University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2023

Combating Antibiotic Resistance: Part One: Novel Antibiotic Isolation from Co-culture, Part Two: Adjuvant Chemistry

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Abstract

A steady decline in new antibiotic development coupled with reduced accumulation of antibiotics in Gram-negative bacteria has exacerbated antibiotic resistance resulting in a need for new methods of antibiotic development. Two methods for treating antibiotic resistant bacteria are to 1) find new antibiotics with novel mechanisms of action and 2) reactivate current antibiotics by overcoming resistance mechanisms using adjuvants. Natural products and their derivatives are the largest source of antibiotics with 73% of approved antibacterial agents between 1981-2014 being natural product production or natural product derivatives due to their unique structural motifs. Specifically, non-pathogenic rhizosphere soil bacteria, such as *Streptomyces griseus* that produces the popular antibiotic streptomycin, are an important source of novel natural products. Part 1 of this work examines the co-culture of Gram-negative 415 and 565 to exploit novel natural products to inhibit pathogenic growth of *Staphylococcus aureus* (SA) and *Escherichia coli* (EC). Part 2 focuses on improving antibiotic uptake by Gram-negative bacteria using adjuvant chemistry. The additional lipopolysaccharide outer membrane (OM) of Gram-negative bacteria increases antibiotic resistance due to impermeability and accumulation issues. It is of particular interest to target EC and ESKAPE pathogens such as *Pseudomonas aeruginosa* (PA) as they account for a majority of nosocomial infections. Permeability can be improved through covalently linking antibiotics to adjuvant molecules that can cross the OM. Part 2 will evaluate the use of cleavable adjuvant/antibiotic hybrids to improve antibiotic activity against EC and PA.

1. Introduction

As reported by the CDC in 2019, there are 2.8 million cases of antibiotic resistant bacterial infections each year in the US with 35,000 associated deaths.[1] In addition to high death totals, there has been a steady decline in novel antibiotics within the past 50 years developed to treat these increasingly resistant bacteria, exacerbating this crisis.[2] It is projected that by 2050, resistant bacterial infections could result in 10 million deaths in the US if current trends continue in antibiotic resistance and lack of drug development.[3] Antibiotic resistance has proven itself to be a global threat to public health, therefore actions must be taken to promote the well-being of humanity. Two promising methods of combating antibiotic resistance reside in using isolation of novel antibiotic compounds from co-culture and adjuvant chemistry.

Several resistance mechanisms for Gram positive and negative bacteria consist of antibiotic efflux and antibiotic inactivation.[4,5] Efflux pumps permit microorganisms to eject toxic substances through single or multi-constituent proteins to regulate internal environments.[6] Thus efflux pumps enable bacteria to eject foreign antimicrobial compounds and metabolites, reducing antibiotic efficacy and increasing resistance. Antibiotic inactivation occurs as enzymes within the bacterial cell irreversibly modify antibiotics and inactivate its function. Prevalent bacterial enzymes that exhibit this function include: β-lactamases, aminoglycoside-modifying enzymes, or chloramphenicol acetyltransferases.[5]

While both Gram positive and negative bacteria fuel the antibiotic resistant crisis, treating Gram negative bacterial infections are of particular interest. Drug development to eradicate Gram negative bacteria has proven to be more difficult as many antibiotics possess the inability to penetrate the cell membrane. Gram positive bacteria possess a peptidoglycan layer composed of sugar and amino acids that encompasses the cytoplasm, serving as a protective barrier (**Figure 1)**. Gram negative bacteria have an extra lipid layer made up of negatively charged lipopolysaccharide components that drastically decrease permeability of antibiotics across the cell membrane.[2,5,7,8]

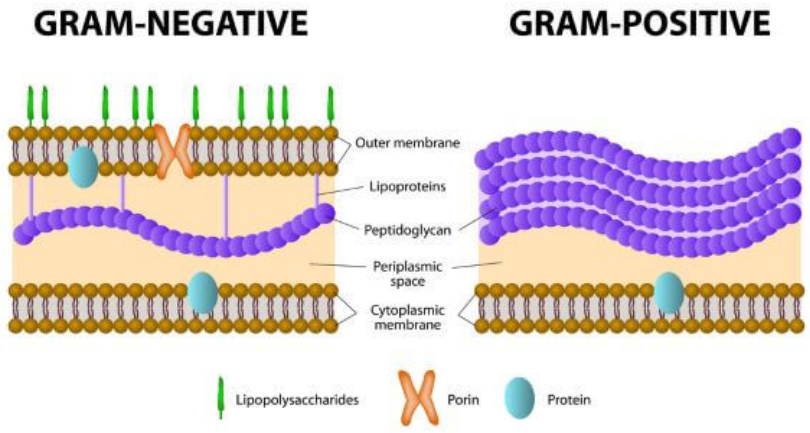


Figure 1. Gram-negative bacteria (left) and Gram-positive cell membrane[9]

It is of particular interest to target ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species) as they are multi-antibiotic resistant bacteria that account for the majority of lethal nosocomial infections.[5] It is crucial that remedies are developed as hospitals have a high prevalence of immunocompromised patients in which antibiotic resistant infections can be fatal.

1.1 Part One

An approach to combating antibiotic resistance is through the isolation of novel antibiotic compounds from co-culture.[10–13] Natural products and their derivatives are the largest source of antibiotics with 73% of approved antibacterial agents developed between 1981-2014 being natural products or natural product derivatives. Natural

products make promising scaffolds for antibiotic drug development due to their unique structural motifs and their ability to be replicated in the lab. Specifically, non-pathogenic rhizosphere soil bacteria, such as *Streptomyces griseus* that produces the popular antibiotic streptomycin, is an important source of novel natural products.

One challenge with bacterial natural products is that many have been previously identified. To overcome re-isolation issues, the Wolfe lab developed an optimal screening method by utilizing a bacteria's ability to produce novel natural products in coculture when under a competitive environment with minimal media conditions.[11] Co-culture more closely resembles the natural environment of bacteria leading to an increased possibility of secondary metabolism activation that can trigger synthesis of novel natural products. Additionally, Onaka found that inoculating actinomycetes with mycolic acid-containing bacteria may be useful to stimulate silent pathways in a variety of actinomycete classes and could be used to increase the discovery of new, novel natural compounds.[14]

The Wolfe lab maintains a library of over 400 bacterial samples from pitcher plant water and rhizosphere soil from locations in Western North Carolina. The harvested bacteria were then screened for inhibition of *Staphylococcus aureus* and *Escherichia coli* in mono-culture. Of 302 bacteria screened in monoculture, 38% produced antibiotic activity against *S. aureus*. Of 267 bacteria screened in monoculture, 34% produced antibiotic activity against *E. coli*. Bacteria that did not produce inhibition in mono-culture were screened in co-culture. Of 303 bacterial pairs screened against S. aureus, 14% showed activity and of 278 bacterial pairs screened against E. coli, 30% showed activity.[11] Former student Clair Huffine in Wolfe lab identified the production of natural products affective against *S. aureus* from the co-culture of Gram-negative 565 under the *Flavobacterium* genus and Gram-negative 415, genus *Pararheinheimera* **(Figure 2)**. Gram stain tests were used to determine the generic identity of the bacteria, i.e. Gram-positive vs. Gram-negative. Purple indicates Gram-positive, whereas a reddish pink indicates Gram-negative. The project was halted due to the COVID-19 pandemic and the structural characterization of isolated natural products were not determined.

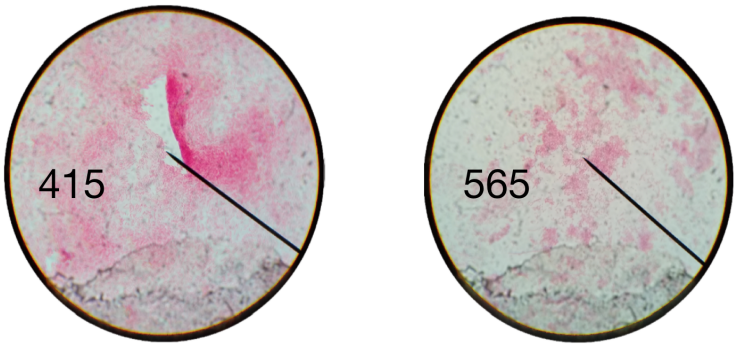


Figure 2. Gram stain images of SS 415 (left) and SS 565 (right) indicating both as gram negative.[15]

Manipulation of parameters such as growth time and media concentration allow this method to be easily adaptable to varying species of bacteria. A limitation of this method is that only bacteria culturable in liquid media can be screened; however, its promising applications outweigh this concern and further research is warranted for the progression of antibiotic discovery.[11]

This project will examine the exploitation of novel natural products via co-culture of bacteria 415 and 565 to combat antibiotic resistance of SA and EC. Natural products derived from 415/565 co-culture will be isolated and purified from growth media by liquid-liquid extractions, solid-phase extractions, and flash chromatography. The compounds will be assessed with inhibition assays against *Staphylococcus aureus* and *Escherichia coli* and structurally characterized using spectroscopy.

1.2 Part Two

ESKAPE pathogens can be targeted through adjuvant chemistry. Adjuvants are molecules that when in tandem with an antibiotic, can increase antimicrobial effectiveness; however, they are ineffective on their own. To combat antibiotic membrane impermeability in Gram negative bacteria, Hergenrother et al. established eNTRy rules that maximize drug uptake.[8] The researchers found that manipulating antibiotics to contain ionizable nitrogenous

functional groups, low globularity or three-dimensionality, and less than five rotatable bonds drastically increased accumulation uptake in Gram negative wild-type (WT) *E. coli*. Small amphiphilic molecules, presence of polar and nonpolar regions, (mw≲500 amu) in accompaniment with eNTRy rules increased uptake. Within the compounds tested in Hergenrother et. al's study, 85% of the guanidine derivatives accumulated in WT *E. coli* to a statistically significant degree **(Figure 3)**. This shows promise for guanidine derivatives to act as adjuvants for known antibiotics.[8,16]

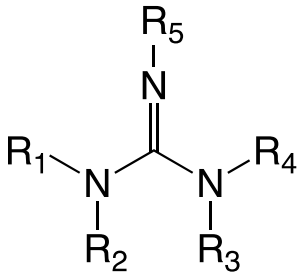


Figure 3. Skeletal structure of guanidine with functionalizable R groups.

Current research in adjuvant chemistry has primarily focused on antibiotic manipulation to increase drug uptake in the Gram negative WT. *E coli*.[8] It is important to extend this research to ESKAPE pathogens, specifically *Pseudomonas aeruginosa (P. aeruginosa),* as it landed on the CDC's serious threat list in 2019, with 32,600 cases in hospitalized patients in 2017.[1] To expand on the literature, our research question examines whether we can covalently link a small molecule guanidine derivative to known antibiotics through a cleavable linker to improve *P. aeruginosa* outer membrane penetration. In this research project, we will attach nitrogen-containing antibiotics via a cleavable linker using acylation chemistry. The antibiotic will ideally detach from the guanidine derivative and linker via a carboxylase enzyme present within the pathogen periplasm that will allow the antibiotic to travel and treat the infection site. Unlike Hergenrother et al., the antibiotics used will not be modified as the issue lies within membrane permeability and not drug efficacy. Antibiotics to be analyzed against *P. aeruginosa* and *E. coli* include trimethoprim, sulfamethoxazole, sulfadiazine, erythromycin, kanamycin, and ampicillin.

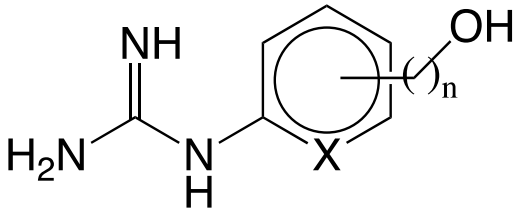


Figure 4. General proposed synthetic structure for adjuvant chemistry. n = 0, 1. X = CH2, N. Hydroxyl group is para or meta to the guanidinium group.

The proposed synthetic compounds are small molecules that align with the eNTRy rules derived by Hergenrother et al. **(Figure 4)**. [16] The first adjuvant compound includes a pyridine core with a para methyl hydroxyl functional group. The second adjuvant compound includes a benzyl core with an ortho hydroxyl functional group. Both adjuvants contain amine groups, polar and nonpolar regions, minimal rotatable bonds, and low globularity. After antibiotic coupling, the antibiotic activity of the hybrids will be analyzed via an IC50 cell death assay against *P. aeruginosa* and *E. coli*. The chemical and enzymatic cleavage will additionally be analyzed with accumulation assays. This compound will be compared to two other guanidine derivatives with slight modifications synthesized by other Wolfe Laboratory members. Specific comparisons to be evaluated include a pyridine versus benzene core ring, meta versus para positioning of the alcohol functional group in proximity to the guanidine, and direct alcohol core attachment versus a one carbon branch alcohol attachment. These parameters will be evaluated to determine the optimal adjuvant structure for accumulation in PA.

2. Methodology, Part One

All bacterial work was done under a flame using sterile conditions. All media and pipet tips were autoclaved under liquid A and plastics respectively (121 °C). All bacteria used were stored in 50% glycerol stock at -80 °C and streaked out onto 10% Tryptic Soy Agar (TSA) (3 g Tryptic Soy Broth and 20 g Agar per 1 L deionized (DI) H_2O plates for laboratory use. Upon bacterial growth, streaked plates were stored in refrigerator for two weeks before being discarded and remade. Pathogenic bacteria used included *E. coli* (ATCC 25922), *S. aureus* (ATCC 29213).

2.1 Co-Culture Screening and Scale Up (1-6L)

A singular bacterial colony of 415 and 565 was inoculated in 1/100th the desired 10% Tryptic Soy Broth (dTSB) (3 g per L DI H_2O) volume (1 – 6L) and shaken (130 RPM on an Excella E25 Incubator Shaker) for 24 hours to allow oxygen exchange at 26 °C. The samples were transferred to a dTSB solution of 1/10th the desired volume and shaken for 24 hours at 26 °C. The samples were transferred to a dTSB solution of the desired volume and shaken for 48 hours at 26 °C. Half of the final desired volume of each bacteria solution was combined and shaken for 48 hours at 26 °C.

2.2 Antibiotic Extraction

2.2.1 solid-phase extraction

Upon completion of the scale up procedure, bacterial solutions were centrifuged at 4,000 rpm and 26 °C for 20 minutes. The supernatant was collected for solid-phase extraction. Diaion HP20 resin beads (2% w/v) were rewetted in excess methanol (MeOH) and allowed to soak for approximately 15 minutes before decanting off the excess MeOH to remove excess grime. The beads were poured into the scale-up supernatant and agitated for 3 hours in the incubator. The beads were loaded onto a 1-L column and six fractions were collected with the mobile phases as follows: DI H_2O (1 L), 20% MeOH (1 L), 40% MeOH (1 L), 60% MeOH (1 L), 80% MeOH (1 L), and 100% MeOH (1 L). Active fractions were determined through a cell death assay described below.

2.2.2 cell death assay

Crude product from each organic solvent was diluted with 10 µL of dimethyl sulfoxide (DMSO) per 1 mg of crude. The dissolved product, 1 µL, was added to a 96-well plate in triplicate and further diluted with 89 µL of Full-Strength Tryptic Soy Broth (FS TSB) (30 g in 1 L) and 10 µL of FS TSB overnight cultures containing either *S. aureus* or *E. coli*. Chloramphenicol and DMSO only columns were used as positive and negative controls. The plate was shaken for 24 hours at 36 °C to resemble a human environment. Antibiotic activity for each organic solvent was determined using a Biotek plate reader (OD 590) after 24 hours of incubation as a measurement of the density of cells within the liquid culture. An active compound would inhibit pathogenic growth and kill cells resulting in lower absorbance values.

2.3 Antibiotic Purification and Characterization

2.3.1 reverse phase high-performance liquid chromatography (RP-HPLC) purification

Active fractions identified from the cell death assay underwent further isolation and purification via a Shimadzu LC-20AR with a SPD-40V UV-vis detector and c18 column. The 20 to 80% MeOH:H_2O gradient method file was used to obtain separation. All eluted fractions were tested on the previously described cell death assay to determine antibiotic activity. The elution time and UV-vis peaks for active compounds were recorded. Collected samples displaying similar elution times and visualization peaks from all scale ups were combined, concentrated, and weighed.

2.3.2 antibiotic characterization

A significant quantity of antibiotic extract (approximately 20 mg) must be obtained prior to characterization. ^{1}H – Nuclear Magnetic Resonance (NMR) and ^{13}C-NMR were analyzed on an Oxford Varian-400 spectrophotometer at 298K to determine the structure of the natural product antibiotic compounds.

2.4 Microbial Characterization

2.4.1 genomic purification

Bacteria samples were harvested from freshly streaked culture plates with a scraper loop. Using Wizard® Genomic DNA Purification Kit and protocol, bacterial DNA was extracted, and Gran-negative procedures were followed to isolate the genome. The purified bacterial DNA was stored at -20°C.

2.4.2 pcr procedure

To identify the genus of 415, a 16S rRNA gene was amplified and sequenced using the universal bacterial primers, 27F (27F: 5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-GGY TAC CTT GTT ACG ACT T-3′). To identify bacteria at the genus level, a 16S rRNA fragment was amplified and sequenced using the universal 16S rRNA primers, 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (CGG TTA CCT TGT TAC GAC TT). The OneTaq Quickload polymerase chain reaction (PCR) procedure was performed with this isolated DNA. The PCR vials contain One Taq Polymerase (0.125 µL), 5x OneTaq Standard Reaction Buffer (5.0 µL), 10 µM Universal Primers (0.5 µL each, 27F and 1492R), 10mM dNTP (0.5 µL), template DNA, and de-ionized H_2O to final volume of 25 µL. The PCR sample was loaded into the thermocycler and initially denatured at 98 °C (3 min). The following parameters were followed: 20 cycles of 98 °C (10 sec), 68 °C (30 sec) (ΔT= -0.5°/cycle), and 72 °C (45 sec), then 15 cycles of 98 °C (10 sec), 58 °C (30 sec), and 72 °C (45 sec). The PCR ended with a final extension period at 72 °C (2 min) before being held at 4 °C continuously until retrieval.

2.4.3 preparing agarose gel

A 1% agarose gel was prepared by microwaving 1x TAE Buffer (50 mL) and agarose (0.5 g) until boiling. The mixture was stirred until dissolved. GelGreen (2.5 µL) was added to the slightly cooled solution and mixed. The melted agarose was poured into a gel casting tray and a well comb was inserted. The gel was left to solidify for ~15-20 minutes. The hardened gel was covered completely with TAE buffer.

2.4.4 gel electrophoresis

PCR sample (5 µL) prepared from the PCR Procedure above was mixed with loading dye (2 µL) and distributed into the wells of the agarose gel. Leftover PCR sample was stored at 4 °C. Quick-Load® Purple 2-Log DNA Ladder (5 µL) was loaded into the gel for size comparison (0.1 - 10.0 kb). After running the gel at 100 V for approximately 30 minutes for suitable separation, PCR fragments were visualized under blue light illumination. The successful reaction was purified using IBI Scientific clean up kit and procedure and DNA concentration was measured using the NanoDrop and stored at -20 °C.

3. Methodology, Part Two

Reagents and solvents used were purchased reagent-grade and used without further purification. All synthetic work was done under inert conditions, Ar (g), with flamed flasks. The following solvent and reagent abbreviations are used: (DMF) dimethylformamide, (DCM) dichloromethane, (MeOH) methanol, (DMSO) dimethyl sulfoxide, (THF) tetrahydrofuran, ($CDCl_3$) chloroform, and (DIPEA) N,N-diisopropylethylamine. Thin-layer chromatography was performed using precoated SiO_2 60 F2 54 glass plates from EMD with visualization by UV light (254 nm). NMR (^1H or ^{13}C) were analyzed on an Oxford Varian-400 spectrophotometer at 298K. Residual solvent peaks were used as an internal reference. Coupling constants (J) (H, H) are given in Hz. Coupling patterns are designated as singlet (s), doublet (d), triplet (t), doublet of doublets (dd), and multiplet (m). IR spectra were recorded on a Thermo Scientific Nicolet iS10 FT-IR spectrophotometer. Low-resolution mass spectral data were measured from a Shimadzu single quadrupole LCMS-2020.

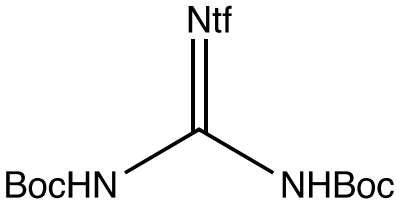


Figure 5. Guanidinylation reagent structure. Tf: trifluromethanesulfonyl, Boc: tert-butyloxycarbonyl.

3.1 Guanidinylation Reaction Conditions

3.1.1 2-aminopyridin-4-yl)methanol

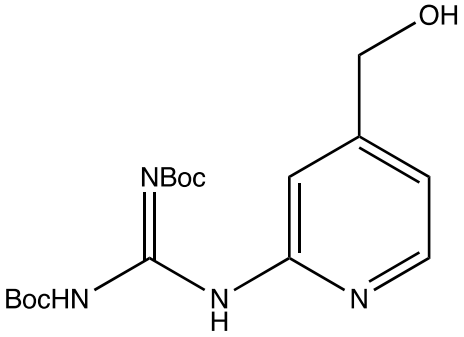


Figure 6. Desired guanidinylated adjuvant 1

(2-aminopyridin-4-yl)methanol (1 equiv, 4.03 mmol, 0.500 g) and triethylamine (3.1 equiv, 12.49 mmol, 1.74 mL) were dissolved in DCM (0.05 M, 81 mL). Guanidinylation reagent (1.5 equiv, 6.04 mmol, 2.36 g) **(Figure 5)**, was added, and the reaction mixture was stirred at room temperature for 16−24 h. The crude reaction mixture was passed through a plug of silica between 1 inch of sand wet with CH_2Cl_2. The column was eluted with 100% DCM followed by 5% MeOH/DCM, and 50% MeOH/DCM, eluting in 5% MeOH/DCM with 43 **(Figure 6)**. ^1H NMR (DMSO, 400 MHz): δ 10.14 (s, 1H), 8.77-8.75 (m, 1H), 7.79-7.78 (m, 1H), 7.30-7.23 (m, 1H), 6.43-6.39 (m, 3H), 5.94 (s, 1H), 5.25 (t, *J* = 5.6 Hz, 1 H), 4.66 (d, *J* = 4.8 Hz, 1H), 4.35 (d, *J* = 6.0 Hz, 3H), 3.37 (s, 18H). HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{17}H_{26}N_4O_5$, 366.19; found, 366.19.

3.1.2 4-aminophenol

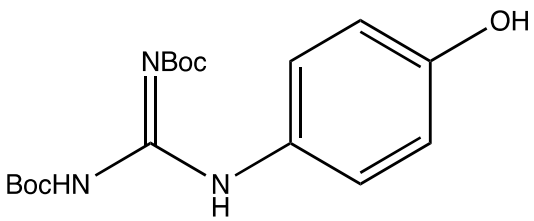


Figure 7. Desired guanidinylated adjuvant 2

4-aminophenol (1 equiv, 4.58 mmol, 0.500 g) and triethylamine (3.1 equiv, 13.76 mmol, 1.92 mL) were dissolved in DCM (0.05 M, 92 mL). Guanidinylation reagent (1.5 equiv, 6.87 mmol, 2.69 g), **(Figure 5)**, was added, and the reaction mixture was stirred at room temperature for 16−24 h. The crude reaction mixture was passed through a plug of silica between 1 inch of sand wet with DCM. The column was eluted with 100% DCM followed by 5% MeOH/DCM, and 50% MeOH/DCM. The guanidinylated adjuvant molecules were confirmed by LCMS characterization of all fractions. **Adjuvant 2** was detected in PIM 352 m/z, eluting in 5% MeOH/DCM with 87% yield **(Figure 7)** as a white solid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 10.01 (s, 1H), 6.64 (dd, J_1 = 6.8 Hz, J_2 = 1.6 Hz, 2H), 1.54 (s, 9H), 1.46 (s, 9H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 163.27, 155.39, 154.69, 153.20, 127.65, 125.84, 115.96, 28.12. HRMS (ESI) *m/z*: $[M + H]^+$ calcd for $C_{17}H_{25}N_3O_5$, 351.18; found, 351.18.

3.2 Coupling Trials

3.2.1 nitrophenyl chloroformate conditions

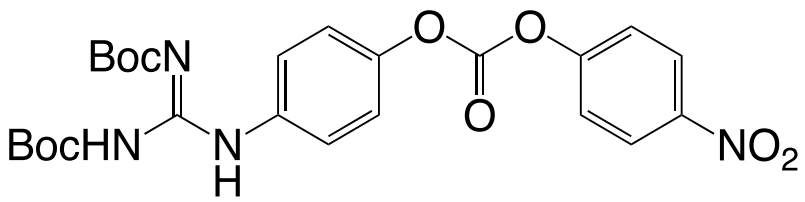


Figure 8. Desired nitrophenyl-adjuvant 2 coupling product.

Adjuvant 2 (1 equiv, 0.28 mmol, 0.100 g) was dissolved in two-thirds of (1:1 v/v, 4.0 mL) THF/DCM under argon, then cooled to 0 . $Li(OH)_2$ (1 equiv, 0.28 mmol, 0.012 g) was added and stirred for 30 minutes. 4-Nitrophenyl chloroformate (1 equiv, 0.057 g) was dissolved in remaining one-third of (1:1 v/v, 2.0 mL) THF/DCM) and added dropwise into reaction flask over ten minutes. Reaction was left to stir for 2 hrs and was monitored with TLC and LCMS. Desired product **(Figure 8)** was detected in PIM 352 and 517 m/z and used as an intermediate in ampicillin coupling.

3.3 Ampicillin Coupling

To probe different antibiotic coupling schemes, only one antibiotic (Ampicillin) was used. Ampicillin was chosen for its nucleophilic primary amine that is predicted to favor the reaction to synthesize the desired product **(Figure 9)**. The following three methods to be discussed were adapted from the literature and all produced trace amounts of product.[17–19]

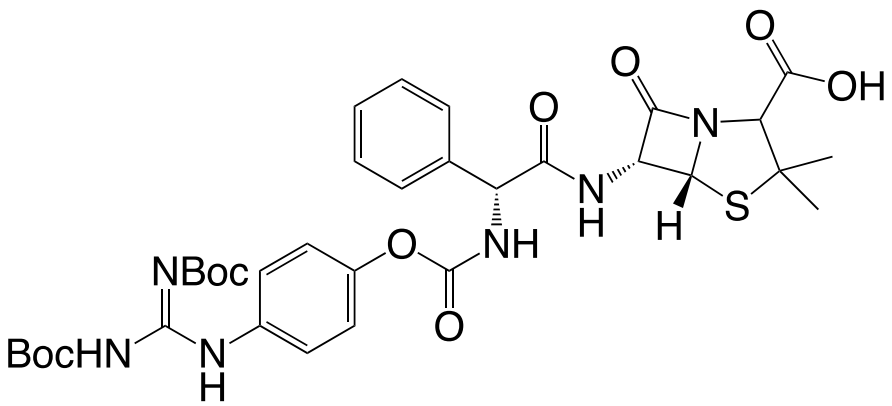


Figure 9. Desired ampicillin-adjuvant 2 coupling product, methods 1-3.

3.3.1 method 1[17]

Ampicillin (1.05 equiv, 0.29 mmol, 0.105 g) was added portion wise to nitrophenyl-adjuvant 2 coupling intermediate from 3.2.1 (6 mL) at 0 °C, and the mixture was warmed to room temperature and then stirred for 4 h. The reaction was quenched with DI H_2O (50 mL) and extracted with ethyl acetate (2 x 50 mL). The combined organic extracts were washed with saturated aqueous NaCl, dried over $MgSO_4$, filtered, and evaporated. Trace amounts of adjuvant-ampicillin hybrid **(Figure 9)** molecules was detected by LCMS in PIM 727 and 365 m/z.

3.3.2 method 2[18]

Nitrophenyl-adjuvant 2 coupling intermediate from 3.2.1 (6 mL) was allowed to warm to rt. Ampicillin (0.5 equiv, 0.14 mmol, 0.050 g) was dissolved in DIPEA (40 µL) and dry DMF (2 mL). This mixture was added to the coupling intermediate, and the reaction was left to stir for 1 hr at rt. The mixture was concentrated under vacuum. Trace amounts of adjuvant-ampicillin hybrid **(Figure 9)** molecules was detected by LCMS in PIM 727 and 365 m/z.

3.3.3 method 3[19]

Ampicillin (1 equiv, 0.28 mmol, 0.100 g) and 4-dimethylaminopyridine (1 equiv, 0.28 mmol, 0.035 g) were dissolved in DI H_2O (20 mL) at 0 °C. This mixture was added to the nitrophenyl-adjuvant 2 coupling intermediate from 3.2.1 (6 mL) and stirred for 24 hrs, allowing to warm to rt. The reaction was quenched with DI H_2O (50 mL) and extracted with ethyl acetate (3 x 50 mL). Aqueous extracts were combined, acidified to pH 2 with 1M HCl, and extracted with ethyl acetate (3 x 50 mL). Organic phase was washed with saturated aqueous NaCl, dried over Na_2SO_4, filtered, and evaporated. Trace amounts of adjuvant-ampicillin hybrid **(Figure 9)** molecules was detected by LCMS in PIM 727 and 365 m/z.

4. Results and Discussion, Part One:

4.1 Microbial Characterization

Colony PCR was initially used using Q5 polymerase and resulted in no amplification. Genomic DNA purification was performed and an overnight culture of 415 isolate was prepared. The overnight culture was centrifuged for five minutes at 5,000g. The supernatant was disposed, and the pellet was used for PCR with the Q5 polymerase method above. This reaction was unsuccessful. Switching to One Taq Polymerase using Genomic DNA purification for Gram-negative bacteria resulted in a successful PCR reaction. Successful PCR products exhibited the desired 16S ribosomal subunit length of 1450 base pairs (bp), as shown in **(Figure 11)**. Genus level identification was performed using the Basic Local Alignment Search Tool (BLAST) publicly available from the National Center for Biotechnology Information with results designated by < 3% variation.

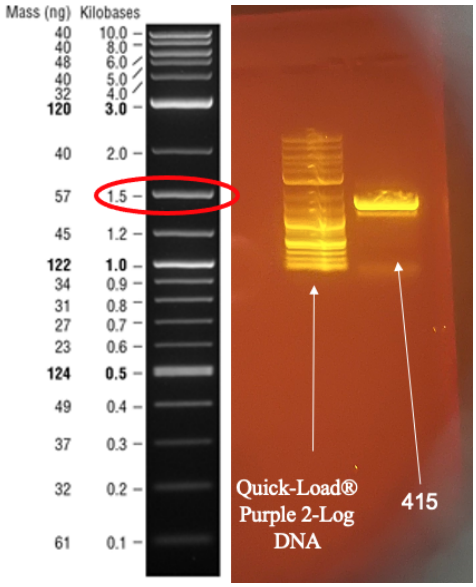


Figure 11. Gel electrophoresis of OneTaq Quickload PCR (left) with Quick-Load® Purple 2-Log DNA ladder reference (middle). The desired product is visualized at 1450 bp (right).

4.2 Antibiotic Isolation

4.2.1 415/565 co-culture

Bacterium 415 stains as a Gram-negative rod and was identified as a bacterium from the genus *Pararheinheimera* using 16S rRNA sequencing. This bacterium grows in a filamentous/rhizoid manner in dTSB with a yellowish appearance. Clair Huffine determined that this bacterium requires 48 h at 30 mL stage to reach exponential growth.[15] 565 stains as a Gram-negative rod and was previously identified by Clair Huffine as a bacterium from the genus *Flavobacterium*. This bacterium grows in a cloudy manner in dTSB with a white/yellowish appearance. Clair Huffine determined that this bacterium required 24 h at the 30 mL stage to reach exponential growth. The high emulsion of this co-culture has reduced scale up volumes to 1L-3L. This was done to reduce column clogs during solid-phase extraction.

4.2.2 solid-phase extraction and RP-HPLC

Solid-phase extraction was performed to extract natural products produced through 415/565 co-culture in the supernatant into the pores of the non-polar resin beads. Natural products were flushed from the column by increasing non polarity from 0% MeOH:100% H_2O to 100% MeOH:0% H_2O and collected in six fractions. Nonpolar compounds will elute in later fractions due to its high attraction to the stationary phase. Since Clair Huffine found antibiotic activity in the 40, 60, and 80% MeOH:H_2O fractions, 40, 60, 80, and 100% fractions were kept. Fractions 0% and 20% were discarded immediately after the solid-phase extraction column. MeOH was evaporated off using a rotary evaporator and the remaining H_2O was blown down overnight with air. The solid products were dissolved in minimal MeOH, transferred into weighed vials, blown down, and stored until use for preliminary cell death assays.

Preliminary cell death assays were conducted to confirm antibiotic activity against *S. aureus* and *E. coli* in fractions 40, 60, and 80% MeOH:H_2O. Activity against *E. coli* was found in all fractions, whereas activity against *S. aureus* was found in only fractions 60% and 80%. To further isolate the target compounds, RP-HPLC was performed on these three fractions to separate compounds based off hydrophobicity using UV-vis detection. Due to the nonpolar nature of the c18 column, polar compounds elute first and thus have a shorter elution time (et). To flush nonpolar compounds from the column, a gradient mobile phase increasing in nonpolarity is required. The elution times and UV-vis peaks were recorded for all eluted compounds and all fractions with similar retention times were combined. All fractions were blown down and screened for activity in cell death assays.

4.2.3 cell death assays and active compounds

Cell death assays described in methodology revealed strong activity (32% inhibition) against *S. aureus* and strong activity (35% inhibition) against *E. coli* by Fraction D **(Table 1)**. Fraction D was isolated from 60% and 80% MeOH and determined by elution time (et) of ~18.823 minutes. Moderate activity (22% inhibition) against *E. coli* was found in Fraction L (40% MeOH, 12.973 min et). Antibiotic activity of Fraction L was deemed inconclusive due to mixed pathogen contamination, thus the assay will need to be re-run.

415/565 Fraction Vial	**Antibiotic Activity Against *S. aureus***	**Antibiotic Activity Against *E. coli***	**Percent Inhibition of *S. aureus***	**Percent Inhibition of *E. coli.***
D	0.462	0.703	32 %	35 ± 3.73%
L	inconclusive	0.862	-	22%

Table 1. Active Fractions from 415/565 Co-culture Cell Death Assay.

4.3. Antibiotic Characterization and Future Directions

Due to the small amount of active compounds isolated and slight impurities (<20 mg) from co-culture, spectroscopic analysis using ^{1}H NMR and ^{13}C-NMR cannot yet be definitively used to determine the structural compositions. More scale ups will be performed in the future to build a stock of active compounds. ^{1}H NMR, ^{13}C-NMR, and Mass Spectrometry characterization will then be performed to identify the structures and determine whether the compounds are novel.

5. Results and Discussion, Part Two:

5.1 Triphosgene Coupling Trials

5.1.1 2-aminopyridin-4-yl)methanol coupling

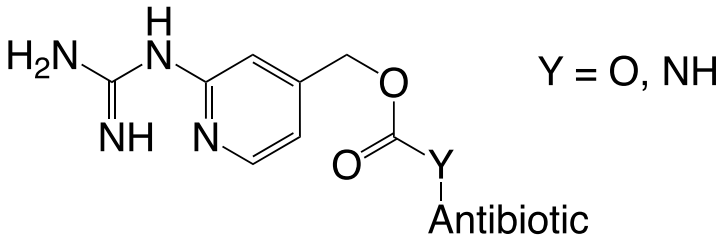
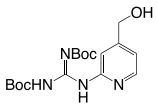


Figure 12. Desired antibiotic-adjuvant 1 coupling product

The desired antibiotic-adjuvant 1 coupling product above was not detected via LCMS characterization when using the reaction parameters below **(Figure 12, Table 2).** Antibiotics tested were tedizolid and trimethoprim. It is possible that these reaction conditions were unsuccessful in product formation because of potential degradation of **Adjuvant 1** stock. Absence of Boc protection groups could result in undesired product formation due to the increased nucleophilic nature of amines over alcohol functional groups.

Adjuvant	Trial	Antibiotic/ Coupling	Base	Triphosgene equivalents	Solvent	Temperature	Time	Yield
	1	trimethoprim (1 eq)	Et_3N (2.5 eq)	1.0	THF (0.1M)	20 °C	48 hrs	–
	2	trimethoprim (1.1 eq)	pyridine (8 eq)	0.4	THF (0.1M)	0 °C to 40 °C	48 hrs	–
	3	trimethoprim (1.1 eq)	pyridine (8 eq)	0.4	THF (0.09M)	0 °C to 23 °C	48 hrs	–
	4	trimethoprim (1 eq)	pyridine (8 eq)	1.5	THF (0.09M)	0°C to 23 °C	48 hrs	–
	5	tedizolid (1 eq)	pyridine (8 eq)	1.5	THF (0.09M)	0 °C to 23 °C	48 hrs	–

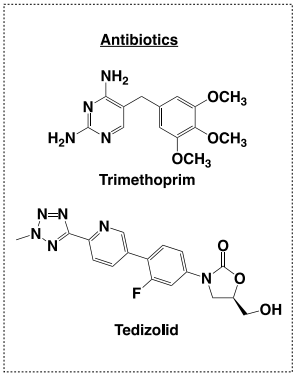


Table 2. Triphosgene reaction conditions for adjuvant 1.

5.1.2 benzyl amine coupling

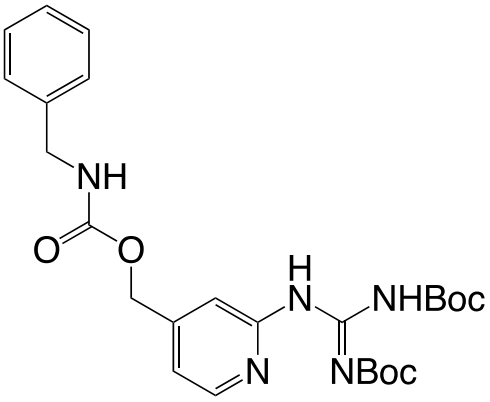


Figure 13. Desired benzylamine-adjuvant 1 coupling product.

To determine whether triphosgene could be used as a linker, coupling trials were conducted using benzyl amine **(Figure 13)**. Benzyl amine was chosen because it is a smaller and less bulky than ampicillin or other antibiotics; therefore, it is predicted that lower steric hinderance would not impede the desired reaction from occurring. The following equivalents and parameters are described below **(Table 3)**.

Condition 1 and Condition 2 reactions differ in the order of addition. Condition 1 added benzyl amine to **Adjuvant 1**, whereas condition 2 added **Adjuvant 1** to the benzyl amine. In trial 6, **Adjuvant 1** was semi-soluble in THF so a solvent trial was performed with acetonitrile (ACN), dichloroethane, and dioxane. The adjuvant was insoluble in all three solvents, so triethylamine (NEt_3) was added dropwise to each solvent. **Adjuvant 1** was soluble in ACN with NEt_3 so the base equivalent was doubled and the solvent was changed to ACN in trial 7. Due to unforeseen circumstances, trial 7 reactions were unable to be stopped at 48 hrs. All coupling trials using benzyl amine were deemed unsuccessful as the desired product was not detected via LCMS characterization.

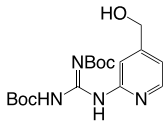
Adjuvant	Trial	Condition	Coupling	Base	Triphosgene equivalents	Solvent	Temperature	Time	Yield
	6	1	benzyl amine	pyridine (10 eq)	0.8	THF (0.05M)	0°C to 60 °C	48 hrs	–
	6	2	benzyl amine	pyridine (10 eq)	0.8	THF (0.05M)	0°C to 60 °C	48 hrs	–
	7	1	benzyl amine	pyridine (20 eq)	0.8	ACN (0.05M)	0°C to 60 °C	240 hrs	–
	7	2	benzyl amine	pyridine (20 eq)	0.8	ACN (0.05M)	0°C to 60 °C	240 hrs	–

Table 3. Triphosgene coupling trial conditions for adjuvant 1.

5.1. 4-aminophenol coupling

It is predicted that triphosgene coupling trials with benzyl amine were unsuccessful due to the high reactivity of triphosgene and the side-generation of HCl that quenched the reactions with prevented desired product formation. To accommodate for triphosgene's reactivity and generation of undesired HCL, the equivalence of triphosgene was reduced two-fold, base equivalence was increased to deprotonate hydrochloric acid, and triphosgene was added at 0 using **Adjuvant 2** to generate the desired product **(Figure 14)**.

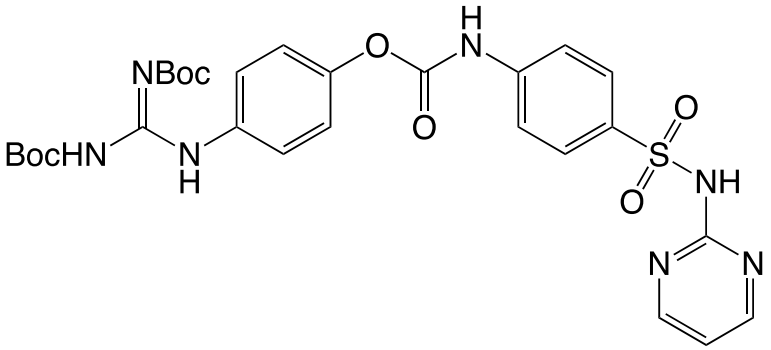


Figure 14. Desired sulfadiazine-adjuvant 2 coupling product.

With the modifications described above, the sulfadiazine-adjuvant 2 coupling desired product was not detected via LCMS characterization. The major peak detected with LCMS characterization was **Adjuvant 2** starting material (PIM 352 m/z). Due to the large presence of unreacted starting material, the order of addition of the adjuvant and antibiotic was reversed in a separate reaction using the same equivalents and conditions above. Adding sulfadiazine to **Adjuvant 2** did not improve product formation as the desired product was not detected via LCMS characterization.

5.2 Nitrophenol Chloroformate Coupling

5.2.1 2-aminopyridin-4-yl)methanol

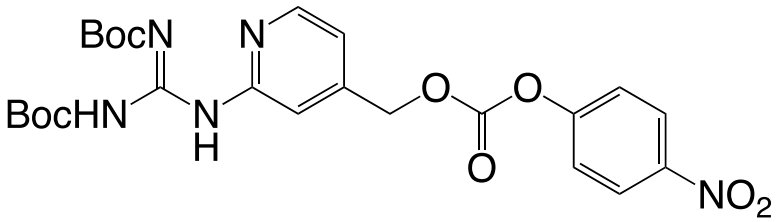


Figure 15. Desired nitrophenyl-adjuvant 1 coupling product.

A coupling trial to generate an intermediate linker to **Adjuvant 1** using nitrophenol chloroformate was performed. The desired product **(Figure 15)** was not detected in LCMS.

5.2.2 *4-aminophenol*

The nitrophenol chloroformate coupling reaction using adjuvant 2 was successful and the desired product was detected by LCMS in PIM 352 and 517 m/z **(Figure 8)**. Due to the unstable nature of this compound, the product was used as a crude intermediate for the receding ampicillin coupling reactions described in methods. It is hypothesized that evaporating off the solvent from this reaction before ampicillin coupling would increase yield of ampicillin-adjuvant 2 hybrid. This alteration will occur in the next reaction scheme.

6.1 Conclusions

Exploiting natural products from the co-culture of non-pathogenic rhizosphere soil bacteria and expanding the efficacy of current antibiotic through adjuvant chemistry are two novel ways to combat antibiotic resistance. In Part One, strong and weak antibiotic activity was found against *S. aureus* and *E. coli*. In future work, the structure of these promising compounds will be elucidated through spectral characterization and their novelty will be determined by literature screens. In Part Two, antibiotic coupling of adjuvant 1 with triphosgene and nitrophenol chloroformate was deemed unsuccessful. Trace amounts of ampicillin-adjuvant 2 hybrids using a nitrophenol chloroformate linker were detected via LCMS. Future work will focus on using adjuvant 2 to optimize the ampicillin reaction and extend the antibiotic coupling library to kanamycin, sulfadiazine, trimethoprim, etc. Assays assessing the hybrid's and the dimer of **adjuvant 2**'s ability to accumulate, permeate, and induce cell death are necessary to examine the applicability of this project.

7.1 Acknowledgements

The author wishes to express their appreciation to Dr. Amanda Wolfe and all members of the Wolfe Laboratory for their encouragement and assistance, Dr. Melinda Grosser and Claudia Prieto for aiding with microbial characterization, Bea Bullard, and all professors in the Department of Chemistry and Biochemistry at UNC Asheville. An additional thank you is extended to the Research Corporation for Science Advancement for funding these projects.

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9.1 Supplemental, Part One:

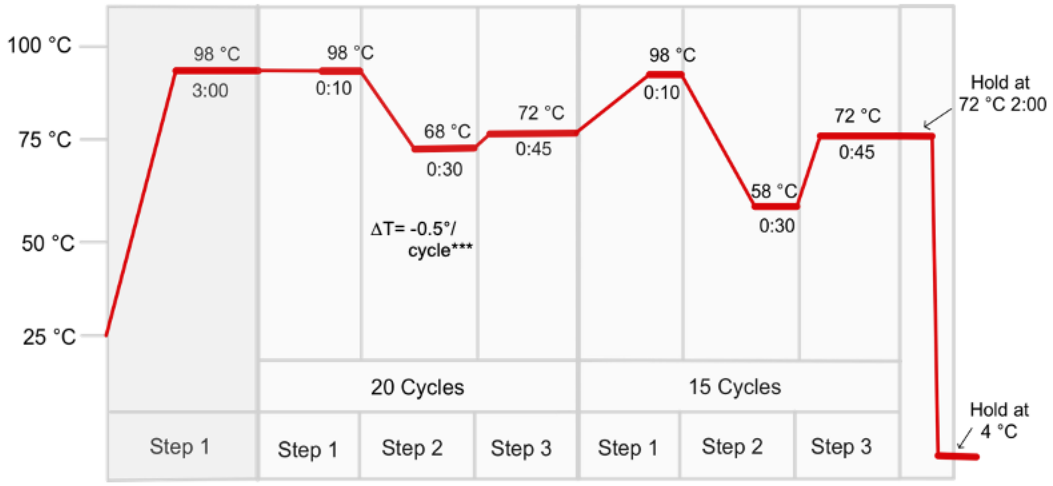

Figure 14. Thermocycler conditions for microbial identification PCR.

9.2 Supplemental, Part Two:

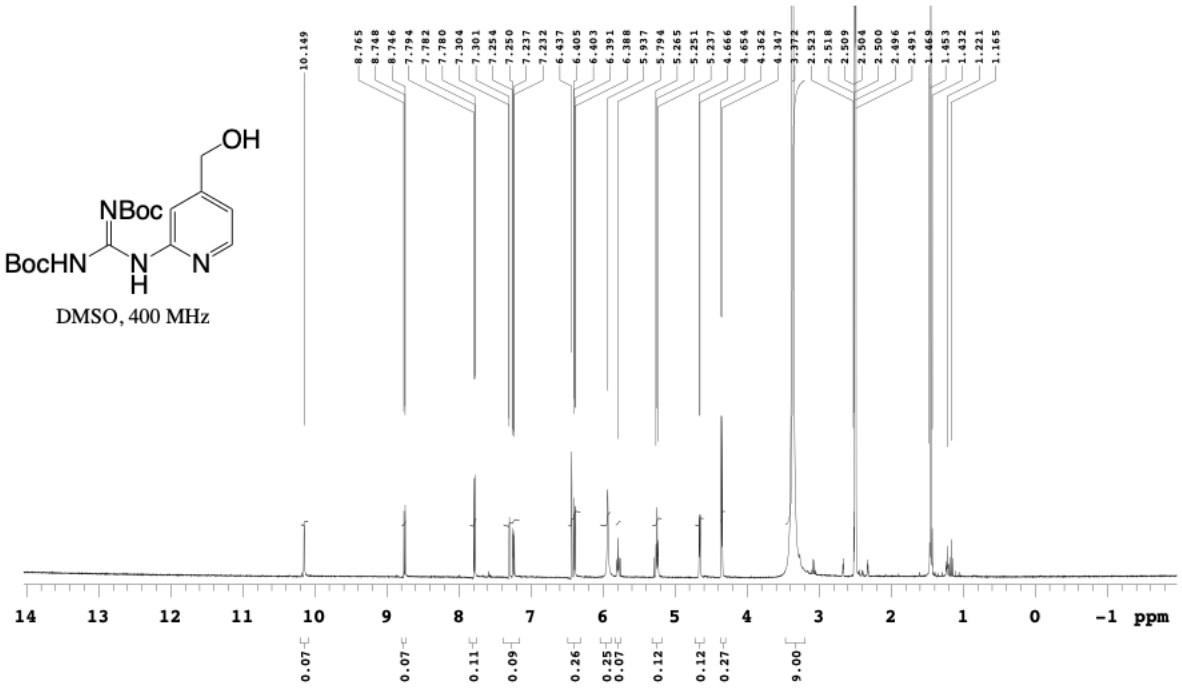

Figure 15. H^1-NMR of Guanidinylated adjuvant product from 04/12/2022.

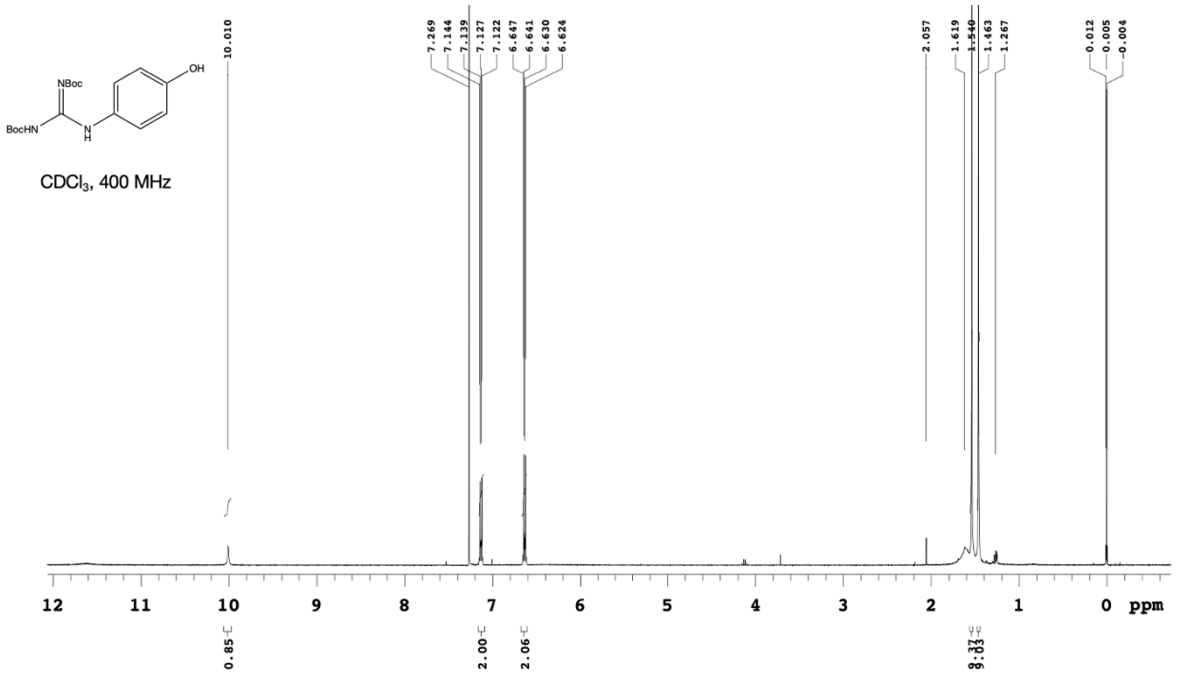

Figure 16. [1]H-NMR of Adjuvant 2

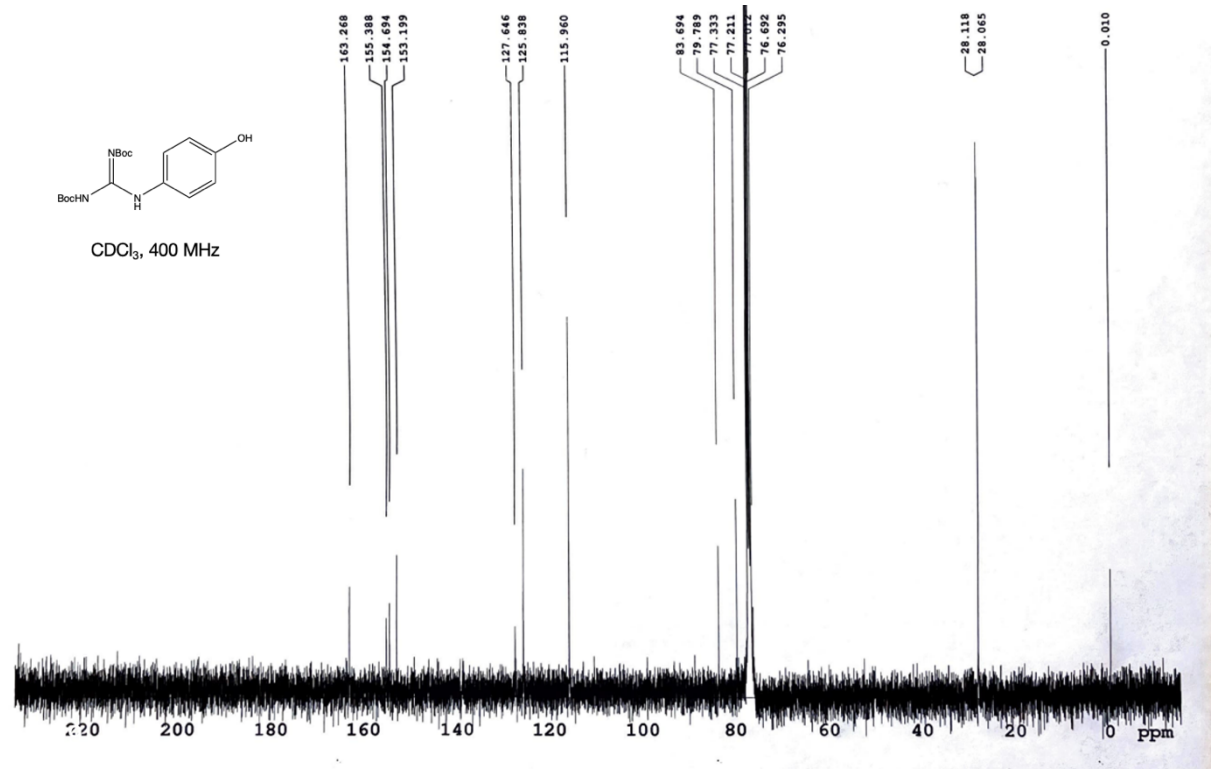

Figure 17. [13]C-NMR of Adjuvant 2