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# Synthesis and Evaluation of Guanidinium-Based Antibiotic-Adjuvant Hybrids and Their Antibiotic Properties

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Abstract

Antibiotic resistance is becoming an increasing threat to our livelihood, and the CDC estimated in its 2019 annual threat report that by 2050, there will be over 10 million deaths due to antibiotic-resistant bacteria. Novel antibiotics effective against Gram-positive (G+) bacteria are not effective against Gram-negative (G-) bacteria due to their additional outer membrane (OM). Research done by Hergenrother et. al. proved that adding nitrogenous groups to antibiotics that are effective against G+ bacteria increased their accumulation in G- bacteria. From this data, they developed the rules of eNTRy, which outline the requirements for a small molecule to penetrate the OM of a G- cell. Previous research has shown that addition of guanidinium functionality to novel antibiotics effective against G+ bacteria increase their effectiveness against G- bacteria

by allowing them to penetrate the OM. For this research, we synthesized a phenyl-guanidine base adjuvant and explored linking conditions in two synthetic routes to synthesize antibiotic-adjuvant hybrids bonded together by a carbonyl-based cleavable linker molecule, all following the aforementioned rules of eNTRy. The adjuvant was successfully linked to ampicillin, sulfadiazine, and trimethoprim in synthetic route 1 with low conversion rates determined by mass spectroscopy. Successful linkage was also observed with sulfadiazine and sulfamethoxazole in synthetic route 2, with high conversion rates and lower yields calculated by mass. These products were tested against different pathogens in cell death assays, and significant inhibition was observed by the sulfadiazine hybrid molecule against multidrug-resistant PA, with an $IC_{50}$ of >32 μg/mL. Future work will involve testing more combinations of antibiotics and different reaction conditions with the developed phenyl-guanidine molecule to yield hybrids in both routes, as well as increasing purification to better isolate the products.

# Introduction

Antibiotic resistance is becoming an increasingly large threat to human health, and this is mainly due to the rise of multidrug resistant bacteria.[1] According to the CDC's 2019 threat report, over 2.8 million antibiotic resistant bacterial infections occur each year in the US alone, leading to over 35,000 deaths. The CDC also estimated that by 2050, over 10 million deaths worldwide each year will be caused by antibiotic resistant bacteria.[1] Strains of bacteria that are known to be the leading cause of nosocomial antibiotic resistant bacterial infections have been dubbed the ESKAPE pathogens. These bacteria include *Enterococcus faecium*, *Staphylococcus aureus* (SA), *Klebsiella pneumoniae* (KP), *Acinetobacter baumannii* (AB), *Pseudomonas aeruginosa* (PA), and *Enterobacter* sp.[2] Specifically, PA is responsible for over 32,000 infections in 2017 that lead to 2,700 deaths. PA typically infects patients with weakened immune systems in hospital settings, and this proves to be dangerous for patients with chronic lung diseases.[1] Pathogens like PA also thrive due to the misuse of the methods used to treat these infections by healthcare providers and patients alike, such as physicians overprescribing antibiotics and patients not taking the full course of prescribed medication. Research into antibiotic discovery done by pharmaceutical companies has slowed down due to poor financial returns, causing very few significant developments in the field since the "golden age" of discovery in the 1970's.[3,4] With bacteria growing

more resistant to known antibiotics and possibly developing resistance to newer compounds over time, a new way of solving the issue needs to be studied.[4]

Bacterial strains can be divided into two different types, Gram positive (G+) and Gram negative (G-) bacteria. G+ bacteria contain a semipermeable membrane and a large peptidoglycan layer, making the process of treatment and inhibition relatively simple. G- bacteria have the same layers as G+ bacteria, but with a thinner peptidoglycan layer. What makes G- bacteria different is a thick negatively-charged outer membrane (OM) on top of all , made up of lipopolysaccharides and phospholipids. Efflux channels and multidrug efflux transporters are all embedded within the OM, and these work to pump out any antibiotics that would harm the cell. This is one of the main effectors as to why G- bacteria is hard to treat compared to G+ bacteria, all of these channels work together to keep specific molecules like nutrients inside and push molecules like antibiotics out.[5] Approaches described by Zgurskaya et. al. that attempt to get past the OM include searching the membrane for new targets that could be interrogated with new drug discovery efforts, as well as the trojan horse method and efflux pump inhibitors have all been proposed. Each method exploits areas of the OM to degrade its functionality. For example, the inhibition of efflux pumps can allow antibiotics to accumulate inside the cell, since the main mechanism of resistance past the OM is inhibited.[5]

Trends and rules for the entry of molecules into *Escherichia coli* (*E. coli*, EC), a G- bacteria, have been developed. These rules, known as the “Rules of eNTRy” favor molecules with a relatively small size, low flexibility, the inclusion of an ionizable nitrogen or amine group, and that have a low globularity. Molecules that fit these trends have a higher chance of accumulating in G- bacteria than molecules that do not.[3,6,7] Successful results have been obtained by using the adjuvant method of antibiotic production, and this was done by Hergenrother et. al. Using the eNTRy rules, three antibiotics that only worked against G+ bacteria were modified to work against G- bacteria as well, making them broad spectrum by adding amines to the structure that do not interfere with the method of inhibition. This addition increased the antibiotics accumulation in G- bacteria and exhibited antibacterial effects against them, showing that modifications to novel antibiotics can increase their effectiveness against G- bacteria.[6] Hergenrother et. al. also studied the effects of adding guanidinium and pyridine groups to novel antibiotics utilizing the aforementioned rules of eNTRy, since these groups

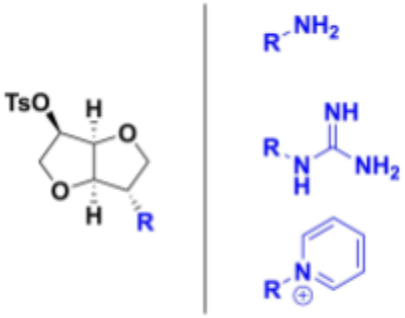


Figure 1: Compound 14 modified by Hergenrother et. al. to include an amine, guanidinium, or pyridine

contain amines with varied charge distributions that differ from simple primary amines. Initially, they edited 20 different compounds, attaching either a primary amine, a guanidinium, or a pyridine, synthesizing a total of 60 compounds (Figure 1). Each compound was then tested for their accumulation in wild-type EC. It was found that 85% of the compounds that had guanidinium or primary amines attached to them were able to accumulate in the EC, while only 25% of the pyridine compounds were able to accumulate. After this, the researchers chose 6 G+ effective antibiotics that had significantly different structures and targets in the cells, and each one was modified to include a primary amine, guanidinium, or pyridine in the same position. Each compound and its 3 derivatives were tested against wild-type EC, and their amounts of accumulation were observed and recorded. All guanidine and primary amine derivatives showed an increase in accumulation compared to their normal counterparts, while 4 out of 6 pyridine derivatives showed an increase in accumulation. More importantly, 5 out of the 6 drugs showed increased inhibitory effects against G- wild-type EC, showing that adding nitrogenous groups to antibiotics can increase their effectiveness against G- bacteria.[6]

Recently, the rules of eNTRy into PA have also been explored by the Hergenrother research group, since getting past PA utilizing the eNTRy rules developed for EC was particularly challenging. After screening 345 individual compounds for accumulation in PA and EC, each one a base antibiotic structure modified with amine groups with differences in length or location, trends were determined for eNTRy. In addition to having a positive charge, low globularity, and ≥5 rotatable bonds, it was found that having a positive polar surface area, a formal charge above 1.0, and a high hydrogen bond donor (HBD) surface area increased a molecule's accumulation in PA. To confirm that these additions were specifically helping with bypassing the OM and not accumulating in other unpredictable ways, a subset of compounds were screened for accumulation in PA as well as while in the presence of $MgCl_2$, which indicates if a molecule utilizes the self-promoted uptake pathway. Compounds modified with monoamines, diamines, and guanidinium groups showed very little accumulation in PA when in the presence of $MgCl_2$, proving that they made it past the OM using the self-promoted uptake pathway. Accumulation in PA was also tested with antibiotics modified with amines, guanidiniums, and pyridine groups, mirroring the researcher's own studies with EC. Results also mirrored those of the EC accumulation trials, with increased accumulation of amines (63% accumulators) and guanidinium groups (69% accumulators) in PA.[8]

Methods of inhibiting G- bacteria have been theorized and tested, with the main goal of either weakening or getting through the tough phospholipid bilayer. One

method of phospholipid bilayer weakening that has been theorized by MacNair et. al and studied by Katsu et. al. involved the addition of amidine groups to the cellular environment, resulting in the permeability of the membrane increasing, potentially allowing antibiotics through the membrane that, under normal conditions, would not be able to penetrate into the cell. This was studied using a new assay method in which the permeability of G- bacteria was measured by the efflux of potassium ions when the bacteria was in the presence of diamide molecules. In addition, antibiotics novobiocin and tyrocidine A were added to the assay plates and, combined with the effects of diamide molecules, they were able to penetrate and inhibit the G- bacteria.[9,10]

Another main method of formulating antibiotics that are able to get through the phospholipid bilayer is the addition of an adjuvant to a known antibiotic that works well against G+ bacteria. Adjuvants are molecules that can be attached to antibiotics to increase their effectiveness against G- bacteria, increasing the overall longevity of that antibiotic's use and possibly decreasing the amount of it needed to treat an infection, theoretically decreasing the chances of the bacteria gaining resistance.[4] Methods proposed by Schweizer et. al. involve adjuvants that also worked as antibiotics, referred to as pharmacore molecules, and these were linked to antibiotics proven effective at inhibiting G+ bacteria. The linkage between the two molecules can be either cleavable and non-cleavable, and they can be linked together by a specific linking molecule. Carbamate-based cleavable hybrids are meant to travel throughout the body linked together and, once they enter the cell, cleave apart when exposed to esterases and release the antibiotic. This strategy allows the antibiotic to get past the phospholipid bilayer in G- bacteria and inhibit the pathogen like it was a normal G+ bacteria.[11] Non-cleavable hybrids are meant to stay together throughout their course in the body and the cell, allowing the pharmacore molecules to pass through the phospholipid bilayer and attack the pathogen.[12] The main problem with the adjuvant approach is that identifying the correct adjuvant to use with the correct antibiotic is very difficult since there are an infinite number of combinations. Another challenge to overcome is that the adjuvant and antibiotic cannot have negative interactions with one another that would decrease the antibiotics effectiveness or inhibitive properties. The creation of a good linking molecule is another challenge with

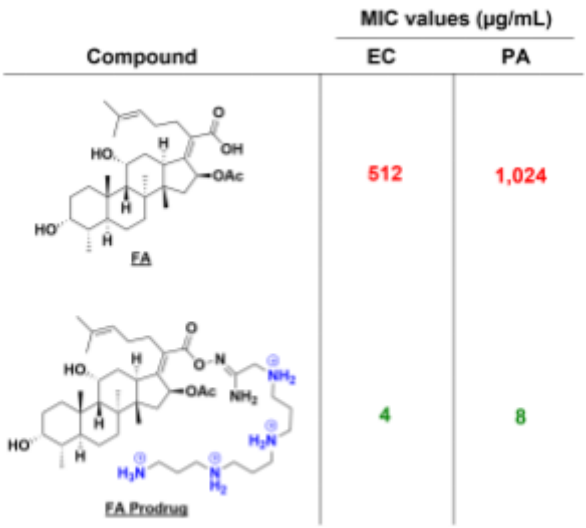


Figure 2: MIC values of FA and the FA prodrug gathered by Hergenrother et. al.

this method of attack, since the linking molecule must survive the body's metabolic enzymes and reach the bacteria intact to inhibit it.[4,12]

Recently, Hergenrother et. al. have published research in which they prove that a cleavable adjuvant attached to a G+ effective antibiotic increases accumulation in PA and drastically decreases MIC values against the pathogen. For their study, the researchers modified the G+ effective antibiotic FA with an amidoxide adjuvant that contained a long carbon-amine chain, increasing the HBD surface area and positive polar surface area of the molecule to get past the PA OM. The nitrogenous adjuvant was designed to be cleaved off inside the cell, releasing free FA to inhibit PA. When tested against PA, the FA prodrug had a 128-fold increase in effectiveness at inhibiting the pathogen than its unmodified form (Figure 2), showing that an adjuvant approach to inhibiting PA and other G- bacteria is possible.[8]

Further research into this topic will be done utilizing the research done by Hergenrother et. al., Schweizer et. al., Melander et. al., and Katsu et. al. to design and synthesize a nitrogenous adjuvant that is capable of linking to an antibiotic, then hopefully is able to bypass the OM of G- bacteria in the form of a hybrid prodrug. The adjuvant will contain a benzene ring with a primary amine and a guanidinium group attached at the para positions of the ring. This adjuvant will covalently bond to a cleavable linker, and then that complex will covalently bond to an antibiotic effective against G+ bacteria. To not hinder the mechanism of action of the antibiotic, the two molecules will be linked with a cleavable carbonyl linker, so that once the prodrug passes through the OM, it can be broken apart by an enzyme to release the antibiotic and allow it to function properly (Figure 3). Once synthesized, the product antibiotic-adjuvant hybrid will be tested against different G- bacterial strains, including multidrug-resistant PA, in a cell death assay to test for inhibitive properties.

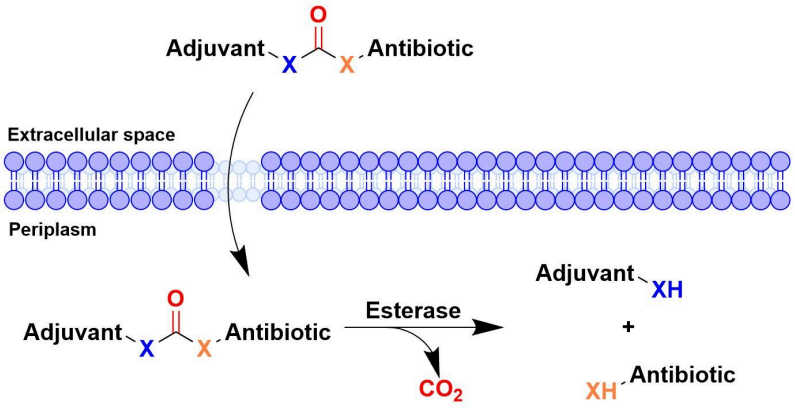



Figure 3: Visualization of how the antibiotic-adjuvant hybrid will be cleaved at the carbonyl group inside of the cell

# Experimental

## General

All reagents were purchased and used without prior purification, and anhydrous solvents dichloromethane (DCM) and tetrahydrofuran (THF) were obtained from an inert solvent system. All reactions were run in an argon atmosphere and flame-dried flasks since the reactions are air and water-sensitive. Evaporation and condensation of solvents were done in vacuo at 40 °C. Thin-layer chromatography was done using $SiO_2$-coated glass plates and visualized using UV light. Nuclear Magnetic Resonance data ($^1H$ or $^{13}C$) was gathered using a Bruker Ascend 400 spectrometer at 298 K. Solvent peaks present were used as an internal reference for other peaks. Coupling constants (*J*) (H, H) are reported in Hz. Coupling patterns are designated as singlets (s), doublets (d), triplets, (t), doublet of doublets (dd), broad (br), and multiplet (m). All steps performed for biological assays were performed using sterile techniques. Glassware used for biological assays were sterilized in an autoclave at 121 °C overnight. Tryptic soy broth (TSB) used for bacterial growth was prepared by dissolving 30 grams of BD Bacto TSB powder into 1 L of deionized water. Bacterial strains purchased or acquired for this study were *E. coli* (EC 25922), drug-resistant *P. aeruginosa* (PA 2108), and drug-resistant *A. baumanii* (AB 179178).

## Safety Statement

Solvents used in the experiments include ethyl acetate (EA), hexane (HX), dichloromethane (DCM), methanol (MeOH), tetrahydrofuran (THF), and diethyl ether. Each solvent was used with caution. In synthetic route 2 of this paper, Triphosgene was used to link the adjuvant and antibiotic together. When this chemical comes into contact with water, triphosgene releases toxic gas that causes burns in skin, eyes, and mucous membranes, and it is fatal when inhaled. Proper handling of triphosgene involves wearing PPE and only exposing it to air under a fume hood, and these precautions were taken in this experiment.[13] When handling multidrug-resistant PA and AB during bioassays, BSL 2 safety protocol was used.

## Guanidine Reagent Synthesis

Commercial grade p-phenylenediamine (0.50 g, 1.0 eq, 3.458 mmol) was added to a round-bottom flask with triethylamine (1.93 mL, 4.0 eq, 13.831 mmol) and dissolved in dichloromethane (DCM) (6.92 mL, 0.5 M) under argon. Once the reagents

were dissolved, 1,3-di-Boc-2-(trifluoromethylsulfonyl) guanidine (1.35 g, 1.0 eq, 3.458 mmol) was added to the mixture, and this was stirred at room temperature for 16 hours. The reaction mixture was passed through a silica plug eluted with DCM, then eluted with 5% MeOH/DCM. If the product was not isolated fully with the first silica plug, it was passed through multiple full-sized columns using 2.5% MeOH/DCM until the product was fully isolated. All fractions were monitored by TLC, and the fractions containing desired product were concentrated under reduced pressure to yield a dry solid product, now referred to as, "Guanidine reagent." Characterization was performed on the guanidine reagent, involving $^{1}$H-NMR, $^{13}$C-NMR, and LC-MS (SF1-SF2).
Guanidine Reagent: $^{1}$H-NMR ($CDCl_3$, 400 MHz): δ 10.05 (s, 2H), 7.29 (d, J=0.02, 2H), 6.65 (d, J=0.02, 2H), 5.30 (s, 1H), 1.50 (d, J=0.05, 18H), 1.27 (s, 1H). $^{13}$C-NMR ($CDCl_3$, 100 MHz): δ 163.59, 154.05, 153.35, 144.02, 127.76, 124.57, 115.34, 83.49, 79.54, 28.20, 28.11. LCMS: expected M+1: 351.42; observed: 351

## Synthetic Route 1: 4-Nitrophenyl Chloroformate

The guanidine reagent (0.10 g, 1.0 eq, 0.2854 mmol) was added to a round-bottomed flask and dissolved in ⅔ of a 1:1 THF/DCM mixture (0.05 M), then the mixture was cooled to 0°C under argon. Once this was cooled, triethylamine (39.8 µL, 1.0 eq, 0.2854 mmol) was added to the mixture, and this was stirred for 10 minutes. While the main reaction mixture was stirring, commercial grade 4-nitrophenyl chloroformate (0.05 g, 1.0 eq, 0.2854 mmol) was added to another round-bottom flask and dissolved in ⅓ of the THF/DCM mixture. After stirring for 10 minutes, the 4-nitrophenyl chloroformate mixture was added to the guanidine reagent/triethylamine mixture dropwise over 5 minutes. This mixture was then stirred for 1 hour at 0°C then 1 hour at room temperature while being monitored by mass spectroscopy. Once the desired product was formed, the reaction mixture was used in the second step of the reaction scheme, making this process a one-pot reaction due to concerns of the product dissociating during purification.

A chosen antibiotic (1.0 eq) was added to a round-bottom flask and dissolved in a THF (5.7 mL, 0.3 M) and $H_2O$ (1.9 mL, 0.3 M) mixture, then triethylamine (59.7 µL, 3.0 eq, 0.428 mmol) was added under argon. Once everything was dissolved in the round-bottom flask, the reaction mixture of step 1 (2.0 eq) that contained the desired intermediate was added to the antibiotic/triethylamine mixture dropwise over 5 minutes, and this was left to stir overnight.

## Route 1 Product Purification:

The reaction mixture was checked by mass spectroscopy to determine if the desired product was present, and once it was confirmed, the reaction mixture was added to a separatory funnel with ethyl acetate and deionized water. After filtering the ethyl acetate layer with deionized water twice, the ethyl acetate layer containing the product was dried with sodium sulfate ($Na_2SO_4$), reduced under pressure, and checked with mass spectroscopy to determine if the product was present. Once the product was determined to be there, the ethyl acetate was passed through different columns to separate the product from any impurities, each column using a mixture of ethyl acetate and hexane, ranging from 5% EA/HX to 25% EA/HX. This process was monitored using TLC, and once the product was successfully isolated, it was reduced under pressure to yield a solid product.

## Synthetic Route 2: Triphosgene

A chosen antibiotic (1.0 eq, 0.2854 mmol) was added to a round bottom flask and dissolved in ⅓ of a total of 10 mL of DCM (0.3 M), then the mixture was cooled to 0℃ under argon. Once this was cooled, triethylamine (0.2 µL, 5.0 eq, 1.427 mmol) was added to the mixture dropwise over 5 minutes under argon. While the main reaction mixture was stirring, commercial-grade triphosgene (33.87 mg, 0.4 eq, 0.11416 mmol) was added to a separate round bottom flask and dissolved in another ⅓ of DCM under argon. Once this was dissolved, the triphosgene mixture was added to the antibiotic/triethylamine mixture dropwise over 5 minutes under argon, and this was left to stir for 25 minutes. While the main reaction mixture was stirring, guanidine reagent (0.10 g, 1.0 eq, 0.2854 mmol) was added to another separate round bottom flask and dissolved in the last ⅓ of DCM. After the main reaction mixture stirred for 25 minutes, the guanidine reagent mixture was added to the main reaction mixture dropwise over 5 minutes. Once all reagents were added, the main reaction mixture was left to stir for 18 hours, warming from 0℃ to room temperature overnight.

## Route 2 Product Purification:

The reaction mixture was checked with mass spectroscopy to determine if the desired product was present, and once it was confirmed, the reaction mixture was added to a separatory funnel with DCM and a saturated sodium bicarbonate ($NaHCO_3$) solution. After filtering the DCM layer with the saturated $NaHCO_3$ solution twice, the DCM layer containing the product was dried with sodium sulfate ($Na_2SO_4$), reduced

under pressure, and checked with mass spectroscopy to determine if the product was present. Once the product was determined to be there, the DCM was passed through different columns to separate the product from any impurities, each column using a mixture of methanol and DCM, ranging from pure DCM to 25% MeOH/DCM. This process was monitored using TLC, and once the product was successfully isolated, it was reduced under pressure to yield a solid product.

Once the solid product was isolated, it was exposed to 5-10 mL of 4M HCl in dioxane in a round bottom flask to deprotect the Boc groups on the adjuvant, and this was left to stir overnight. After stirring overnight, the mixture was blow dried down to a solid and checked with mass spectroscopy to determine if the deprotected product was present, and when it was confirmed, 5-10 mL of diethyl ether was added to the round bottom flask to neutralize the product. The flask was left to stir overnight, and the next day, the diethyl ether was blow dried down to a solid and checked again by mass spectroscopy to confirm if the product was present. Once the deprotected product was confirmed to be present, more washes with diethyl ether and hexanes were done to isolate the product from any leftover impurities, and the product was checked again by mass spectroscopy to study this. Once it was confirmed to be pure and present, the final product was dried under reduced pressure and weighed in a vial to determine the yield. After weighing, characterization was performed on the product to confirm its structure, and this involved studying with $^{1}$H-NMR, $^{13}$C-NMR, and LC-MS (SF3-SF6).

Guanidine Reagent + Sulfadiazine: $^{1}$H-NMR (DMSO, 400 MHz): δ 9.86 (s, 2H), 9.65 (s, 1H), 8.50 (s, 2H), 7.89 (d, J=0.02, 2H), 7.61 (d, J=0.02, 2H), 7.53 (d, J=0.02, 2H), 7.38 (s, 1H), 7.16 (d, J=0.02, 2H), 7.04 (s, 1H), 3.55 (br, 1H), 2.99 (s, 1H), 1.23 (s, 1H). $^{13}$C-NMR (DMSO, 100 MHz): δ 158.83, 157.44, 156.72, 152.77, 144.42, 138.76, 132.77, 129.54, 129.22, 126.57, 119.45, 117.41. LCMS: expected M+1: 427.69; observed: 427

Guanidine Reagent + Sulfamethoxazole: $^{1}$H-NMR (DMSO, 400 MHz): δ 11.27 (s, 1H), 9.84 (s, 1H), 9.61 (d, J=0.02, 1H), 7.76 (d, J=0.02, 2H), 7.64 (d, J=0.02, 2H), 7.53 (d, J=0.02, 2H), 7.32 (s, 1H), 7.17 (d, J=0.02, 2H), 6.13 (s, 1H) 3.38 (s, 1H) 2.99 (s, 1H), 2.30 (s, 3H), 1.23 (s, 1H) $^{13}$C-NMR (DMSO, 100 MHz): δ 170.69, 158.09, 156.68, 152.73, 144.75, 138.70, 131.89, 129.29, 128.67, 126.58, 119.52, 117.92, 95.84, 12.54. LCMS: expected M+1: 430.69; observed: 430

## Biological Assays

To test the hybrid molecule's effectiveness at inhibiting growth of the target bacteria, cell death assays were run with the synthesized molecules against multidrug

resistant strains of PA and AB, as well as a wild-type strain of EC. Inhibition testing was run in triplicate using the broth microdilution method outlined by the Clinical and Laboratory Standards Institute. To determine minimum inhibitory concentrations (MIC) and inhibitory concentrations at 50% growth ($IC_{50}$), 96-well microtiter plates were prepared with 2-fold serial dilutions of the compounds from 0 to 128 μg/mL (final assay concentrations) dissolved in biological-grade DMSO. Each well contained 1 μL of compound in DMSO, 89 μL of TSB, and 10 μL of bacteria inoculum. Bacteria were grown from a single colony in 10 mL of TSB for 4 to 6 hours. After incubating the plates for 12 to 15 hours at 37°C, absorbance at 590 nm was read using a Biotek Synergy HTX Multimode plate reader to observe growth in each well. Data from the scans were processed by subtracting the background of the media absorbance and normalizing the data to full bacterial growth with DMSO only. MIC is defined as the lowest concentration of any hybrid molecule that achieves ≥85% growth inhibition.

# Results and Discussion

Yields of the different synthetic routes have been determined in two ways. For reactions that produced a product but were unable to be purified into a dry solid, the yield was determined in the form of percent conversion. This involved comparing the relative peak heights of the guanidine reagent and the desired product in a mass spectroscopy scan, and the resulting estimation of conversion is reported. For reactions that were able to produce a dry and purified product, the yield is reported traditionally by product mass.

## Guanidine Reagent Synthesis

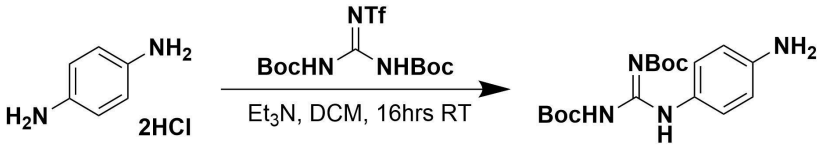



Figure 4: Detailed route of the Guanidine Reagent synthesis reaction

| Trial # | Temperature | Time | % Yield (by mass) |
|---|---|---|---|
| 1 | RT | 16hr | 58% |
| 2 | RT | 16hr | 74% |

Table 1: Reaction conditions and yields of the guanidine synthesis

The first trial of the guanidine synthesis reaction (Table 1) was done 3 times to yield a large amount of product, weighing in at 2.1095g (58% yield). This trial involved only one round of column chromatography using 5% MeOH/DCM, leading to a lot of lost product being impure. A few days after synthesis, the dried product seemed to oxidize to a light brown color, showing that a few impurities might be present. The second trial of the guanidine synthesis was performed when the product of the last reaction was depleted, and this trial had a much higher yield of 74%, mainly due to the multiple rounds of column chromatography, with 4 total rounds eluding multiple different impurities. A few days after synthesis, not much oxidation was observed, with the product turning a white/tan color and impurities that were saved from purification turning a jet black color. This could indicate a higher purity of product due to the byproducts of the reaction being filtered from the desired product, but no quantitative results have been collected to compare these two products.

# Synthetic Route 1: 4-Nitrophenyl Chloroformate

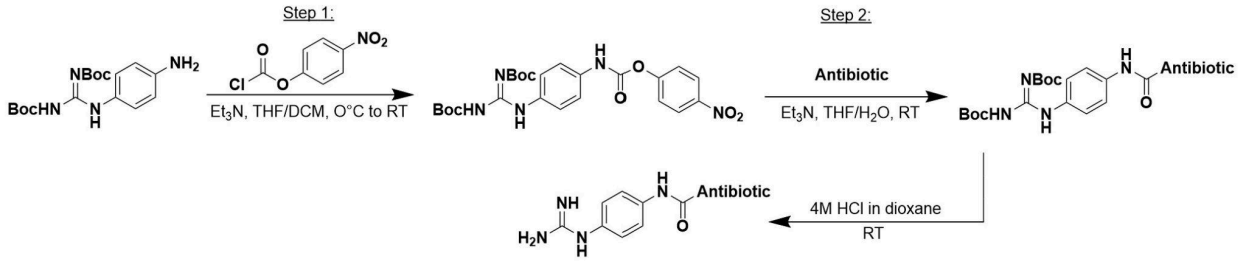



Figure 5: Detailed synthetic route 1 using 4-Nitrophenyl Chloroformate as a linker

| Trial # | Temperature | Time | % Conversion (MS) |
|---|---|---|---|
| 1 | 0℃ to RT | 24hr | 85% |
| 2 | 0℃ to RT | 2.5hr | 85% |
| 3 | 0℃ to RT | 2hr | 85% |
| 4 | 0℃ to RT | 24hr | 0% |
| 5 | 0℃ to RT | 2hr | 0% |
| 6 | 0℃ | 2hr | 50% |
| 7 | 0℃ | 2.5hr | 50% |
| 8 | 0℃ | 2hr | 50% |
| 9 | 0℃ | 1.75hr | 50% |
| 10 | 0℃ | 1hr | N/A |
| 11 | 0℃ | 2.5hr | 50% |

Table 2: Reaction conditions and yields for step 1 of the 4-Nitrophenyl Chloroformate scheme (Figure 2)

| Trial # | Antibiotic | EtN (eq) | Temperature | Time | % Conversion (MS) | De-Boc Conversion (MS) |
|---|---|---|---|---|---|---|
| 1 | Ampicillin | 3.0 eq | RT | 24hr | <5% | Detected |
| 2 | Ampicillin | 3.0 eq | RT | 24hr | <5% | Detected |
| 3 | Trimethoprim | 3.0 eq | RT | 24hr | Trace | Detected |
| 4 | Sulfadiazine | N/A | N/A | N/A | N/A | N/A |
| 5 | Sulfadiazine | N/A | N/A | N/A | N/A | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6 | Sulfadiazine | 3.0 eq | RT | 24hr | Trace | Detected |
| 7 | Trimethoprim | 3.0 eq | RT | 24hr | Trace | Detected |
| 8 | Sulfadiazine | 3.0 eq | RT | 24hr | 0% | N/A |
| 9 | Tedizolid | 3.0 eq | RT | 24hr | 0% | N/A |
| 10 | Tedizolid | N/A | N/A | N/A | N/A | N/A |
| 11 | Tedizolid | 4.0 eq | 75℃ | 24hr | N/A | N/A |

Table 3: Reaction conditions and yields for step 2 of the 4-Nitrophenyl Chloroformate scheme (Figure 2)

Steps 1 and 2 of the 4-nitrophenyl chloroformate reaction (Figure 5) had varying temperatures and reagent quantities, and these values are listed in Tables 2 and 3. Issues that occurred through synthesis began with the percent conversion of the second reaction decreasing from 85% to 50%, with 0% conversion in between, and the reasoning behind this reduction in yield is unknown. Successfully linking the adjuvant-linker molecule to the chosen antibiotic proved to be mostly unsuccessful, with the only cases of the reaction actually working were the trials testing with ampicillin with a very low conversion rate before deprotection. No dried and purified products were isolated from these reactions, but deprotection was still performed on the impure products to ensure they were somewhat present.

## Synthetic Route 2: Triphosgene

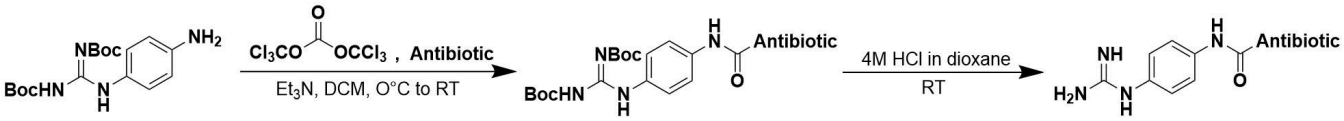



Figure 6: Detailed synthetic route 2 using Triphosgene as a linker

| Trial # | Antibiotic | Temperature | % Conversion (MS) | % De-Boc Conversion (MS) | % Yield (by mass) |
|---|---|---|---|---|---|
| 1 | Sulfadiazine | 0℃ to RT | 0% | N/A | N/A |
| 2 | Sulfadiazine | 0℃ to RT | 0% | N/A | N/A |
| 3 | Sulfadiazine | 0℃ to RT | <5% | N/A | N/A |
| 4 | Sulfamethoxazole | 0℃ to RT | 40% | 0% | N/A |
| 5 | Sulfamethoxazole | 0℃ to RT | 70% | 99% | 15.9% |
| 6 | Ampicillin | 0℃ to RT | 0% | N/A | N/A |
| 7 | Sulfadiazine | 0℃ to RT | 10% | N/A | N/A |
| 8 | Sulfamethoxazole | 0℃ to RT | 90% | 99% | 50.4% |
| 9 | Ampicillin | 0℃ to RT | 0% | N/A | N/A |
| 10 | Ampicillin | 35℃ to 0℃ | 0% | N/A | N/A |
| 11 | Ampicillin | 35℃ to 0℃ | 0% | N/A | N/A |
| 12 | Ampicillin | 0℃ to 35℃ | 0% | N/A | N/A |
| 13 | Sulfadiazine | 0℃ to RT | 30% | 99% | 19.9% |

Table 4: Reaction conditions and yields for synthetic route 2

The second synthetic route using triphosgene (Figure 6) proved to have a few issues during synthesis. Very little conversion was observed in the trials using sulfadiazine, but an increasingly large amount of conversion was observed in trials 4, 5, and 8, all using sulfamethoxazole. The increase in percent conversion reported is due to the mass spectroscopy scans being performed at different steps in purification for each product. As trials for sulfamethoxazole were done, more purification techniques, such as column chromatography, were performed on each to get a larger amount of pure product isolated. For trial 4, there is no reported de-Boc conversion, and this is due to the product being preemptively discarded by human error. A strange dimer

formation between two guanidine reagent molecules was observed in the second trial with sulfadiazine, and it was observed through mass spectroscopy. No other quantitative data has been gathered on this dimer product to confirm the product, but a possible structure has been theorized in Figure 7. The same dimer side product was formed in trail 7 with sulfadiazine along with the desired product, but both compounds were lost in purification. For a brief time, linking the adjuvant with ampicillin became a main priority to show that these methods can be used with β-lactam drugs. These trials were unsuccessful with heat experimentation, and no product was observed to be forming in mass spectroscopy scans. Adjuvant linkage and deprotection was successfully performed with sulfadiazine and sulfamethoxazole in trials 5, 8, and 13, with relatively high yields calculated by mass. These products were dried and used in biological testing.

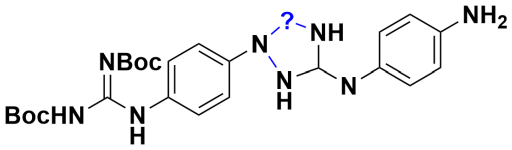



Figure 7: Theorized structure for the guanidine dimer side product

# Biological Assays

| | Positive Control | | | Trial 5 Product | | | Trial 8 Product | | | Trial 13 Product | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | | | | 128 | 128 | 128 | 128 | 128 | 128 | 128 | 128 | 128 |
| **B** | | | | 64 | 64 | 64 | 64 | 64 | 64 | 64 | 64 | 64 |
| **C** | | | | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 | 32 |
| **D** | | | | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 |
| **E** | | | | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| **F** | | | | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| **G** | | | | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| **H** | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** |

| | |
|---|---|
| | = **Growth Media** |
| | = **Growth Media + Bacteria** |
| | = **Growth Media + Bacteria + Trial 5 Product (µg/ml)** |
| | = **Growth Media + Bacteria + Trial 8 Product (µg/ml)** |
| | = **Growth Media + Bacteria + Trial 13 Product (µg/ml)** |

Figure 8: Master plate layout used for all cell death assays

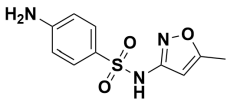
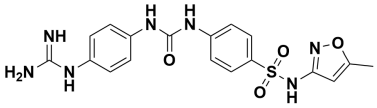
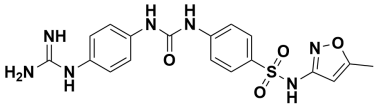
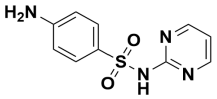
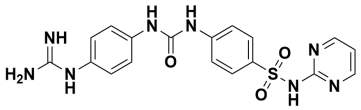
| **Compound Name** | **Chemical Structure** | **PA $IC_{50}$ (µg/mL)** | **AB $IC_{50}$ (µg/mL)** | **EC $IC_{50}$ (µg/mL)** |
|---|---|---|---|---|
| Sulfamethoxazole |  | >128 | >128 | >128 |
| Trial 5 Product |  | ≥128 | N/A | >128 |
| Trial 8 Product |  | >128 | N/A | >128 |
| Sulfadiazine |  | >128 | >128 | >128 |
| Trial 13 Product |  | **>32** | N/A | >128 |

Table 5: Results of the cell death assays, reported as inhibitory concentration at 50% growth ($IC_{50}$)

A few errors occurred while performing biological assays. The cell death assay ran with AB against the synthesized hybrid molecules contained multiple pipetting errors, so no data detailing inhibition of the pathogen was gathered. When PA and AB were transferred to their respective assay plates, all wells were inoculated with bacteria, leaving no plates to calculate a background absorbance for. Correct pipetting

was performed on the EC plate, which produced a very consistent background signal, and this background calculation was used for the other plates for processing. Although Trial 5 and Trial 8 products were structurally the same, they were tested separately in the cell death assays due to concerns over their individual purities.

Significant inhibition was observed with all products against multidrug-resistant PA, with the Trial 13 product indicating an $IC_{50}$ value of >32 µg/mL against the pathogen and over 60% inhibition at 64 µg/mL (Figure 9). The Trial 13 product showed an increased percent growth at 128 µg/mL compared to 64 µg/mL, and this was caused by the opacity of the product at a higher concentration. Trail 5 product showed increased inhibition of PA compared to the Trial 8 product, and this is theorized to be caused by the Trial 8 product being not as pure as its counterpart.

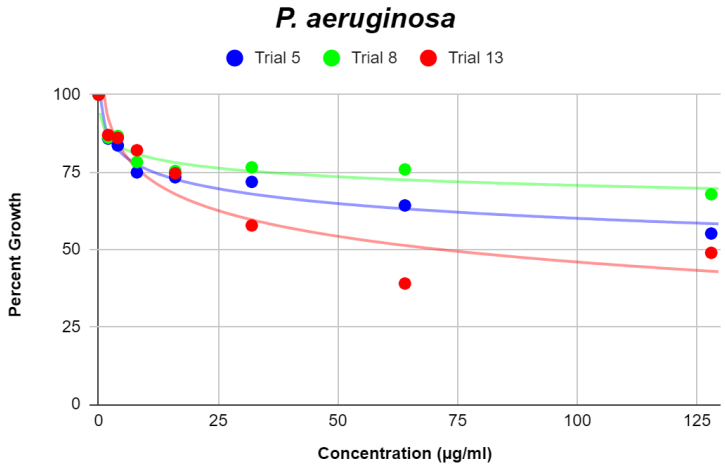



Figure 9: Percent growth of PA against different concentrations of synthesized products

# Conclusion/Future Work

Overall, successful synthesis was observed to produce two hybrid molecules composed of a guanidine-based adjuvant and a G+ effective antibiotic, covalently linked together by a cleavable carbonyl linker. These products were characterized using $^{1}$H-NMR, $^{13}$C-NMR, and LC-MS to confirm their structures, then they were used in biological assays to study their antibiotic effects. Out of the two products tested, the Trial 13 product combining the adjuvant with sulfadiazine showed significant inhibition of multidrug-resistant PA, with an $IC_{50}$ of >32 μg/mL. Future work will involve using different antibiotics and reaction conditions in both synthetic routes to create new hybrid molecules, as well as designing a more effective purification scheme for each product to properly isolate them. Investigating linkage conditions between other sulfa drugs would be desirable, since successful linkage was observed with sulfadiazine and sulfamethoxazole. B-lactam linkage is also a desirable goal for this research in the future, since almost all ampicillin trials in both synthetic routes were unsuccessful.

# Acknowledgements

I would like to thank the University of North Carolina at Asheville for the opportunity to perform undergraduate research on campus. I would also like to thank all faculty and staff from the Department of Chemistry and Biochemistry at UNCA for providing a helpful and friendly environment for learning and growing. I specifically would like to thank Dr. Amanda Wolfe and the students/colleagues I have worked with in the Wolfe lab for all the help I have received while doing research.

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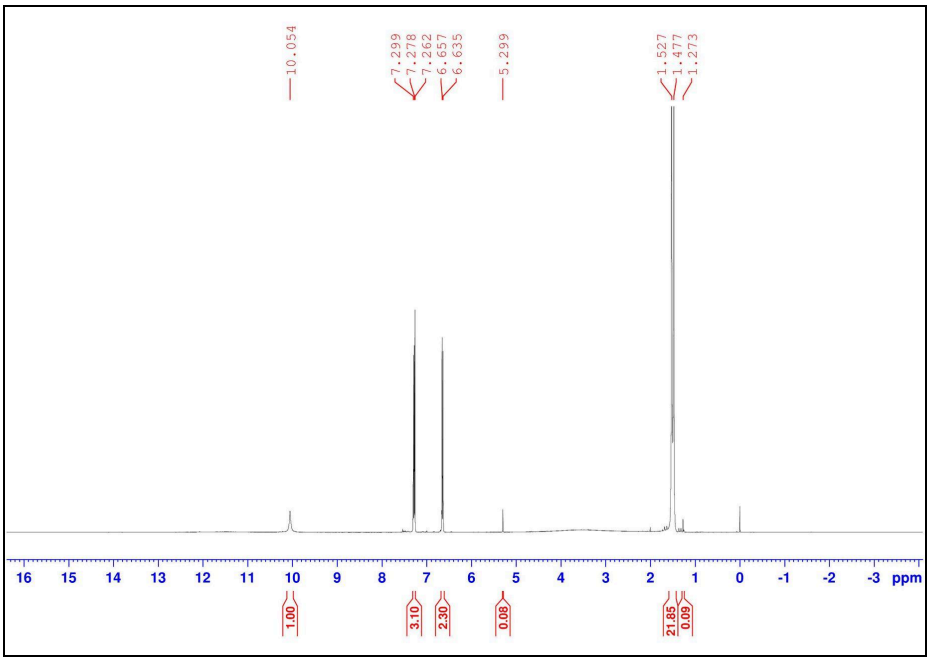
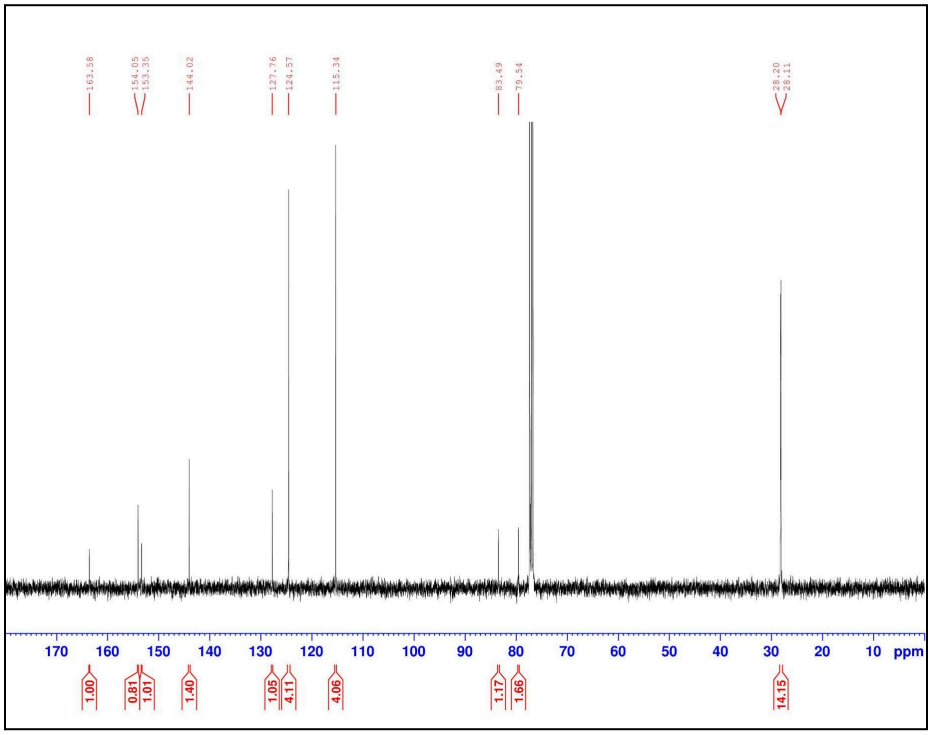
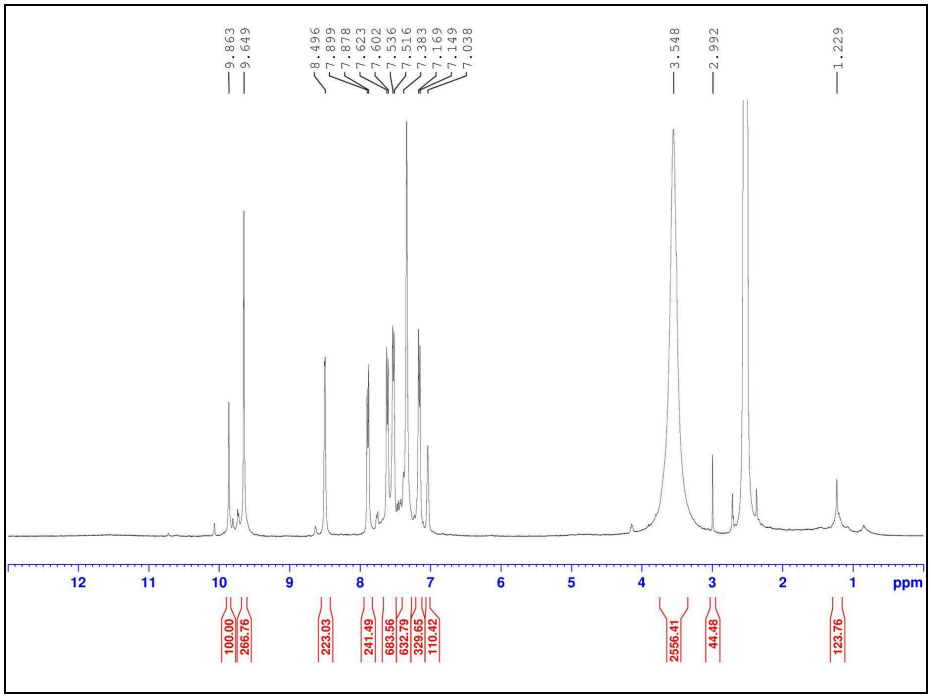
# Supplemental Data



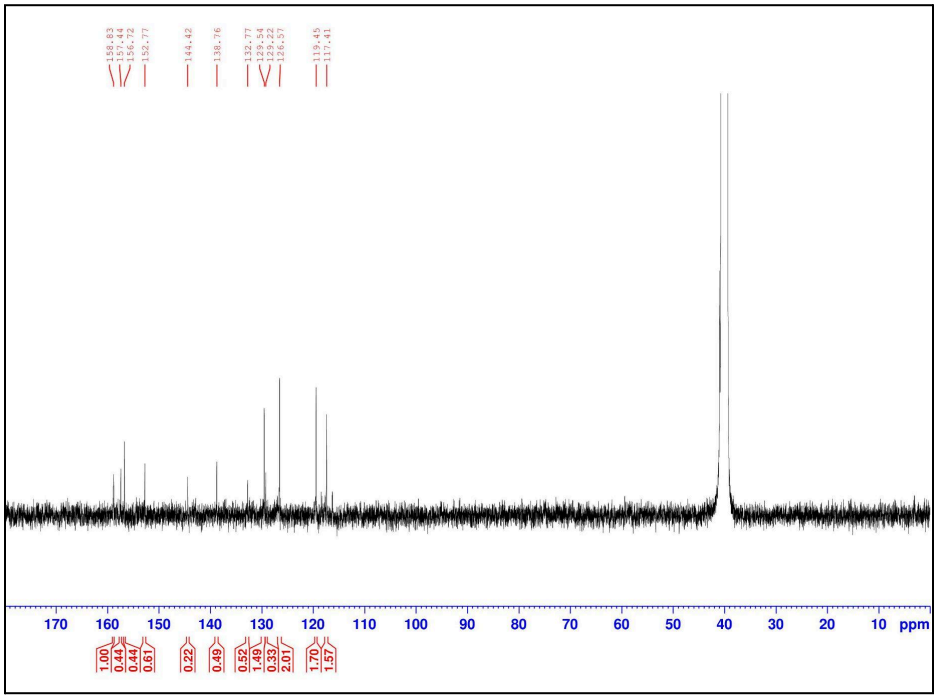

Supplemental Figure 1: $^{1}$H-NMR of the Guanidine Reagent



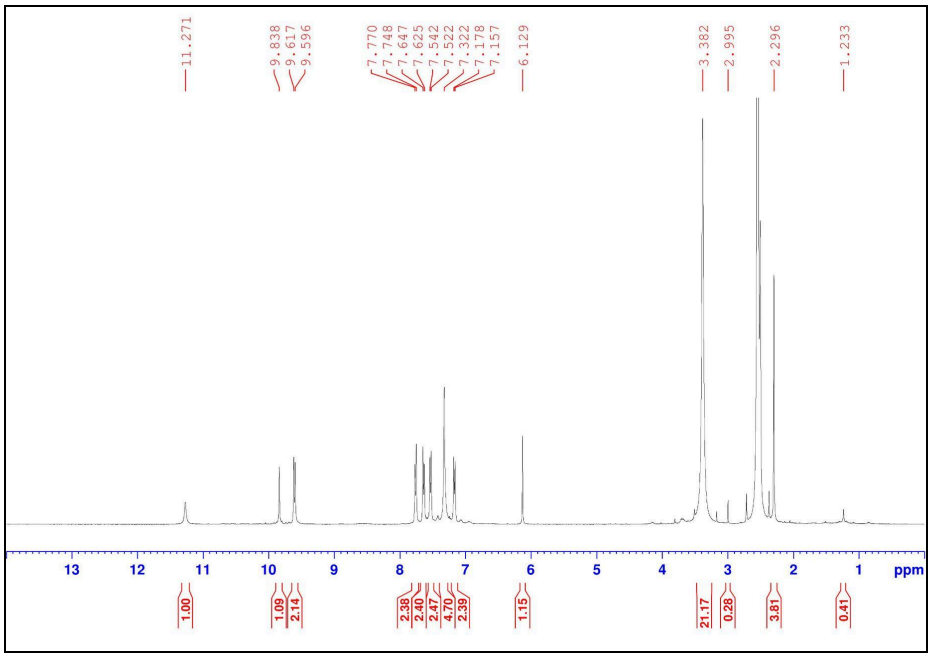

Supplemental Figure 2: $^{13}$C-NMR of the Guanidine Reagent



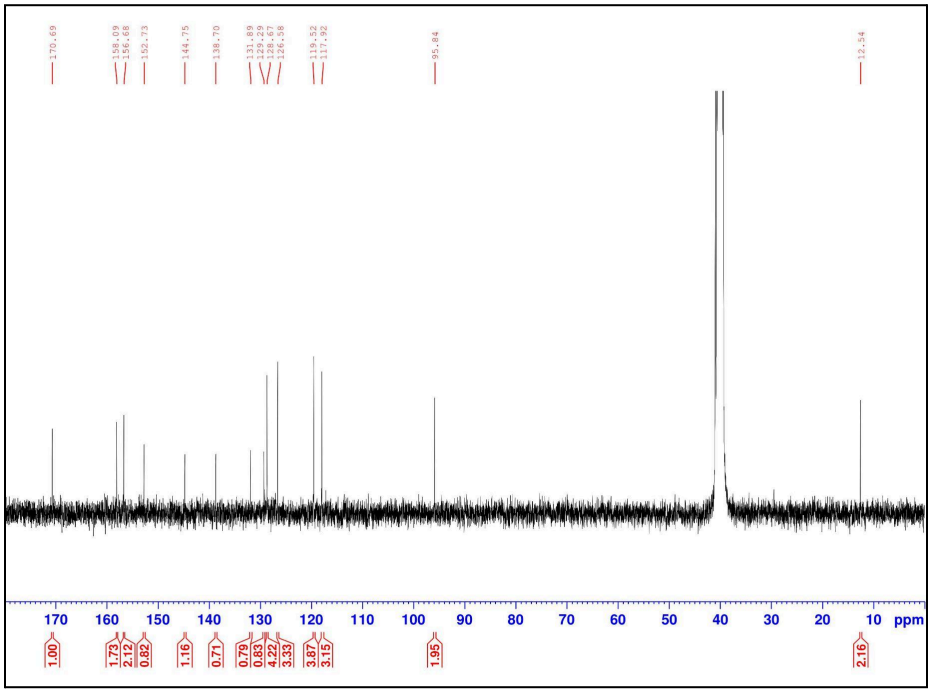

Supplemental Figure 3: $^{1}$H-NMR of the Guanidine + Sulfadiazine product




Supplemental Figure 4: $^{13}$C-NMR of the Guanidine + Sulfadiazine product




Supplemental Figure 5: $^{1}$H-NMR of the Guanidine + Sulfamethoxazole product




Supplemental Figure 6: $^{13}$C-NMR of the Guanidine + Sulfamethoxazole product