sterile Petri dishes. Solidified plates were stored at 4 °C until used.

Lysogeny Broth (LB)

Lysogeny Broth was used in order to create a seed culture of *Shigella*, and for subsequent dilutions for each antimicrobial assay.

Solvents

All solvents used were HPLC grade obtained from Fisher Chemical unless otherwise stated. ≥99.9% ethyl acetate ($C_4H_8O_2$) was used for extraction and reconstitution of KCHXC3 compound of interest. For extraction trials and LH-20 chromatography, 99.9% methanol (CH_3OH) was used. For C18 HPLC column run, dH_2O in tandem with 99.9% acetonitrile (CH_3CN) was used. The NH_2 HPLC column run discussed used 98.5 % hexanes (C_6H_{14}) and Pharmco 200 proof ethyl alcohol (C_2H_5OH). Washing the NH_2 HPLC column was done with ≥99.5% acetone (C_3H_6O), acetonitrile, and hexane. Fraction was dissolved in deuterated chloroform ($CDCl_3$) for structural study.

Obtaining the Antibiotic-Like Compound

Growing the KCHXC3

A loop of KCHXC3 was inoculated into 2.5 mL of RM broth and grown in a 28 °C bath for 24 hours. This seed culture was then used to inoculate 150 mm RM agar plates. These plates were grown at room temperature for 2 weeks, and then put into a 4 °C refrigerator for 3 months.

Extraction of Compound from KCHXC3

After growing, the bacteria were washed off of the agar plates. The agar was then cut into approximately 2 cm x 2 cm chips and soaked in 100 mL of ethyl acetate per every half plate. After 24 hours of stirring, the agar chips were removed from solution and discarded. The resulting solution was pooled and left to dry for another 24 hours. The solid left after drying was dissolved in the minimum amount of ethyl acetate and stored for future purification. Other extraction trials were performed in the same manner utilizing methanol to confirm the best solvent to obtain the inhibitory compound (**Figure 3**).

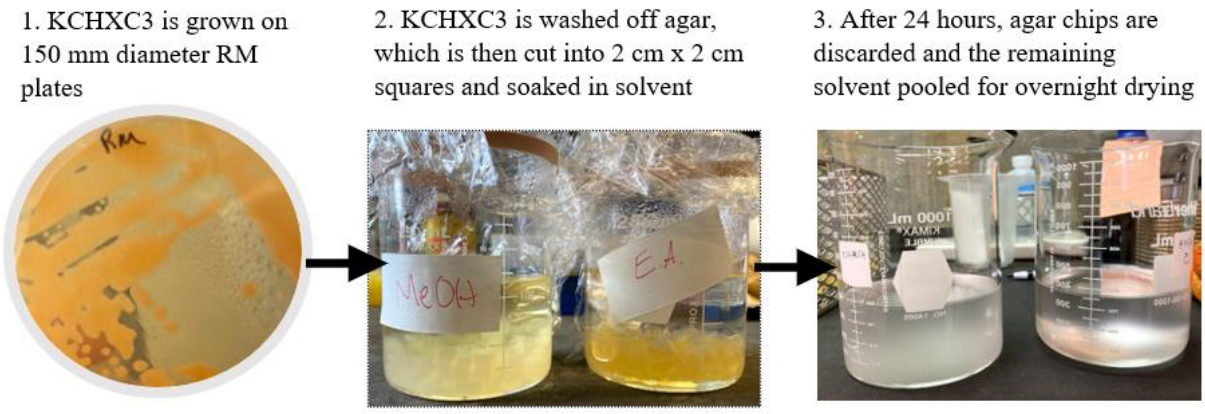

Figure 3. Extraction of compound from KCHXC3. Each 250 mL beaker had one standard size plate or 0.5 large plate in 100 mL of ethyl acetate.

Antimicrobial Activity Test

Shigella sonnei was inoculated into 2 ml of LB broth and grown at 37 °C for 24 hours. It was then diluted to 0.5 McFarland turbidity standard. A cotton swab saturated with this seed culture was then used to inoculate a 37 °C warmed 100 mm diameter MH plate. 50 µL total of extract dissolved in ethyl acetate was applied onto absorbent paper disks in 25 µL increments and allowed to dry. The disks were made using a standard hole punch with Whatman Blotting Paper GB004. These disks were then added to the MH plate with the *Shigella sonnei*. After 24

hours of growing, the assay was checked for positive activity, indicated by a nice large zone of inhibited growth. This was done after initial extraction and throughout the purification process to ensure retention of the antimicrobial compound.

Chromatography Methods

LH-20 Size Exclusion

In preparation for size exclusion chromatography, the crude extract from multiple rounds of growth plates was pooled and dissolved in a minimum amount of 1:1 methanol: ethyl acetate solvent. This solution was then filtered through 13 mm diameter, 0.45 µm pore size Millex®-HP syringe filter to remove large particles left from agar extraction. This was then added to the approximately 200 mL volume size exclusion column with LH-20 stationary phase and MeOH mobile phase. Flow rate was set to approximately 8 ml/min, and 40 mL fractions were taken every 5 minutes and then tested for activity.

High-Performance Liquid Chromatography (HPLC) Chromatography

HPLC Chromatography is useful for detecting small quantities of analyte that is common for natural products. Shortcomings of HPLC are that it requires a large amount of solvent, making it fairly costly. HPLC can also have low sensitivity for certain compounds, meaning that it may not detect all of the compounds within a sample[12]. HPLC utilizes high pressure to push the mobile phase through the stationary phase, resulting in separation of analyte. A UV-Vis detector is then used to measure absorbance of the solution, resulting in peaks on the chromatogram. This current work utilizes a Shimadzu Prominence HPLC instrument equipped with a solvent system, detector, controller, manual injector, and column.

Solvent System. The HPLC instrument was equipped with a two solvent system labeled solvent A and solvent B. Two separate pumps (Shimadzu LC-10AS) were used in order to vary

the concentrations of the two solvents into the column. The ratio of the solvents was kept the same for isocratic methods but was changed for gradient methods. The maximum pressure allowed for the column was 4000 psi.

Vacuum Degassing. Because the presence of air bubbles results in peaks on the chromatogram, degassing of the solvents is necessary before HPLC. Solvents were placed in an HPLC solvent container with a stir bar and connected to a Buchi V-700 Vacuum Pump. The solvent was then stirred on high speed while the vacuum was on until there were no more small air bubbles, which was about 10-15 minutes.

UV-Vis detector. A UV-Vis SPD-10A Shimadzu detector was used in order to measure absorbance in the HPLC system. The highly sensitive (10^{-10} – 10^{-11}g analyte/s) detector is essential in order to quantify the small concentration of analyte present. Normal wavelengths used for UV-Vis of organic compounds range from 210-254 nm. Hydrocarbons absorb well at 254 nm; however, many organic compounds also absorb around 225 nm. Peptide bonds also absorb light in this range[13].

Analytical Columns. Besides changing the solvents, utilizing a different column for HPLC purification is the best way to get better separation between peaks in the extract. The polarity of different column stationary phases allows for the analyte to be retained in the column longer, while other useless cellular debris can flow quickly through the column. In these trials, a standard C18 column, polar C18 column, and a NH_2 column were used to attempt purification. Notable trials involved the C18 column and the NH_2 column (**Figure 4**).

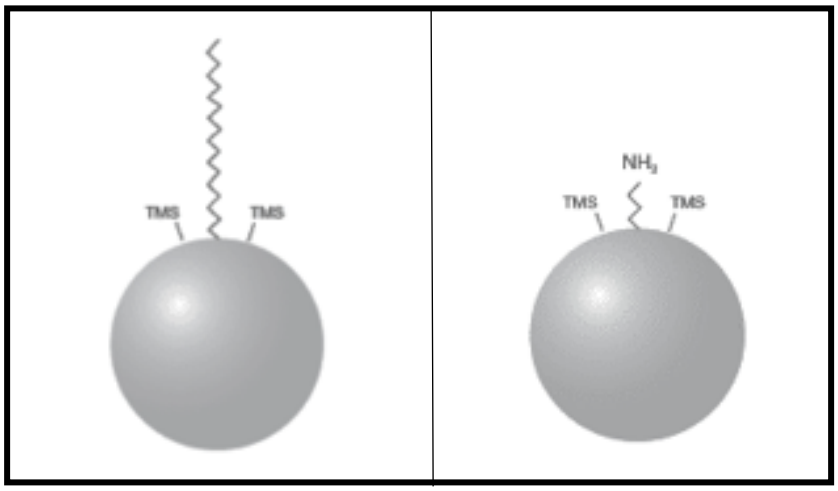

Figure 4. HPLC stationary phases in the C18 (left) and NH_2 (right) analytical columns. TMS stands for trimethylsilyl, which is commonly used to line particles in HPLC columns[14].

Methods Used for Partial Purification. Initial purification attempts were performed utilizing a Kinetix 150 x 4.6 mm C18 column. Most trials utilized water and acetonitrile (ACN), as seen in the notable trial below (**Figure 5**). In this gradient method, the flow rate was set to 1 mL/min and the detector was at 225 nm.

Time (min)	ACN Concentration (%)
10.20	1
40.20	30
42.20	1
60.20	1

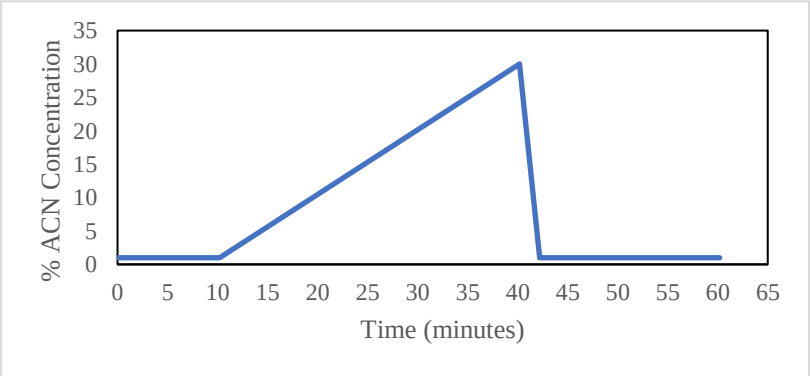

Figure 5. Change in ACN gradient for C18 column run. Pumps kept the concentration of ACN at 1% for first 10 minutes, and then increased the concentration to 30% the next 30 minutes before returning to 1%.

Due to previous unsuccessful attempts to purify with a standard nonpolar C18 and a polar C18 column, normal-phase chromatographic purification was attempted utilizing a Luna Omega 150

x 4.6 mm NH_2 HPLC column. One notable trial utilized both hexane and ethanol in a gradient method with a flow rate of 3 mL/min, with the detector set at 225 nm (**Figure 6**).

Time (min)	Ethanol Concentration (%)
2.20	2
5.20	15
15.20	30
18.20	98
28.20	98
30.20	2

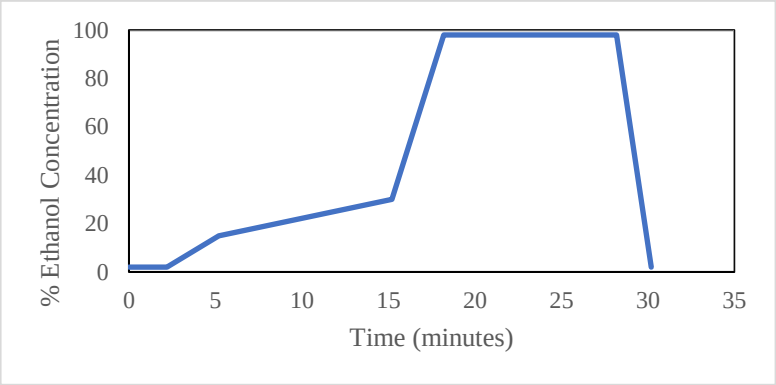

Figure 6. Gradient method for changing ethanol concentration for NH_2 column run. Rate of change in ethanol concentration fluctuated throughout the method before returning to 2% ethanol, 98% hexane for the next run.

Washing the NH_2 HPLC column was done utilizing 1 ACN: 1 hexane: 3 acetone mixture in an isocratic method at a 3 mL/min flow rate.

Structural Analysis of Crude Product After Washing

H-NMR.

A 400 MHz Oxford AS400 Magnet NMR was used in order to determine possible functional groups on the active compound and qualitatively determine purity. A major limitation for NMR is the concentration of analyte needed for accurate quantification,[15] which limits its usefulness for natural product research due to very low concentration of analyte. After drying under a vacuum, the fraction studied was dissolved in deuterated chloroform and put into an NMR tube. An NMR spectrum was generated after 180 scans of the sample.

Gas-Chromatography/Mass Spectrometry (GC-MS).

A Shimadzu GCMS-QP2010 Plus Gas Chromatographic Mass Spectrometer was used in order to attempt to determine the structure and purity of the crude product. After HPLC, 5 µL of sample dissolved in the minimum amount of ethyl acetate was injected into the column. The

column was run for a total of 40 minutes with Helium carrier gas. Samples best analyzed by GC-MS are small, volatile molecules, possibly limiting the usefulness for quantifying larger natural products[16].

RESULTS AND DISCUSSION

A variation of extraction times and solvents were used; however, the best method for extraction was determined to be the one described above utilizing ethyl acetate. Overall, extract used for future purification and structural elucidation described was the result of 106.5 standard plates. This extract was combined and redissolved in minimum amound of ethyl acetate and methanol, and then put through a 0.45 mm, 13 mm filter before added to LH-20 column.

Results of LH-20 Chromatography

Half of the crude extract was added to the Sephadex LH-20 column with a methanol mobile phase. A vacuum was utilized to set a flow rate of approximately 3 mL/min through the column. Fractions were collected at every 40 mL through the column. These fractions were dried overnight and then reconstituted in 2 mL of ethyl acetate. 100 µL of this was added to disks for the antimicrobial assay (**Figure 7**).

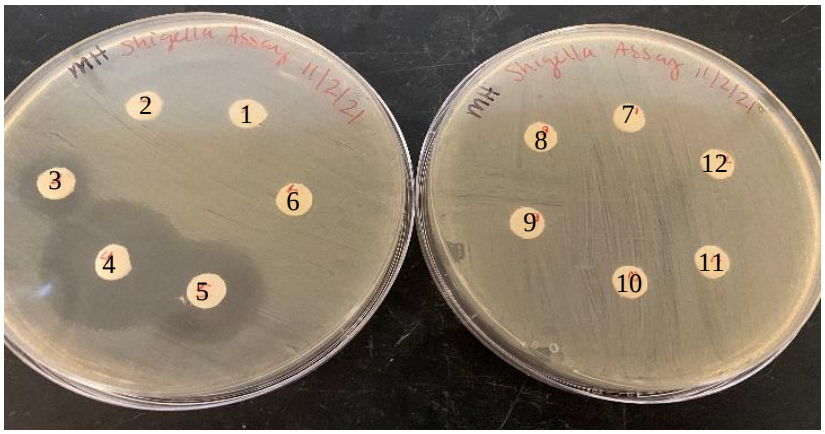

Figure 7. Assay results from size exclusion LH-20 chromatography. Activity is evident in fractions 3-5. This preliminary purification technique largely retained activity in the extract. The resulting solution after purification was used for HPLC.

Fraction 4 had the greatest zone of inhibition: 27.5 mm, while fraction 3 had a 13.5 mm zone and fraction 5 had a 17.0 mm zone. This was repeated with the other half of the crude extract, which produced similar results. Active fractions were combined and concentrated utilizing ethyl acetate.

HPLC Trials

C18 Column.

The best separation trial utilizing the C18 column consisted of a gradient method with ACN and water (**Figure 5**). Initial purification attempts utilizing a standard C18 column indicated the compound of interest was polar, which was consistent with past study[5]. Run KCH0023 Method 15 showed the best separation of peaks with injection of 100 µL of crude extract (**Figure 8**). Crude extract consisted of 6 100 mm diameter plates and 2 150 mm diameter plates, approximately 12 standard plates in total.

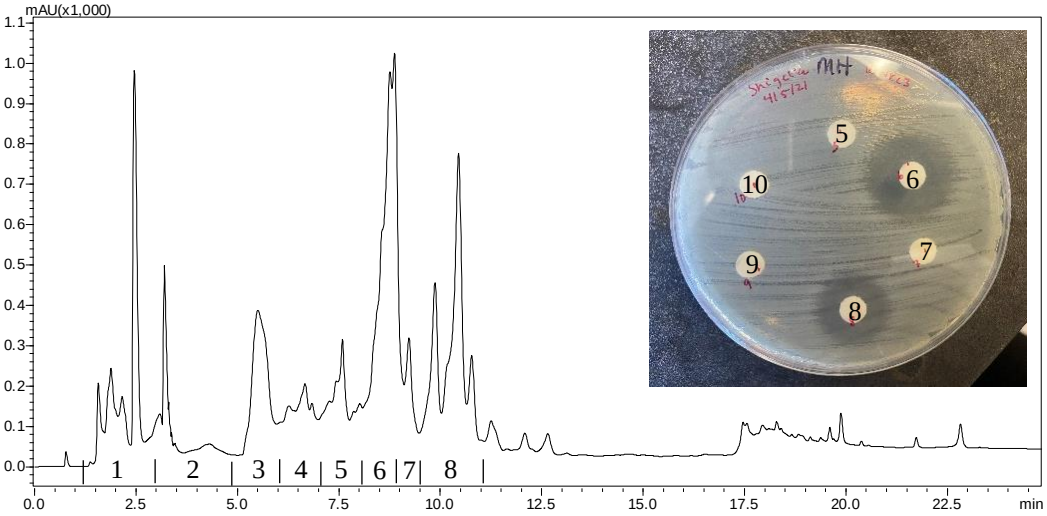

Figure 8. HPLC chromatogram of crude extract purification utilizing a C18 column. Active fractions are visible in the 6 and 8 fractions. Fractions 1-8 are marked on chromatogram.

As evident above, this HPLC run revealed two active compounds eluding from the column at different times. This indicates that KCHXC3 produces at least two antibiotic-like

compounds. Fraction 6 produced a zone of inhibition of 17.0 mm, and fraction 8 produced a 22.0 mm zone. These compounds are most likely similar in structure due to similar elution time, which is common in biosynthetic pathways. Further purification attempts were performed with a more polar column, to better match the polarity of the antibiotic-like compound.

NH_2 Column.

Purification utilizing an NH_2 column was used to hold the compound for longer to allow cellular debris to flow through. 100 µL of extract after LH-20 purification was injected into the HPLC. Run KCHa23 (**Figure 9**) utilized a gradient method with hexane and increasing ethanol solvent (**Figure 6**). The flow rate was 3 mL/min, and the UV detector was set to 225 nm.

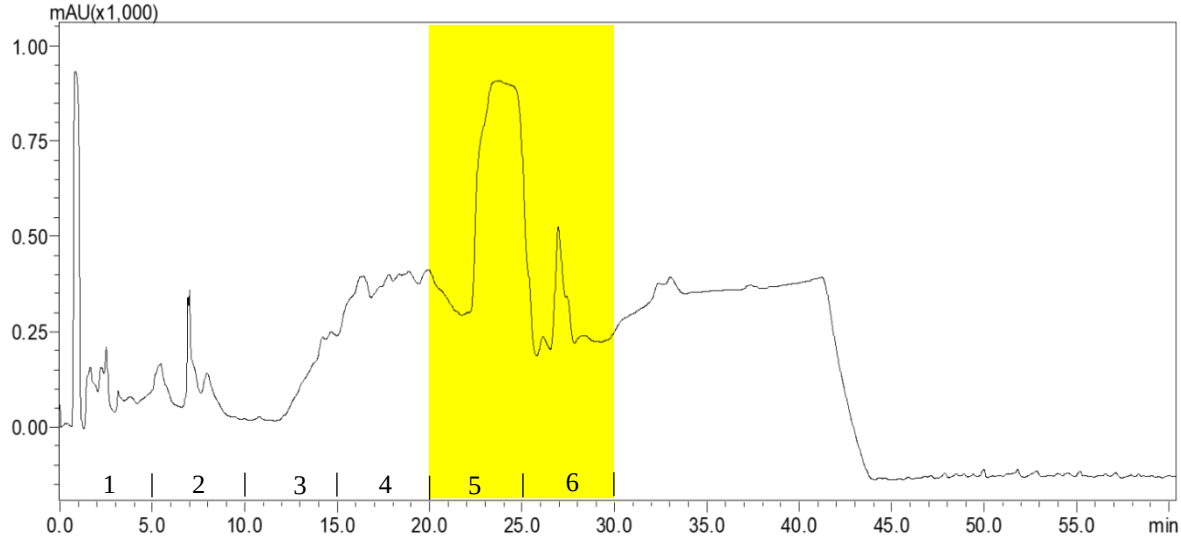

Figure 9. HPLC Run KCHa23 with NH_2 column. Yellow highlighted region showed activity against *Shigella sonnei* and produced a white precipitate after drying. First six fractions are shown on the chromatogram.

Fractions were taken every 5 minutes for a total of 40 minutes. Interestingly, one of the active fractions after evaporation produced a white, needle-like precipitate (**Figure 10**).

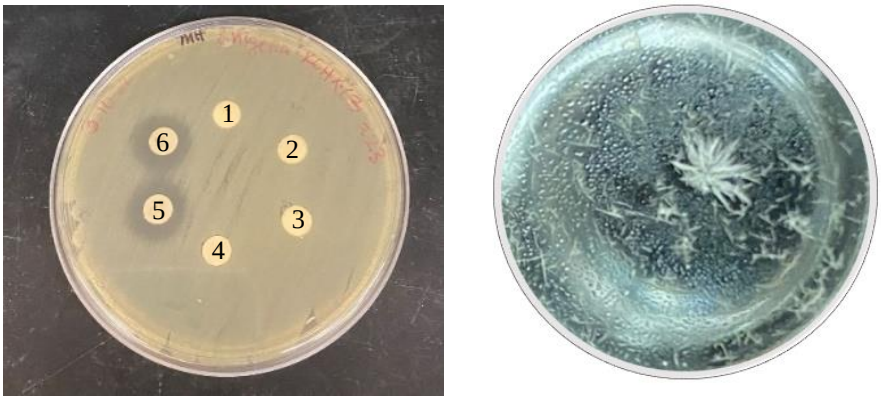

Figure 10. Active fractions from NH_2 column run KCHa23 (left**) and crystal-like precipitate from fraction 5** (right)**.** Precipitate may indicate some sort of pre-purification.

The active fractions 5 and 6 had zones of 16.0 and 13.5 mm in diameter, respectively. These fractions are highlighted in yellow on the chromatogram seen above. The crystal-like solid active fraction eluded at a solvent mixture of 16% ethanol and 84% hexane. This may be evidence of pre-purification of the active compound. More trials need to be conducted in order to get more of this solid to then be used in another round of purification for HPLC. It is unlikely that the precipitate is a pure compound due how broad the peak is on the HPLC chromatogram.

Structural studies

H- NMR. Proton NMR was run on the sample obtained by washing the NH_2 column with 1:1:3 acetonitrile: hexane: acetone solvent. This was done because none of the fractions from run KCHa10 to KCHa18 had activity. Washing with this solvent resulting in a white solid which is partially pictured below with an estimated 1 mL total of crude extract after LH-20 purification (**Figure 11**).

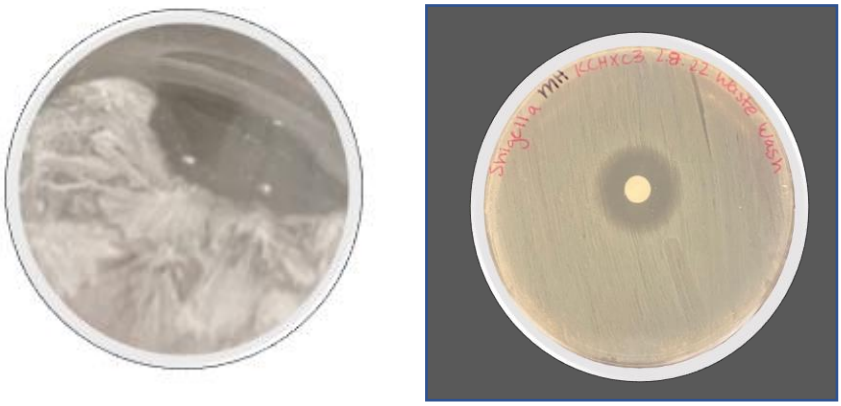

Figure 11. Vial with white crystals that washed off column (left) **and resulting *Shigella* activity** (right). The assay indicates that the vial shown has a high concentration of compound of interest, possibly visibly confirmed by white crystal-like precipitate.

Assay confirming activity was performed utilizing 200 µL 1:1 methanol and ethyl acetate. 50 µL of this was added to the disk, which resulted in a large 23.5 mm zone. This indicates that the extract in the vial is probably relatively concentrated. This extract was dissolved in 2 mL of deuterated chloroform and scanned 180 times in the H-NMR (**Figure 12**).

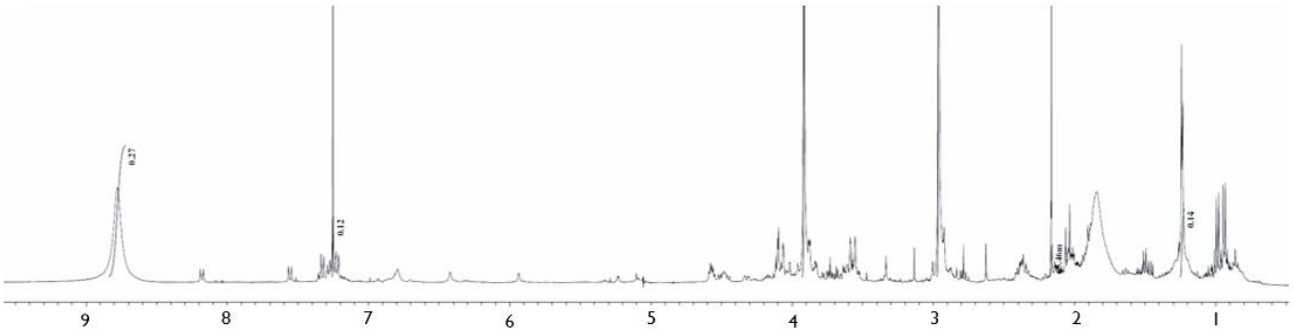

Figure 12. H-NMR of crude white solid that came off of HPLC. Peaks of interest are small peaks downfield in spectrum. Larger peaks are attributed to solvent.

It should be noted that the large peaks in the spectrum are most likely residual solvent peaks and are not related to the compound of interest. The spectrum shows many peaks around 7-8 ppm, indicating the presence of aromatic groups. There are also some smaller singlet peaks from the 5-7 ppm range that also indicate vinylic hydrogens. It cannot be confirmed that all of even the

smaller peaks are attributed to the compound of interest.

GC-MS. The same concentrated vial used for NMR was also used for GC-MS analysis. The precipitate was dissolved in 2 mL of 1:1 ethyl acetate: methanol solvent. 5 µL of this was injected into the column, which ran for 40 minutes total. The chromatogram only had peaks from 7-16 minutes (**Figure 13**).

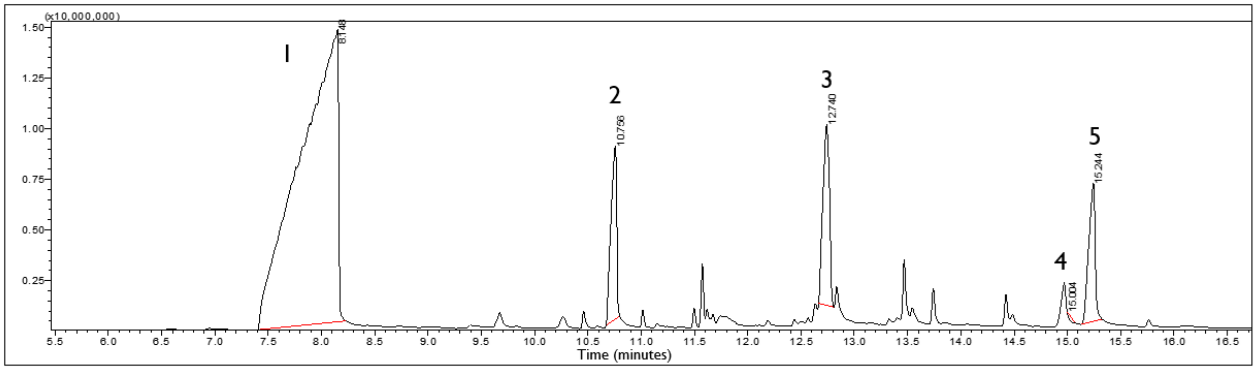

Figure 13. GC-MS results of white crystalline-like solid vial. 5 major peaks analyzed may indicate the partial structure of the compound of interest.

Of the multiple peaks in the chromatogram, five peaks were of interest. The presence of a large shoulder in the chromatogram could indicate that there are multiple compounds in that peak, so the predicted structure may not be very accurate. The peaks identified resemble amino acids bonded together (**Figure 14**), which indicates that the active compound may be a peptide.

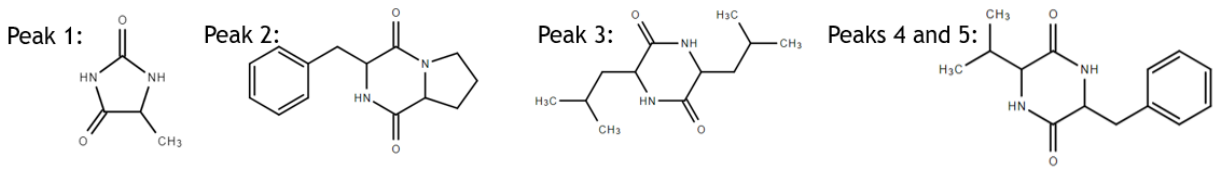

Figure 14. Major peaks identified on GC-MS chromatogram from active vial. Peaks shown resemble amino acids, which indicates that the compound of interest may be a peptide.

Peak 2 consists of a phenylalanine bonded to a leucine. Peak 3 appears to consist of two leucine residues. Peak 4 and 5 consists of a valine and phenylalanine residue. Peak 1 structure

was similar to urea. All structures are estimated by the GC-MS system and may not be accurate. It should also be noted that the compound of interest is probably not just one of these peaks, and the chromatogram may not show all of the structure of the compound if it is a larger, complex molecule.

CONCLUSIONS AND FUTURE WORK

The novel bacterium *Rhodococcus* strain KCHXC3 isolated from soil produces a broad-spectrum antibacterial compound. Purification through LH-20 chromatography and then HPLC NH_2 column chromatography resulted in an impure mixture of compounds that retained activity against the indicator strain *Shigella sonnei*. Purification by HPLC resulted in a clear crystalline-like active solid. This may be indicative of pre-purification of the compound. Structural analysis of the crude product shows that it contains a mixture of compounds. GC-MS and NMR indicate that presence of aromatic rings and amino acids on the compound, indicating that the unknown compound may be a peptide. Past work showed that the compound was not a ribosomally produced protein[5]. This information points to the possibility that the active compound produced by KCHXC3 is a nonribosomal peptide. Future work should focus on producing at least 1 mg of pure compound for structure elucidation study in order to identify the active antibiotic-like compound produced by KCHXC3.

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