Extracts from Soil Samples around Pennsylvania Exhibit Potent Antibacterial Properties against *Bacillus anthracis*

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ABSTRACT
Deadly bacterial infections such as anthrax continue to pose a significant threat to human health worldwide. This disease is caused by *Bacillus anthracis,* which the CDC classifies as a Tier 1 biological agent due to its ability to form spores resistant to severe environmental stress conditions, including antibiotics. Identifying new antibiotics against this pathogen is therefore crucial for combatting anthrax infections. In this research, crude extracts from Pennsylvania soil were purified using various chromatography methods, resulting in natural products, which were assessed for their antimicrobial properties. After performing minimum inhibitory and bactericidal concentration assays, two compounds, AMS002 and AMS003, exhibited growth inhibitory and killing activity against *B. anthracis* at 0.8 mg/ml and 0.2 mg/ml, respectively. Both compounds inhibited greater than 80% of protein synthesis relative to the control samples in cell-based and *in-vitro* fluorescent reporter assays, suggesting that these compounds may target the bacterial protein synthesis pathway as their primary mode of action. The novelty of this discovery is vital due to the resistant nature of *B. anthracis* spores and their use as a potential weapon in bioterrorism.

KEYWORDS
Antibiotics; Resistance; Natural Products; Chromatography; *B. anthracis*; Minimum Inhibitory Assay; Minimum Bactericidal Assay; Reporter Assays; Course-based Undergraduate Research Experience (CURE)

INTRODUCTION

Antibiotic resistance continues to pose a significant threat to human health worldwide. There is a substantial shortage of drugs that can fight resistant bacterial infections due to the decline in research aimed at developing novel antibiotics.[1] Over one million people die from bacterial infections every year, including 200,000 newborn babies.[2,3] These alarming statistics are projected to increase to ten million annual fatalities by 2050, creating a significant problem that requires urgent resolution.[2,3]

Pathogenic bacteria develop resistance to antibiotics using different strategies, which enable them to survive and replicate in the presence of antibiotics. Common resistance mechanisms include increased efflux systems that remove the drug from bacterial cells, alterations to the drug's molecular target, and the production of drug-altering enzymes.[4] Antibiotics are critical in providing therapy for infections, but their overuse and misuse contribute to the rise of resistant strains. According to classifications by the Centers for Disease Control and Prevention (CDC), some of the highest risk priority pathogens include *Bacillus anthracis,* vancomycin-resistant *Enterococci* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Clostridium difficile*.[5] The threat posed by these pathogens is accompanied by urgent calls from the CDC for increased drug discovery-related research to identify novel antibiotic candidates to treat deadly infections caused by these pathogens.

Following the discovery of penicillin in 1928, scientists focused on synthesizing derivatives of previously existing antibiotics to combat resistance during the medicinal chemistry era.[6] Since 2017, the Food and Drug Administration (FDA) has approved eight new antibiotics for combating antibiotic-resistant infections, most of which are derivatives of traditional antibiotics.[7] For example, Eravacycline, approved by the FDA in 2018, is a fully synthetic antibacterial agent containing two chemical modifications from tigecycline, a broad-spectrum tetracycline antibiotic.[8] Using derivatized analogs to treat bacterial infections quickly led to resistance, making such drugs ineffective.[9]

As resistant strains of bacteria persist, researchers have turned to natural products as a source of new antibiotics. The environment is a reliable resource for producing potential antimicrobial compounds because of the diversity of microorganisms within that ecosystem. Some microbes produce compounds with potent antimicrobial properties to compete with neighboring organisms for nutrients or fend off adversaries. For example, penicillin was discovered accidentally in 1928 by Sir Alexander Flemming from the fungal species *Penicillium chrysogenum.*[10] Similarly, actinomycin, pro-actinomycin, and streptothricin are potent antimicrobial compounds that vary in structure and function but were isolated from actinomycetes, a soil microbe.[11] Other soil-dwelling bacteria also produce natural products with therapeutic properties, making such an environment the ideal ecosystem for isolating compounds with antibacterial properties. In addition, the advent of sequencing technology also promoted scientists to proactively seek previously untapped biosynthetic pathways in soil microorganisms to produce antimicrobial molecules.[12]

Bacillus anthracis is a Gram-positive, spore-forming bacteria that lives naturally in the soil and causes anthrax infections.[13] Its pathogenicity is linked to the release of three main factors including the lethal factor, edema factor, and protective antigen.[14] The spores formed by *B. anthracis* are highly resistant to temperature, pressure, radiation, and chemical agents such as disinfectants.[14] When inhaled, these spores germinate within the macrophages of the lungs and are carried to the lymph nodes where infection occurs.[15] Because this infection mode occurs within the macrophages, the host's innate immune system does not respond, allowing the bacteria to grow while the host's adaptive immune response develops.[16] The active synthesis and production of virulence factors encoded by plasmids pXO1 and pXO2 from *B. anthracis* within the macrophage inactivates the MAP Kinase signaling pathway, releases adenosine monophosphate, and binds toxin receptors in the host cells.[17–19] These actions cause symptoms in the host, including localized pneumonia, fever, and other flu-like symptoms, which are commonly misdiagnosed.[20] Although recovery rates have increased over the years, inhalation anthrax remains the deadliest form of anthrax infection. Other types of infections by this pathogen include cutaneous, gastrointestinal, and injectional infections, which, although not as lethal, can result in death without early or proper treatment.[21]

Due to its ability to produce spores, *B. anthracis* is considered a weapon of bioterrorism since these spores can be easily aerosolized to cause inhalation anthrax in large populations of people.[22] Despite antibiotic treatment, many people may suffer re-infection due to ungerminated spores surviving the initial treatment.[23] Because the continuation of antibiotic treatment risks the emergence of resistance, a vaccine against anthrax is often also a part of the treatment regimen as it decreases the chances of reinfection from ungerminated spores following an early treatment.[23,24] These reasons underscore the continued efforts to discover and develop novel antibiotics for combating *B. anthracis* infections.

As part of the Course-based Undergraduate Research Experience (CURE) at Penn State[25], we isolated organic compounds from soil samples collected from two locations in Central Pennsylvania to extract and identify natural products exhibiting antibacterial activity against several bacterial species. Initial evaluation of growth inhibitory properties of the crude extracts demonstrated significant activity against Gram-positive bacteria. We continued to characterize the activity of the isolated compounds. Two natural products, AMS002 and AMS003, inhibited the growth of *B. anthracis* at 0.8 and 0.2 mg/ml, respectively. These compounds also exhibited bactericidal properties against this pathogen at the same concentrations. We employed fluorescence and *luminescence*-based reporter assays to elucidate these two compounds' potential mechanism of action. We observed that they inhibited protein synthesis *in vitro* and within bacterial cells. Subsequent mass spectrometry analysis identified the molecular mass and potential identify of one of the promising compounds. This discovery may provide new molecules to add to the declining arsenal of compounds that treat deadly *B. anthracis* infections.

METHODS AND PROCEDURES

Description of strains used in this study

Strain or plasmid	Strain description
DH5α	Host strain for cloning
BL21 (DE3)	Host strain for protein expression
B. subtilis 168	Common laboratory strain
B. anthracis Sterne	Attenuated strain of *B. anthracis*
***E. coli* - Wild Type**	MG1655 (K12) strain containing the TolC efflux pump
E. coli* - Δ*tolC	MG1655 (K12) strain lacking the TolC efflux pump
P. aeruginosa	Strain PAO1 – common laboratory strain

Table 1. Bacterial strains used in the current study.

Soil Sample Collection

The locations where the soil samples were collected were based on areas prone to having a high biodiversity of microbes. The first soil sample was collected at the corner of Fox and Grants Road, Jersey Shore, PA. This sample originated from an Amish field with biannual fertilization using manure rather than an artificial fertilizer. This sample was selected based on the organic nature of manure fertilization for plant growth. Manure can act as a source of nutrients for microbial life forms, which potentially produce compounds to compete with common soil microbes, such as *B. anthracis*. The soil collected from this location was lighter in color and dry, likely due to the lack of rainfall in the weeks before collection. The second sample was collected about 150-200 feet from the banks of the Little Juniata River, Tryone, PA 16686. This location had dense vegetation with low levels of human activity, as human interaction can interfere with the natural soil ecosystem. Soil samples were collected by digging a small hole about 0.5m in diameter and about 15 cm deep. Samples were collected in gallon-sized plastic bags, taken back to the laboratory, and the bags were left open to allow the soil to dry completely prior to extraction.

Solvent Extraction of Natural Compounds

The soil samples were inspected visually, and large particulates, including rocks, leaves, and sticks, were manually removed. The samples were packed halfway into 1 L bottles, and about 500 ml of 100% ethyl acetate was added to the bottles in a chemical fume hood. The contents were mixed gently by inversion with occasional venting to prevent gas build-up inside the bottles. These samples were left to soak for 72 hours to maximize the extraction of small molecules.

Filtration and Solvent Evaporation to Isolate Crude Organic Compound Extracts

A Büchner funnel was placed onto a filtration flask connected to a vacuum source. A Whatman filter paper was placed on the Büchner funnel and soaked with 100% ethyl acetate. Vacuum was applied, and pre-soaked soil samples were filtered. The filtrate was transferred into a round-bottomed flask, and the solvent was removed using a rotary evaporator under reduced pressure at 52 °C. The rotation speed of the instrument was increased gradually to prevent solvent bumping. This process was repeated until all the solvents were removed to yield the crude natural product extract, hereon referred to as the crude extract.

Crude Extract Analysis by Thin-Layer Chromatography (TLC)

The profile of compounds available in the crude extract was assessed by TLC analysis. Pre-cut glass TLC plates coated with a fluorescent indicator detectable with 254 nm or 366 nm wavelength (Millipore-Sigma, Burlington, MA) were used. A plate was marked with a pencil 1 cm from the end of the plate to outline the origin of sample spotting. A small amount of the crude extract was dissolved in the selected mobile phase and spotted onto the TLC plate using thin capillary tubes. The plate was dried and placed in a TLC chamber containing the same mobile phase at the level below the baseline. The mobile phases used were 50% dichloromethane with 50% hexanes, 80% dichloromethane with 20% hexanes, 100% dichloromethane, 100% dichloromethane with a drop of methanol, and 90% dichloromethane with 10% methanol. The solvent traveled by capillary action, and the plate was removed and dried after the mobile phase reached the plate's upper end. The crude profile was visualized with ultraviolet light using a hand-held dual-wavelength UV lamp. This technique determined the solvent system used to purify the crude extract.

Purification of Individual Compounds by Column Chromatography

A silica gel slurry in 100% dichloromethane was poured into a 60 mL chromatography column and allowed to pack by gravity. A thin layer of sand was added on top to protect the silica layer from disturbance. The crude extract was dissolved in minimal amounts of the mobile phase and loaded onto the column, forming a thin layer on top of the sand. This layer was allowed to be absorbed into the silica before more of the mobile phase was slowly added to the layer of sand, ensuring that the top layer of silica was not disturbed. The mobile phase was continually added to elute the compounds in 3 ml fractions. TLC grids were created by spotting the contents of each fraction onto a TLC plate, which was visualized using UV light (254/366 nm) to determine the number of UV-absorbing compounds. Fractions with similar TLC profiles were grouped, pooled, and concentrated for biological analyses or characterization. Because multiple compounds were eluted within the same fractions, further purification to separate all compounds in the crude was achieved using a CombiFlash flash chromatography system (Teledyne ISCO). Crude soil extracts were loaded onto pre-packed columns containing 40 g of silica gel and purified using the same solvent system determined from the TLC analyses. The automated system identified compounds using a UV detector (254 nm) and isolated them based on their polarity. The samples were concentrated on a rotary evaporator to produce five fractions: AMS001-AMS005.

*Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assay*s

A bacterial culture (5 ml) in lysogeny broth (LB) medium was incubated at 37 °C while shaking overnight. The optical density of a 10-fold diluted stationary phase culture was determined at 600 nm on the Spectronic Genesys 5 Spectrophotometer. A bulk working culture was prepared by diluting the overnight culture to an initial OD_{600nm} of 0.002 (lag phase) for subsequent antibacterial growth inhibitory assays. For microplate assays, 96 µl of LB broth was transferred to the first row of wells on a 96-well plate while 50 µl was added to the remaining wells on the plate. Concentrated aliquots (4 µl) of the purified compounds, or the necessary controls [chloramphenicol (positive control), kanamycin (positive control), and DMSO (negative control)], were

added to the first row of wells in triplicate. Two-fold serial dilutions were performed down each column by transferring 50 µl of the mixture from the first row to the second one. This procedure was repeated until the last row, when 50 µl was discarded, leaving all the wells with equal volumes. A 50 µl aliquot of the diluted culture was then added to each well, and the microplates were incubated at 37 °C for 24 h. A qualitative assessment of the plate was performed by visual inspection to determine the MIC values for the corresponding test compounds. The MIC was recorded as the lowest concentration of a test compound lacking visible growth. Further analyses were performed by obtaining the optical densities of samples in the 96-well plate on a SpectraMax i3 Microplate reader (Molecular Devices). The dose response data were plotted relative to the DMSO control. The bactericidal properties of the test compounds were determined from the microplate assays used to evaluate the MICs. Following visual determination of the MICs, spot cultures were created by transferring 5 µl from sample wells in the microplate lacking bacterial growth for each corresponding test compound onto the sectioned quadrants of LB-agar plates. Similar experiments were performed for the controls, and the plates were incubated overnight at 37 °C.

Disk Diffusion Assay

Using aseptic techniques, a saturated liquid culture of bacteria was spread onto an LB plate using a sterile cotton swab and allowed to dry at room temperature. Sterile 6.5 mm disks were soaked in 20 µl of purified natural products, spectinomycin (SPEC), or DMSO. These disks were placed in sectioned quadrants on LB agar plates and incubated at 37°C for 24 hours. At the end of the incubation period, the diameter of the clearance zones for each corresponding sample was measured and used to assess the growth inhibitory effect.

Fluorescence and Luminescence Reporter Strain Development

A pET28a cloning vector containing a gene encoding the Yellow Fluorescent Protein (YFP) was transformed into *E. coli* BL21 (DE3). 100 µl of the cells were plated on kanamycin-containing selective LB agar medium and incubated overnight at 37 °C. Colonies that grew on the selective media were restruck and grown in liquid broth containing kanamycin. The luminescence reporter strain was prepared similarly by transforming a pET28-nano-luc plasmid containing the nano luciferase gene into BL21 (DE3) cells. Transformants containing the pET28a-*nano-luc* were used in cell-based luminescence screening assays to assess translation inhibitors.

Cell-based Fluorescence and Luminescence Translation Reporter Assays

The fluorescence reporter assays were set up by first transferring 96 µl of LB to the top row of wells of a black 96-well plate (Corning, New York), and 50 µl was added to the remaining wells. 4 µl of the purified test compounds, or the necessary controls [chloramphenicol (positive control), and DMSO (negative control)], were then added to the top row in triplicate. Two-fold serial dilutions were performed from rows A – H by transferring 50 µl of culture from the first row to the second one. This procedure was repeated, and 50 µl was extracted from the last rows of the plate. Subsequently, an overnight culture of BL21 (DE3)-pET28a-*yfp* was diluted to an OD_{600} of 0.4 in LB broth containing 50 µg/ml of kanamycin for plasmid maintenance. Isopropyl ß-D-1-thiogalactopyranoside IPTG (1 mM) was then added to the culture to initiate the expression of YFP. 50 µl of this culture was added to each well, and the plate was incubated for 2 hours at 37 °C while shaking at 300 rpm. Fluorescence readings were then obtained to detect the translation of YFP using the SpectraMax i3 (Molecular Devices, $\lambda\ Ex_{468\ nm}/\lambda\ Em_{530\ nm}$). The data was analyzed using GraphPad Prism 9. The luminescence-based reporter assays were set up in a similar manner to the assay above using the BL21 (DE3)-pET28a-nano-luc cells.

Preparation of the Nano-luciferase mRNA Transcript

Preparation of the *nano-luc* mRNA for use as a template in the *in vitro* translation reactions was done by *in vitro* transcription. 1 ml of a 5X high-yield transcription buffer was prepared by mixing 500 µl 1 M HEPES (pH 7.5), 200 µl 1M dithiothreitol (DTT), 100 µl 1M $MgCl_2$, 70 µl 2mg/ml bovine serum albumin, 130 µl RNase-free water. The transcription reaction was initiated by combining 20 µl of 5X transcription buffer, 1.0 µl 1 M DTT, 30 µl 100 mM NTP mixture, 0.2 µg of pET28a-nano-luc plasmid, 0.25 µl inorganic pyrophosphatase (0.03 units), 5 µl T7 RNA polymerase (250 units) (New England Biolabs), 44 µl RNase-free water. This mixture was incubated for 3 h at 37 °C and treated with RNase-free DNase I to degrade the DNA template. Following the inactivation of the enzyme at 75 °C for 10 minutes, the sample was resolved on a denaturing 6 % urea-based acrylamide gel. The synthesized *nano-luc* mRNA was extracted and ethanol precipitated following standard protocols. This mRNA was used as a template in place of the *nano-luc* gene in an *in vitro* translation experiment, as described below.

In-vitro Luminescence-based Translation Assay.

A lysate of 50 ml *E. coli* BL21 (DE3) cells was prepared by sonication as previously described.[26] *In vitro* translation using the nano-Luciferase gene was accomplished by combining an *E. coli* BL21 (DE3) lysate (10 µl), freshly made polymix buffer (10 µl) (final reaction concentrations 5 mM HEPES pH 7.6, 5 mM NH_4Cl, 0.5 mM $CaCl_2$, 1.5 mm $MgCl_2$, 1 mM DTT, 8 mM putrescene, 2 mM ATP, 2 mM GTP, 1 mM CTP, 1 mM UTP, 0.3 mM each amino acid, 3 mg/ml *E. coli* tRNAs), pET28a-*nano-luc* plasmid or

nano-luc mRNA transcript (2.5 µl; ~10 ng), and 10 µl water. Finally, the corresponding inhibitors, DMSO as a negative control, and chloramphenicol as a positive control, were added to designated samples at (2 mg/mL, 0.2 mg/mL, and 0.02 mg/mL) and samples were incubated at 37 °C for 2 h. The Nano-Glo assay system (Promega) was used to detect the activity of the expressed luciferase enzyme in our reactions. Sample preparations were performed according to the manufacturer's instructions. The substrate was mixed with each sample in a 1:1 volume ratio, and luminescence was then recorded at 560 nm using the SpectraMax i3 microplate reader.

Mass Spectrometry and Compound Discoverer Analyses

Mass spectrometry analysis for ASM002 was performed at the Huck Life Sciences proteomic facility (Penn State University). The compound was dissolved in methanol and diluted to a final concentration of 10 µM. Samples were analyzed by liquid chromatography followed by electrospray ionization mass spectrometry in the positive mode (ESI-MS +) on a Waters Q-TOF Premier HPLC coupled instrument to obtain the exact mass and fragmentation profile of ASM002.[27] After obtaining the molar mass of the unknown compound, we used Compound Discoverer software (Thermo Fisher Scientific) to predict its potential molecular formula and structure.

RESULTS

Precise Collection Locations and Yield from Test Soil Samples

The crude extract obtained from the Amish field soil was named Crude 1, and the crude extract from the Little Juniata River was referred to as Crude 2 (**Table 2**).

Soil collection location	GPS location	Name	Antibacterial Compound
Fox and Grants Road, Jersey Shore, PA	Longitude: 41.146314; Latitude: -77.250216	Crude 1	ASM002
Little Juniata River, Tryone, PA	Longitude: 40.66476; Latitude: -78.21656	Crude 2	ASM003

Table 2. Summary details of the soil samples used in the current study.

Soil crude extract exhibits antibacterial properties against Gram-positive bacteria

Working stock solutions of 10 mg/ml of each crude extract were prepared in DMSO for these experiments. To determine the antibacterial activity of the crude extracts, a minimum inhibitory concentration assay was performed, where bacteria were grown in serially diluted concentrations of the crude extracts. The lowest concentration of the crude that could inhibit the growth of bacteria was considered the Minimum Inhibitory Concentration (MIC). Minimum inhibitory assays were performed initially with *Bacillus subtilis*, a Gram-positive species; *Escherichia coli*, a Gram-negative species; and an *E. coli ΔtolC* mutant (a deletion mutant lacking the multidrug efflux pump, *tolC*). The minimum inhibitory concentration (MIC) against *B. subtilis* was observed to be 0.49 mg/mL for crude 1 and 0.24 mg/ml for crude 2. The crude extracts did not display significant activity against *E. coli* and *E. coli ΔtolC*, with MICs greater than 3.92 mg/mL (**Figure 1, A**). This data suggests that the compounds in the crude extracts may not be able to penetrate the outer membrane of Gram-negative bacteria.

Since antibiotics can inhibit either growth or kill bacterial cells, we also evaluated the crude extracts for potential bactericidal properties against *B. subtilis*. 5 µL of culture from the MIC plates were plated onto LB agar plates and incubated overnight. The lowest concentration of the crude extract in which bacterial growth was not observed on the plates was considered the minimum bactericidal concentration (MBC). We recorded an MBC of 0.49 mg/ml for crude extract 1 and 0.24 mg/ml for crude 2, suggesting that they both contained bactericidal compounds. No MBC was observed against the *E. coli* and the *ΔtolC* mutant (**Figure 1, B**).

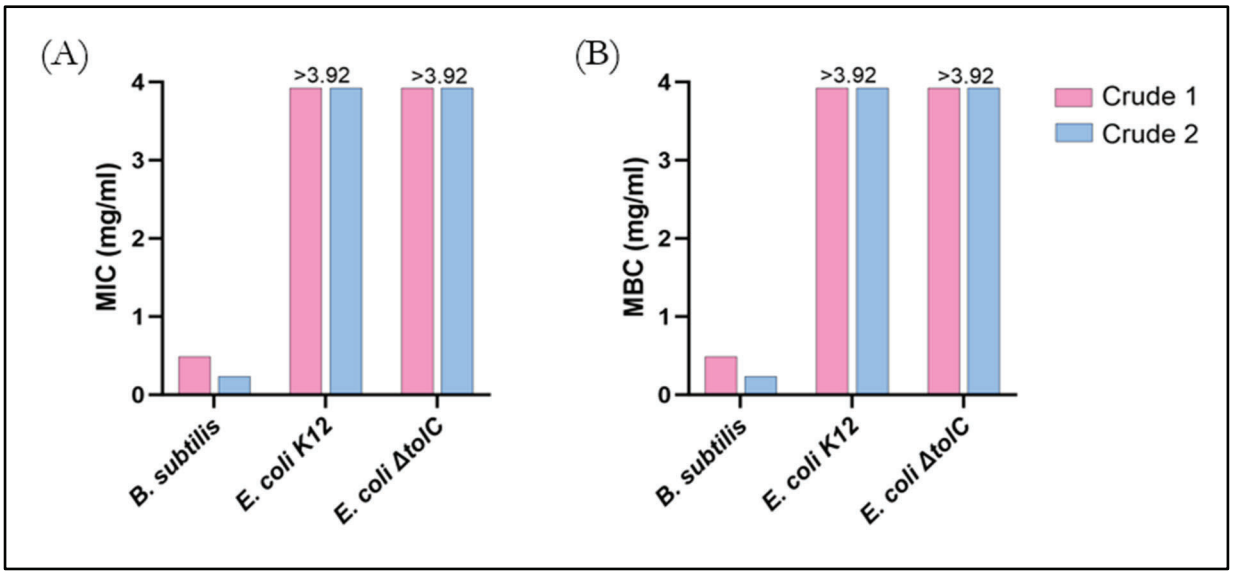

Figure 1. **Crude soil extracts showed bactericidal activity against *B. subtilis*.** **A**). Minimum inhibitory concentrations of crude extracts 1 and 2 against *B. subtilis, E. coli K12, and E. coli ⊿tolC (n=3; SD = 0).* **B**). Minimum bactericidal concentrations of crude extracts 1 and 2 against *B. subtilis, E. coli K12, and E. coli ⊿tolC (n=3; SD = 0).* SD: standard deviation.

Purification of crude extracts by column chromatography yields five compounds

The initial screens on the crude extracts indicated the presence of compounds with antibacterial properties among the mixture. We then isolated the organic compounds from the crude samples to test their individual antibacterial capabilities. First, thin-layer chromatography (TLC) was used to visualize the different compounds within the crude samples and identify the optimal mobile phase to isolate the compounds via column chromatography. Five individual compounds were observed using TLC in the crude extract derived from the Amish soil sample.

Column chromatography was used to separate the compounds in the crude extracts. For Crude 1, the column was run with 80% dichloromethane with 20% hexane before being increased to 100% dichloromethane to flush the most polar sample through the column. To separate the compounds in Crude 2, the column was started with a mobile phase of 90% hexane with 10% dichloromethane, and the polarity was increased to 100% dichloromethane. Then, the column was flushed using 50% dichloromethane with 50% methanol. During this process, the compounds were separated into fractions. Some isolated fractions, however, contained several compounds due to having very similar polarities; as a result, flash chromatography was performed to isolate the compounds with better resolution (**Figure 2, A**). Three compounds were isolated from crude 1 and two from crude 2 (**Figure 2, B**).

Isolated natural products display antibacterial properties against B. anthracis

Although we conducted our preliminary experiments using *B. subtilis*, the promising activity of the isolated compounds prompted us to transition to *B. anthracis,* which is among the most dangerous species of bacterial pathogens. The purified compounds were tested for activity against three pathogenic strains of bacteria: *B. anthracis, E. coli*, and *Pseudomonas aeruginosa*. Two compounds, AMS002 and AMS003, one from each crude extract, had inhibitory activity against *B. anthracis* at 0.8 mg/mL and 0.2 mg/mL, respectively. They did not show any significant growth inhibition activity against *E. coli* and *P. aeruginosa* (MICs > 0.8 mg/mL) (**Figure 3, A**). Experiments assessing these compounds' ability to kill *B. anthracis* cells revealed that they both possessed bactericidal properties at their respective MIC concentration (**Figure 3, B**). These results are consistent with the antibacterial effects observed from our crude extracts 1 and 2, in which only the Gram-positive bacteria such as *B. subtilis* was targeted. In contrast, Gram-negative bacteria such as *E. coli* and *P. aeruginosa* were not affected. All other isolated compounds did not show growth inhibitory activity (MICs > 0.8 mg/mL).

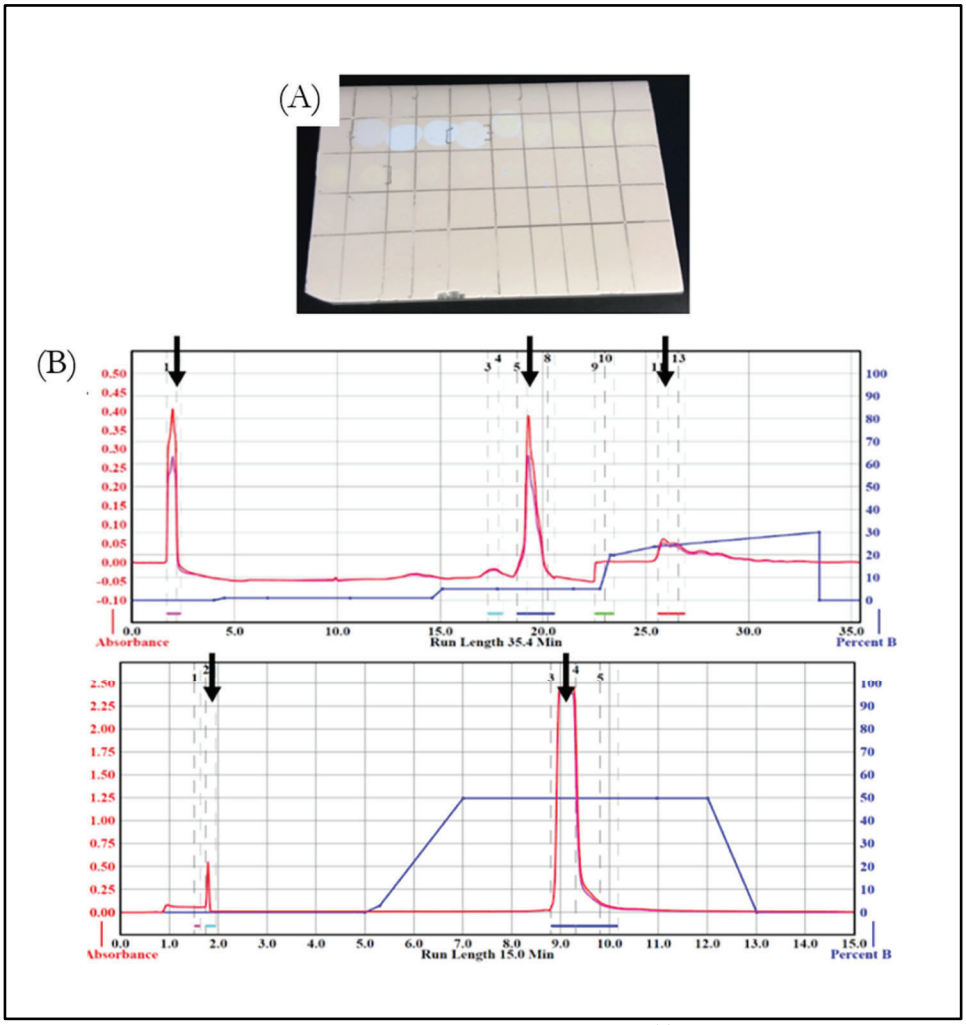

Figure 2. Purification and analysis of compounds using chromatographic methods. A). Sample TLC plate illustrating the separation strategy for crude samples and visualization under UV light. Tints of overlapping blue and green indicate fractions collected from column chromatography that were not pure. The experiment was run with a mobile phase containing 10% methanol in dichloromethane and visualized at 254 nm using a hand-held UV lamp. **B**). Crude extract purification by flash chromatography, resulting in three compounds from Crude 1 (top) and two from Crude 2 (bottom) (compounds indicated by arrows). Crude 1 (top) was purified using dichloromethane with an increasing gradient of methanol. Crude 2 (bottom) was purified using hexane with an increasing gradient of ethyl acetate. ASM002 eluted between 19 – 20 minutes (top chromatogram) while ASM003 eluted between 9 – 9.5 minutes (bottom chromatogram). NOTE: the red trace was collected at 254 nm and the pink trace at 366 nm. The blue trace indicates the concentration of the more polar solvent in the mobile phase.

We used disk diffusion assays as an alternative way to assess growth inhibition for the test compounds. Sterile disks were soaked with 5 mg/mL of AMS002 or AMS003. Bacterial lawn plates were prepared by spreading 200 µl of a *B. anthracis* culture (OD600 nm 0.3) on LB agar plates, and the disks were added on top of the lawn. The diameter of the clearance zones around the disks was measured (**Figure 3, C**). Zones of clearance for the AMS002 and AMS003 are 8.5 mm and 9.5 mm, respectively, compared to 17 mm for the positive control (**Figure 3, D**). The smaller zones of clearance observed in AMS002 and AMS003 may be due to the lack of mobility in diffusing through the agar plate. When determining the growth of bacteria as a dose-response of the inhibitors, inhibition of bacterial growth at various concentrations of AMS002 and AMS003 revealed half maximal inhibitory concentration (IC_{50}) between 10 and 50 µg/ml; data was normalized relative to the untreated controls to achieve dose-response curves (**Figure 3, E and F**).

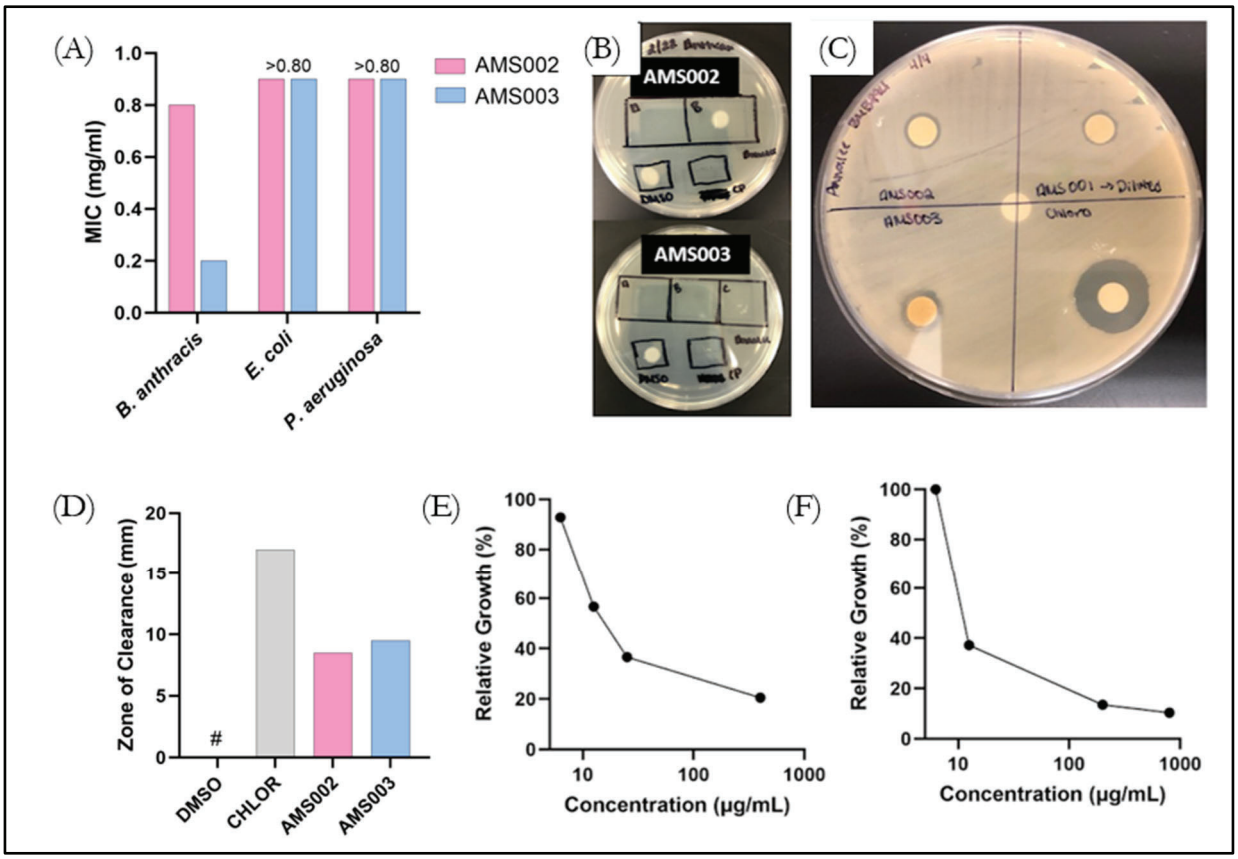

Figure 3. **Two isolated compounds, AMS002 and AMS003, showed antibacterial activity against *B. anthracis*.** **A**). Both AMS002 and AMS003 inhibited the growth of *B. anthracis* but showed no activity against *E. coli* and *P. aeruginosa* (n=1*)*. **B**) MBC assay shows AMS002 at a 0.8 mg/ml concentration was bactericidal against *B. anthracis* (top plate). The top left box is 0.8 mg/ml, top right box is 0.4 mg/ml of AMS002. AMS003 at a 0.2 mg/ml concentration was bactericidal against *B. anthracis* (bottom plate). From the top left to right, the concentrations of AMS003 in each box are 0.8 mg/ml, 0.4 mg/ml, and 0.2 mg/ml. On both plates, the bottom left spot is the negative control DMSO, and the bottom right spot is the positive control, spectinomycin. **C**). Disk diffusion assay showed zones of clearance around inhibitor-soaked disks (top-left: AMS002, bottom-left: AMS003, center: top-right: AMS001, DMSO, bottom-right: Chloramphenicol). **D**). Measured diameters of the zones of clearance around AMS002 and AMS003 along with positive (CHLOR) and negative (DMSO) controls. **E**). Growth of *B. anthracis* was inhibited in a dose-dependent manner when treated with AMS002 (n=1). **F**). Growth of *B. anthracis* was inhibited in a dose-dependent manner when treated with AMS003 (n=1). Note: ASM001 was not included in subsequent analyses due to the low quantity available.

Cell-based reporter assays suggest AMS002 and AMS003 inhibit protein synthesis

We tested whether the compounds could inhibit protein synthesis in bacterial cells to elucidate the potential mechanism(s) of action for AMS002 and AMS003. To achieve this, a YFP gene was transformed into *E. coli* cells. These cells were used in an MIC-type assay where the fluorescence was detected two hours after the inhibitors were added. This assay showed a significant decrease in fluorescence, similar to the positive control, chloramphenicol at 1 mg/ml, a known protein synthesis inhibitor. Both AMS002 and AMS003 decreased the fluorescent signal in the cells to about 20% of the DMSO-treated negative control sample (**Figure 4, A**).

To confirm these results, a similar assay was performed using *E. coli* cells transformed with a *Nano-luciferase* gene, where luminescence was detected after the inhibitors were added. Luminescence produced after the treatment with AMS002 and AMS003 was decreased to nearly 30% of the DMSO-control sample for both samples (**Figure 4, B**). Taken together, both results suggested the AMS002 and AMS003 may have prevented Gram-positive bacterial growth by interfering with transcription and/or translation.

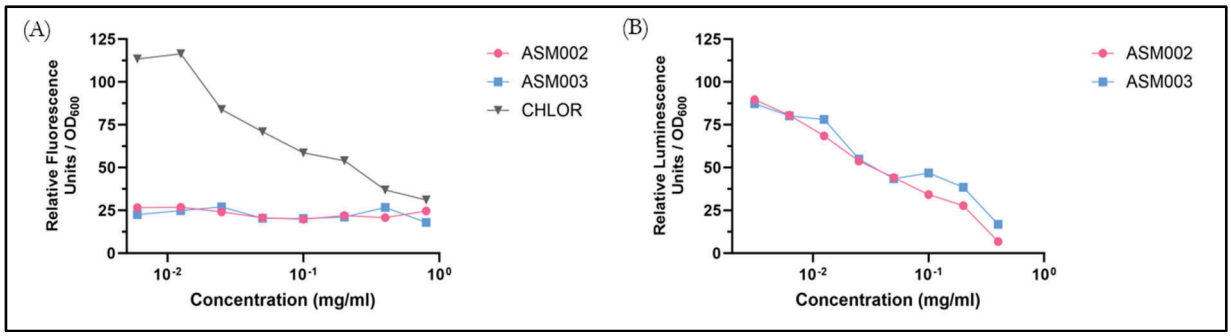

Figure 4. **Protein synthesis may be inhibited after treatment with AMS002 and AMS003**. **A**). Fluorescence-based reporter assay assessing the expression of YFP protein from a plasmid template (n=1). No increase in fluorescence was recorded for all concentrations tested with AMS002 and AMS003 while chloramphenicol reduced the YFP fluorescence signal in a dose-dependent manner. **B**). Luminescence-based assay illustrating a dose-dependent decrease in the luminescent signal by AMS002 and AMS003 from cells transformed with the *Nano-luciferase* gene (n=1). The data was normalized to the cell counts ($OD_{600\ nm}$) and plotted relative to the DMSO-treated control samples, representing 100% growth.

In-vitro luminescence-based screening confirms AMS002 and AMS003 inhibit translation

To distinguish which part of the central dogma AMS002 and AMS003 inhibit, DNA and mRNA templates encoding Nano-luciferase were mixed with an *E. coli* cell lysate and then treated with the inhibitors. Luminescence was measured to determine if the DNA or RNA templates had achieved protein synthesis. When treated with AMS002 and AMS003, synthesis of the Nano-Luciferase enzyme was halted when either the DNA or mRNA templates were added to the lysate. After adding the DNA template, the lack of luminescence suggested that transcription and/or translation was inhibited. In contrast, the lack of a luminescence signal after adding the mRNA Nano-Luciferase template indicated that translation was likely inhibited by AMS002 and AMS003 (**Figure 5, A & B**).

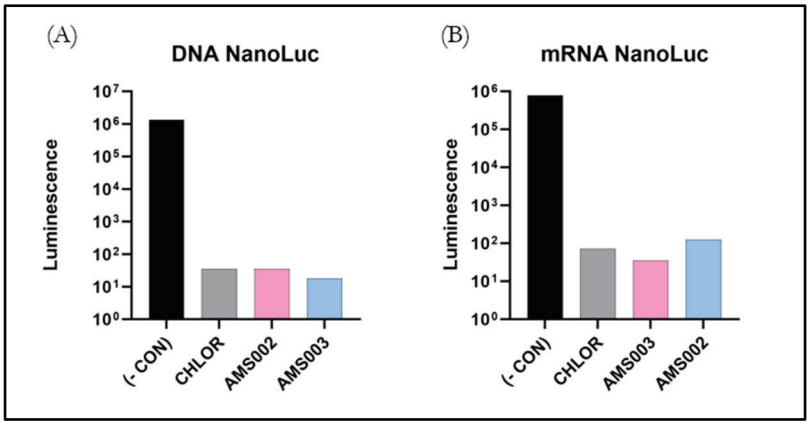

Figure 5. **AMS002 and AMS003 inhibited protein synthesis *in vitro***. **A**). Translation of a Nano-luciferase DNA template using *E. coli* 30S lysates. Chloramphenicol, AMS002- and AMS003-treated lysates show decreased luminescence signals following compound addition relative to the drug-free control (black bar) (n=1). **B**). Translation of a Nano-luciferase mRNA template using *E. coli* 30S lysates. Chloramphenicol (CHLOR), AMS002 and AMS003-treated lysates demonstrated decreased luminescence signals following compound addition relative to the drug-free control (black bar) (n=1).

Mass spectrometry analysis of compound AMS002 suggests a novel natural product

As part of our characterization of the isolated natural products, we wanted to determine whether the compounds exhibiting antibacterial properties were unique compared to known antibiotics. To achieve this, we conducted mass spectrometry and analyzed one of the promising compounds, ASM002, which had >95% purity relative to the other isolated fractions. Following the dissolution of the sample in methanol, it was analyzed by Electrospray Ionization Mass Spectrometry (ESI-MS). The obtained spectrum from the analysis revealed a compound with a base peak at m/z 157 (**Figure 6**). Upon comparing these data to known antibiotics used in the clinic, our findings suggested the discovery of a novel compound. We utilized computational methods to try and discover the potential identity of AMS002. We employed the compound discoverer software (Thermo Fisher Scientific) and searched the database for any small molecules with an identified molecular mass of 157 g/mol exhibiting antibacterial properties. This endeavor led to the identification of 5-Nitrofuran-2-carboxylic acid as a prospective candidate. Despite this finding, additional characterization studies are needed to reveal the identity of this compound. Similarly, more work is required to enhance the purity of AMS003 and determine its structural identity.

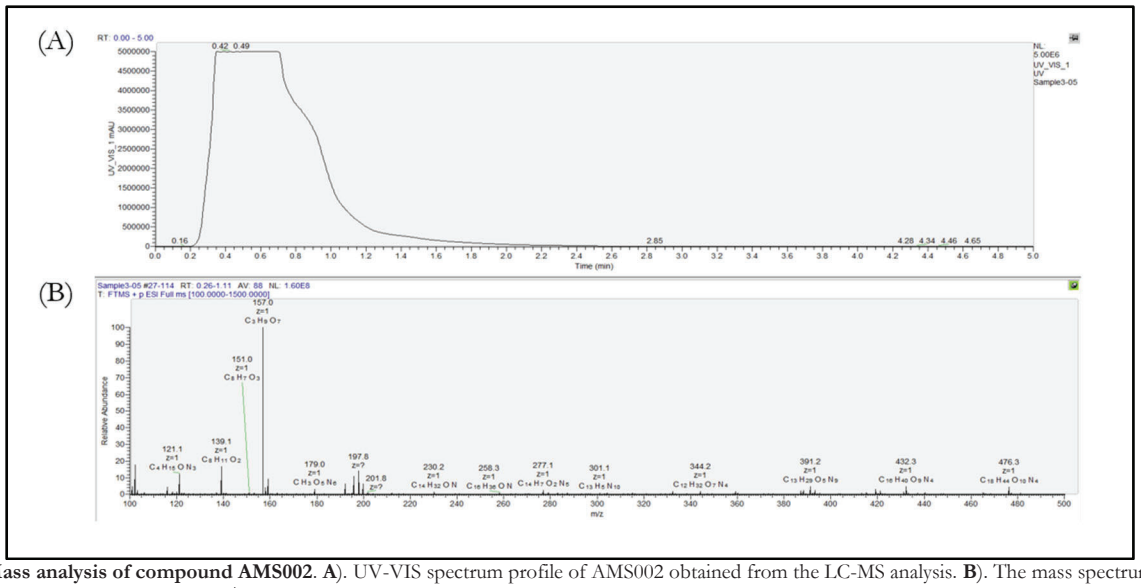

Figure 6. Mass analysis of compound AMS002. **A**). UV-VIS spectrum profile of AMS002 obtained from the LC-MS analysis. **B**). The mass spectrum for AMS002 highlighted the base peak at m/z 157 and the fragmentation pattern.

DISCUSSION

This study began by extracting organic compounds from soil samples in Pennsylvania. The first sample was collected from an Amish field, where manure was frequently applied for soil fertilization. The Amish are known for their all-natural farming, so the potential for synthetically added antibiotics was low. Manure helps facilitate plant growth as it provides a nitrogen source. Soil-dwelling bacteria may also use nitrogen containing compounds as an energy source, producing microbe-rich soil. Because microbes in the soil compete with other microbes for nutrients, inhibitory compounds are likely being produced from these organisms. The second soil sample was collected from a wooded and shaded region where human activity is low. This sample represented another soil ecosystem with a more natural source of microbes. For these reasons, we hypothesized compounds with antibacterial properties would be found in the soil of these locations.

After the isolation of the compounds and initial testing for antibacterial activity, it was found that two of the isolated compounds possessed inhibitory activity against *Bacillus anthracis*, the bacterial species causing anthrax infections. However, the observed antibacterial activity raises the question of where these compounds originated from. It may be possible that the compounds originated from other microbial life forms in the soil or nearby vegetation. Comparison of the mass spectrometry results and compound libraries in the discoverer database provided a prospective identity for AMS002, 5-Nitrofuran-2-carboxylic acid. Derivatives of this compound have been synthesized and explored for their antimicrobial activity against Gram-positive bacterial species and fungi.[28] However, we note that additional characterization experiments are needed to obtain the exact identity of the isolated compounds. Moreover, the purity of the compounds was ~ 90%, suggesting that the observed activity could have been affected by the purity. Planned experiments involve purifying these samples using HPLC to increase the purity and, hopefully, their antibacterial properties.

Our results showed that the soil samples collected from Pennsylvania locations contained potentially novel organic compounds with inhibitory activity against *Bacillus anthracis* by inhibiting translation. Inhibition by AMS002 and AMS003 was observed using MIC and disk diffusion assays. The mechanism of action was evaluated by employing *fluorescent and luminescent* reporter assays. We observed a decrease in protein synthesis after treatment with AMS002 and AMS003, and *in vitro* assays further narrowed down the mechanism as inhibition of translation. Surprisingly, both compounds completely arrested protein synthesis at concentrations below 0.01 mg/ml relative to chloramphenicol (**Figure 4, A**), which had no effect at a similar dose. These data are very promising and suggest these newly isolated compounds could be effective at inhibiting protein synthesis in pathogenic bacteria.

In the future, we would like to further characterize the mechanism of action by discovering the inhibitors' gene target(s). We plan to generate mutants of *B. anthracis* against AMS002 and AMS003 through inducing drug stress. Sequencing the genes that encode proteins involved in translation and comparing the sequences to wildtype genes would allow us to identify mutations that enable the bacteria to survive in the presence of AMS002 and AMS003, thereby identifying gene targets. This would teach us the specific inhibitory mechanisms that can effectively kill *B. anthracis* cells.

CONCLUSION

In this study, organic compounds were isolated from Pennsylvania soil samples using various chromatography methods. Two of the isolated compounds, AMS002 and AMS003, showed potent antibacterial properties against Gram-positive bacteria, including *B. anthracis*. We found that both compounds may exert their antibacterial effect by specifically inhibiting translation when using either a DNA or mRNA template, suggesting that they could target the ribosome as a mechanism of action. Mass spectrometry results indicated that one of the compounds, AMS002, may have structural similarities to a compound previously explored for its antimicrobial activity. However, further characterization of ASM002 is required to validate this hypothesis.

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PRESS SUMMARY

Deadly bacterial infections such as anthrax remain a serious global health concern. Anthrax, caused by *Bacillus anthracis*, is highly dangerous as it forms spores, making it resistant to antibiotics and harsh environmental conditions. Therefore, finding new antibiotics to fight this pathogen is crucial. In this work, we purified soil extracts from Pennsylvania and found two compounds, AMS002 and AMS003, which effectively inhibited and killed *B. anthracis* cells. Our data suggests these compounds target the bacteria's protein synthesis machinery. This finding is crucial as it offers a promising solution for combating infections caused by *B. anthracis*.