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Synthesis and Evaluation of Antibiotics with Cleavable Guanidine Linkers to Combat Gram-Negative Antibiotic Resistance Mechanisms

Jason Dunford
Chemistry and Biochemistry
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty advisor: Amanda Wolfe

Abstract

Antibiotic resistance is a rising global concern with several bacteria having resistance mechanisms to many widely used antibiotics. Particularly, Gram-negative bacteria present a unique challenge because of their second, semipermeable outer membrane (OM) which offers additional protection against antibiotics. Prior studies have shown that penetration of Gram-negative OMs is dependent upon 5 separate features that most current antibiotics do not have. These features include having small molecular masses, high polar surface areas, unsubstituted amines, and low globularity. To improve antibiotic activity and facilitate OM penetration we have modified known antibiotics with guanidinium groups that follow these rules well, which should increase antibiotic effectiveness in host cells and result in cell death when paired with an antibiotic. By attaching the guanidinium through a cleavable linker, the antibiotic is theoretically able to permeabilize the membrane and then, through natural cellular environmental interactions, cleave the bond between the antibiotic and the linker. Cleaving the linkers is important as they likely would lead to reduced potency for the antibiotic. In particular,

sulfamethoxazole was successfully synthesized with the guanidine linker and evaluated for activity within the cell with a cell death assay against wild-type *Pseudomonas aeruginosa*, a *Pseudomonas aeruginosa* efflux knockout strain, and wild-type *Escherichia coli*. There was no visible cell death recorded in any of the assays, however. Further testing with other types of antibiotics to evaluate if this method has potential further antibiotic development capabilities will be done.

Introduction

Bacterial infections due to multidrug resistant bacteria (MDRBs) are causing a worldwide healthcare crisis. According to the CDC in 2019, these bacteria caused 2.8 million bacterial infections resulting in 35,000 deaths in the United States.[1] In particular, ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* (PA), *and Enterobacter* species) are the leading cause of nosocomial infections in the world and have multiple resistance mechanisms that are used to combat antibiotics even in a medical space (hospitals, emergency cares, etc.).[2] Focusing in, pathogens *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Enterobacter* species, and PA are all Gram-negative bacteria as well, meaning they have a characteristic second semipermeable outer membrane (OM) with a periplasmic space and cell wall between the two membranes. The OM of Gram-negative bacteria is made of an outer leaflet of lipopolysaccharides and an inner leaflet of phospholipids. These lipopolysaccharides have negatively charged phosphate groups, similar to phospholipids, that are hydrophilic and bind positive molecules.[11] This second membrane is responsible for a lot of the resistance mechanisms present in these bacteria with many current antibiotics being unable to penetrate both membranes. As well as this, the antibiotic once entering the periplasmic space is also susceptible to efflux pumps that are able to then shuttle the antibiotics back out of the cell, which prevents antibiotic accumulation and eventual death within a cell.[3,4] Figure 1 below demonstrates these mechanisms and more used specifically by ESKAPE pathogens to resist commonly used antibiotics and continue causing infection.

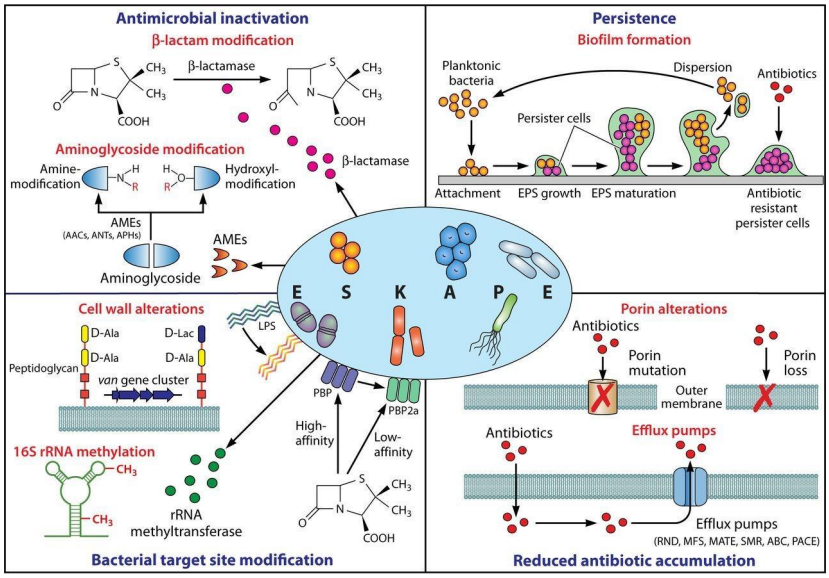


Figure 1. Diagram of antibiotic resistance mechanism commonly used by ESKAPE pathogens in order to evade cell death by antibiotics. Mechanisms specific to Gram-negative bacteria appear in the bottom right quadrant wherein porin concentrations within cell membranes that allow for antibiotics to shuttle in are decreased when antibiotic concentration becomes too high as well as efflux pumps shuttling out absorbed antibiotics back into the extracellular space. Published with permission from De Oliveira, D. M. P.; Forde, B. M.; Kidd, T. J.; Harris, P. N. A.; Schembri, M. A.; Beatson, S. A.; Paterson, D. L.; Walker, M. J. Antimicrobial Resistance in ESKAPE Pathogens. *Clinical Microbiology Reviews* **2020**, *33* (3), e00181-19. https://doi.org/10.1128/CMR.00181-19.[5]

In order to better permeate bacterial OMs, adjuvant antibiotic therapy has been used in recent years in order to increase potency of commonly used antibiotics. The adjuvants themselves usually do not have antibiotic properties, but work in tandem with antibiotics when dosed together.[6] For membrane permeability in particular, groups have found compounds like pentamidine, liproxstatin-1, and metformin act as adjuvants against certain strains of MDRBs when paired with common antibiotics such as novobiocin, vancomycin, and doxycycline.[7] When looking into how small molecules permeate OMs of Gram-negative bacteria, Richter and Hergenrother found that there were certain properties many of these compounds had that allowed them act as effective penetrating agents. These properties were dubbed “eNTRy rules”. These eNTRy rules favor molecules that: have a molecular weight <500 g/mol, a globularity <0.2, a high total polar surface area, a low number of rotatable bonds (<6), and a cLogP between -2 and 0.[8] Using these eNTRY rules, adjuvants can be designed with the properties reflected by these rules in order to make them better able to support antibiotic permeability and increase potency.

With those eNTRy rules in mind, we can apply them to the development and usage of adjuvants in antibiotic treatments both for the future and in current use. Figure 3 below compares this with the three adjuvants talked about previously. All values in green are ones that fit the eNTRy rules well, while values in red are indicative of values that do not. Globularity concerns itself with the shape of the molecule itself in terms of how spherical it is. For these small molecules, more planar configurations are favored for membrane permeability. The amount of rotatable bonds is a question of how the molecule's potential rotational movements are limited. Then, tPSA is a measure of total polar surface area, which, in this circumstance, a higher number is desired to better permeate the OM. Finally, cLogP is a measure of how greasy (lipophilic/nonpolar) the molecule is. With these molecules, a negative number is desired because of the need for high polarity. Pentamidine is found to have a low molecular weight and globularity, but lacks a high polar surface area, has too many rotatable bonds, and is too greasy to be considered a perfect candidate for potentiation. Liproxstatin-1 is a bit better with the same low molecular weight and globularity as well as only 3 rotatable bonds, but still a small polar surface area and not a lot of dipole moments present in the molecule itself. Finally, metformin has low molecular weight, 3 rotatable bonds, a negative value for cLogP, and a small globularity value with its only problem being a low polar surface area. Therefore, metformin is the candidate that was chosen by our group to be derivatized for testing for OM potentiation.

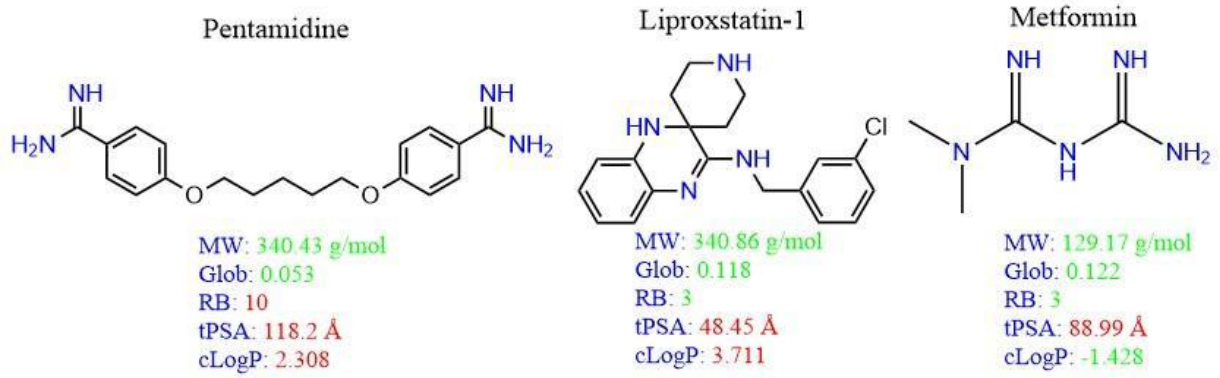


Figure 3. Structure and properties of commonly used adjuvants against Gram-negative bacteria. The eNTRy rules are followed relatively well with many of these containing reactive nitrogen species, having low globularity, and small molecular masses. Values for this figure were generated by www.entry-way.org and ChemDraw (vs. 19.0).

Previous work done by our group has led to the design of small adjuvants designed to be covalently linked to antibiotics. This paper will look at the addition of guanidine groups (which have a similar structure to metformin above, Figure 4 below) to antibiotics in order to ascertain the ability for them to kill Gram-negative bacteria. This guanidine contains many of the same properties of metformin that make it a follower of the eNTRy rules such as having low globularity, molecular weight, rotatable bonds, and cLogP.

Because of these same properties, guanidine can also be tested for good adjuvant activity.

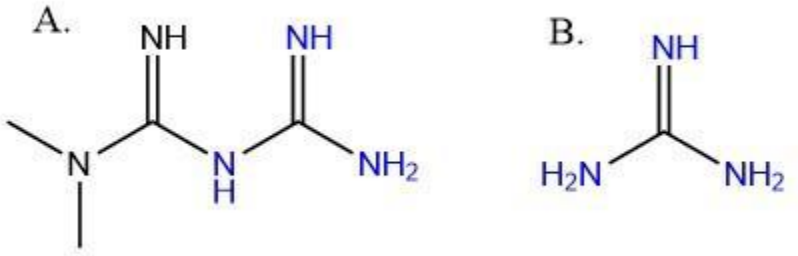
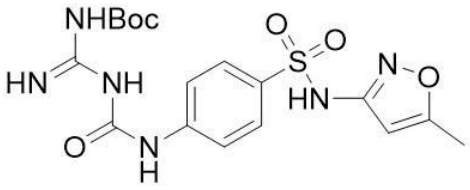


Figure 4. A is a structural representation of metformin, which was mentioned above as an effective adjuvant for Gram-negative bacterial antibiotic treatments and has an excellent fit with the eNTRy rules. B shows the guanidine adjuvant that this study is attaching to several antibiotics to measure its effects on bacterial death.

Experimental

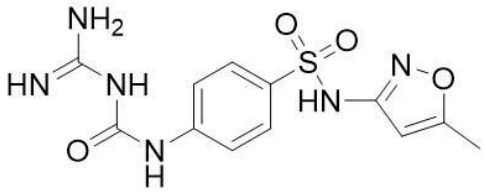
2.1 Synthesis

General. Reagents and solvents were purchased reagent-grade and used without further purification. All reactions were performed in flame-dried glassware under an Ar atmosphere. Evaporation and concentration *in vacuo* was performed at 40 °C. TLC was conducted using precoated SiO_2 60 F254 glass plates from EMD with visualization by UV light (254 nm). NMR (^1H or ^{13}C) were recorded on an Oxford Varian-400 spectrophotometer at 298 K. 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), quadruplet (q), quintuplet (qu), multiplet (m), or broad singlet (br). IR spectra were recorded on a Thermo Scientific Nicolet iS10 FT-IR spectrophotometer and measured neat. Mass spectral data were acquired on a Shimadzu single quadrupole LCMS-2020. The purity of each tested compound (>95%) was determined via ^1H NMR.



Compound 1:

Sulfamethoxazole (0.1 g, 0.40 mmol, 2 equiv.) and di-Boc-guanidine (0.05 g, 0.20 mmol, 1 equiv.) were dissolved in tetrahydrofuran (0.98 mL) with triethylamine (0.08 mL) and refluxed overnight. The crude product was then purified through automatic flash chromatography using a Biotage Isolera Four (SiO_2, 1 × 10 cm, 0−5% CH_3OH/CH_2Cl_2) and evaporated under reduced pressure to yield the product as a white solid (11.8%). ^1H NMR ($CDCl_3$, 400 MHz): δ 7.58 (d, J = 27.2, 2H), 6.60 (d, J = 8.8, 2H), 6.19 (s, 1H), 2.33 (s, 3H), 1.46 (s, 7H, should be 9H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 170.74, 157.62, 151.17, 129.32, 129.14, 126.65, 114.03, 113.36, 95.53, 30.97, 28.06, 27.981, 12.74. $[M+H]^+$: calcd for $C_{17}H_{22}N_6O_6S$ 438.46; found: 439



Compound 2:
Compound 1 (0.01 g, 0.02 mmol, 0.5 equiv.) was dissolved in a 4M HCl/1-4 Dioxane solution and allowed to stir overnight before being purified through automatic flash chromatography using a Biotage Isolera Four (SiO_2, 1 × 10 cm, 0−5% CH_3OH/CH_2Cl_2) and evaporated under reduced pressure to yield the product as a white solid (51%). ^1H NMR (Methanol-d_4, 400 MHz): δ 7.84 (d, J = 9.2, 2H), 7.64 (d, J = 8.8, 2H), 7.52 (d, J = 8.8, 2H), 6.62 (s, 2H), 6.12 (s, 1H), 2.28 (s, 3H), 1.99 (s, 1H). $[M+H]^+$: calcd for $C_{12}H_{14}N_6O_4S$ 338.08; found: 339.

2.2. Biological Evaluation

All compounds evaluated in biological assays were >95% pure.

General Sterilization Procedure. The following are general steps, unless otherwise noted. All steps were completed with aseptic techniques. All media and glassware were sterilized via autoclave at 121 °C for 60 minutes. All agitation occurred at 160 rpm in a temperature-controlled console shaker (Excella E25) at 25 °C. 10% tryptic soy broth (TSB) was made by dissolving 3 g BD Bacto TSB powder in 1 L deionized water. Full strength tryptic soy broth (FSTSB) was made by dissolving 30 g BD Bacto TSB powder in 1 L deionized water. 10% tryptic soy agar (TSA) was made by dissolving 3 g BD Bacto TSB powder and 20 g Bacto agar in 1 L deionized water. Purchased and acquired bacteria strains used were *Escherichia coli* (EC, ATCC 25922) and *Pseudomonas aeruginosa* (PA, ATCC 9027).

Antimicrobial Susceptibility Assay Procedure. Susceptibility testing was performed in biological triplicate, using the micro-dilution broth method as outlined by the Clinical and Laboratory Standards Institute. Briefly, IC_{50} determinations were carried out in 96-well microtiter plates with 2-fold serial dilutions of the compounds in duplicate. Briefly, to each well 1 µL of compound in DMSO, 89 µL of tryptic soy broth (BD Difco), and 10 µL of bacterium inoculum in tryptic soy broth were added. After incubation for 12–15 h at 37 °C, absorbance at 590 nm was read on a Biotek Synergy HTX Multi-mode plate reader. IC_{50} values were then determined by plotting the concentration versus absorbance on Excel and using the trend function to determine the concentration at 50% inhibition. Bacteria strains used were *Escherichia coli* (ATCC 15022) and *Pseudomonas aeruginosa* (ATCC 9027).

	1	2	3	4	5	6	7	8	9
A	12.8 mg/ml	12.8 mg/ml	12.8 mg/ml	12.8 mg/ml	12.8 mg/ml	12.8 mg/ml	12.8 mg/ml	12.8 mg/ml	12.8 mg/ml
B	6.4 mg/ml	6.4 mg/ml	6.4 mg/ml	6.4 mg/ml	6.4 mg/ml	6.4 mg/ml	6.4 mg/ml	6.4 mg/ml	6.4 mg/ml
C	3.2 mg/ml	3.2 mg/ml	3.2 mg/ml	3.2 mg/ml	3.2 mg/ml	3.2 mg/ml	3.2 mg/ml	3.2 mg/ml	3.2 mg/ml
D	1.6 mg/ml	1.6 mg/ml	1.6 mg/ml	1.6 mg/ml	1.6 mg/ml	1.6 mg/ml	1.6 mg/ml	1.6 mg/ml	1.6 mg/ml
E	0.8 mg/ml	0.8 mg/ml	0.8 mg/ml	0.8 mg/ml	0.8 mg/ml	0.8 mg/ml	0.8 mg/ml	0.8 mg/ml	0.8 mg/ml
F	0.4 mg/ml	0.4 mg/ml	0.4 mg/ml	0.4 mg/ml	0.4 mg/ml	0.4 mg/ml	0.4 mg/ml	0.4 mg/ml	0.4 mg/ml
G	0.2 mg/ml	0.2 mg/ml	0.2 mg/ml	0.2 mg/ml	0.2 mg/ml	0.2 mg/ml	0.2 mg/ml	0.2 mg/ml	0.2 mg/ml
H	0 mg/ml	0 mg/ml	0 mg/ml	0 mg/ml	0 mg/ml	0 mg/ml	0 mg/ml	0 mg/ml	0 mg/ml
Labels	Chloramphenicol (+ Control)			Sulfamethoxazole-Guanidine Hybrid			Sulfamethoxazole		

Figure 5. Master plate setup for the cell death assay performed. These concentrations were diluted by 100 in the assay plate. The actual plate had a dilution of pure sulfadiazine present as well for testing of other projects in the lab that is not relevant to this study.

Results

The antibiotics were linked to the guanidine groups through the use of a carbonyl substitution mechanism, which involved a nucleophilic group on the antibiotic itself attacking the carbonyl carbon on a BOC group. The general synthetic scheme is shown below in Figure 6. The points of modification include the atoms acting as the nucleophiles to attach to the guanidine, the antibiotic itself, and the base used.

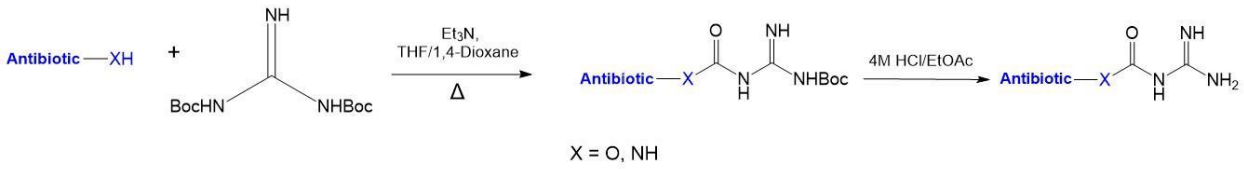


Figure 6. General synthetic scheme for antibiotic-guanidine compounds.

Of the antibiotics that were tried (sulfadiazine, trimethoprim, tedizolid, quercetin, ampicillin, and sulfamethoxazole), only one (sulfamethoxazole) was successfully synthesized, deprotected and isolated. However, over half of the antibiotics tested reacted well with the guanidine using the carbonyl substitution reaction. Figure 6 below shows the mechanism involved for the first reaction.

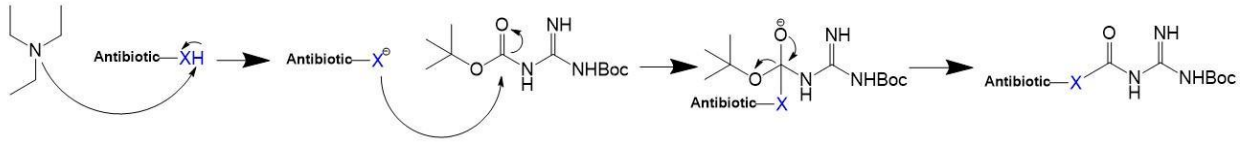


Figure 6. Mechanism of carbonyl substitution using a nucleophilic group on the antibiotic to attach to the guanidine linker.

Of the five tested antibiotics, trimethoprim, sulfadiazine, and sulfamethoxazole were able to complete the first step of the reaction by attaching the antibiotic to the guanidine. Then, upon running the second reaction, sulfamethoxazole and trimethoprim were found to have been deprotected successfully as well. However, purification of the products proved to be a difficult task. Only sulfamethoxazole was adequately purified and isolated from the antibiotics tested. Figure 7 below shows all of the different reaction conditions attempted with the different antibiotics. An “X” over a reaction arrow indicates that this reaction did not take place successfully and the synthesis was restarted under different conditions or abandoned, whereas an “?” over the reaction arrow indicates that the reaction was observed to be successful using LC-MS, but not isolated and purified for biological evaluation due to time constraints.

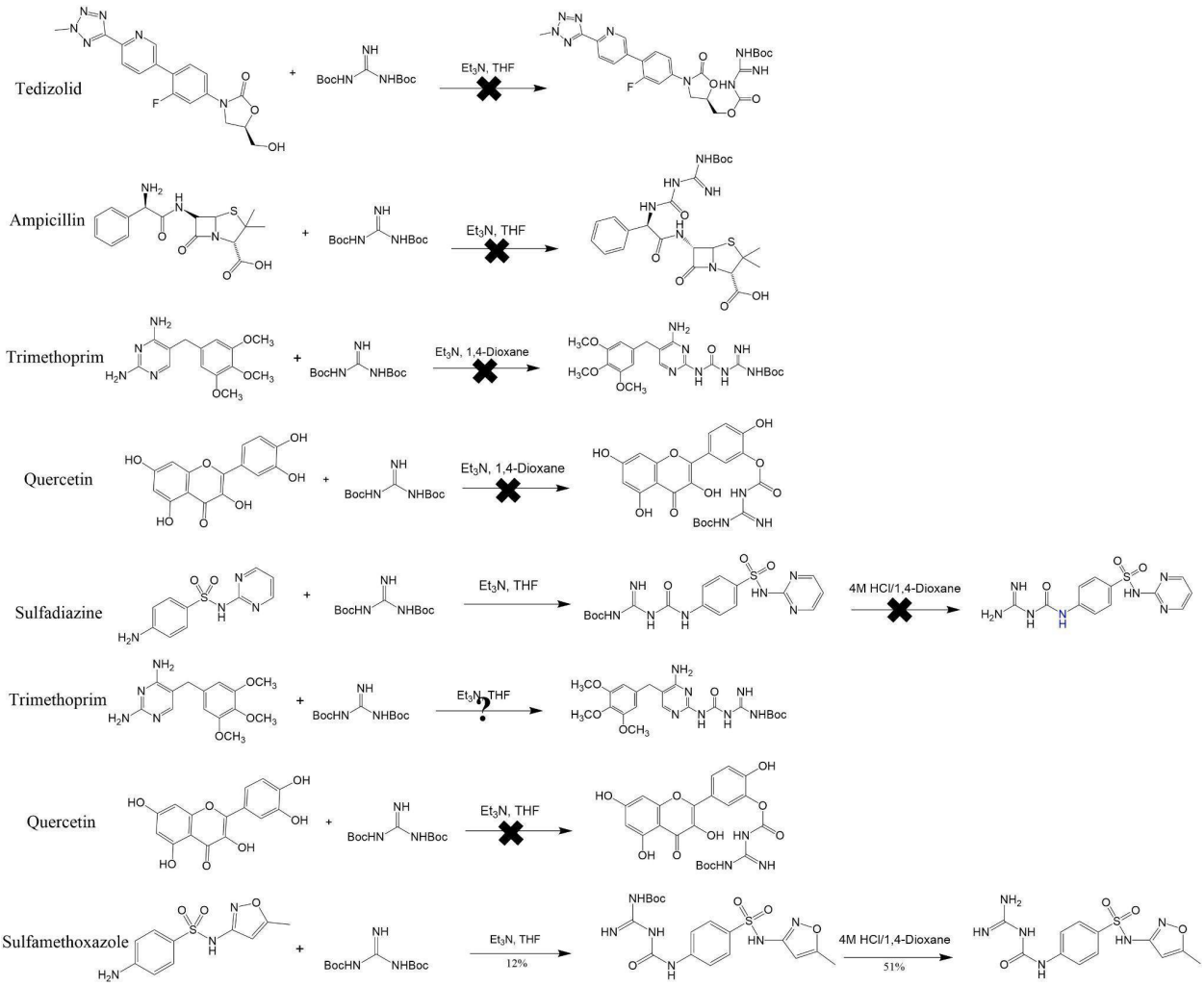


Figure 7. All attempted adjuvant-antibiotic synthesis reactions. The only one to have successfully been completed was the trial with sulfamethoxazole.

After testing the inhibitory properties of the synthesized sulfamethoxazole-guanidine hybrid, it was determined that the MIC of the compound exceeded 128 ug/mL. At the tested concentrations, the sulfamethoxazole-guanidine hybrid failed to inhibit bacterial growth, which is likely due to a number of possible factors. These could include potential for the antibiotic to be too impotent to kill off any of the bacterial strains tested against it or for the antibiotic to still not be able to permeate the OM even with the adjuvant attached. As well as this, the guanidinium cleavage mechanism has not yet been studied, so future work will be done to examine the stability of the linker as well as its cleavage rate and location in the cell.

Conclusion

Currently, the sulfamethoxazole-guanidine hybrid is the only antibiotic-guanidine hybrid that has been successfully synthesized using this method in this study. For future work, an accumulation assay will be performed to determine if the antibiotic is able to permeate the OM of the bacteria to determine the cause of its failure in the cell death assay. As well as this, synthesis of additional antibiotics using this same synthetic method could yield interesting results when coupled with more powerful, traditional antibiotics like ampicillin and penicillin. Finally, more cell death assays could be run at higher concentration to ascertain the MIC of the synthesized hybrid and compare to values obtained from sulfamethoxazole itself.

Additionally, more work will need to be done to determine how the cleavage mechanism occurs in the cell or if it takes place at all, which can be monitored by LC-MS. With all of these future avenues for antibiotic-guanidine adjuvant chemistry, the work to combat a global medical crisis continues on.

Acknowledgment

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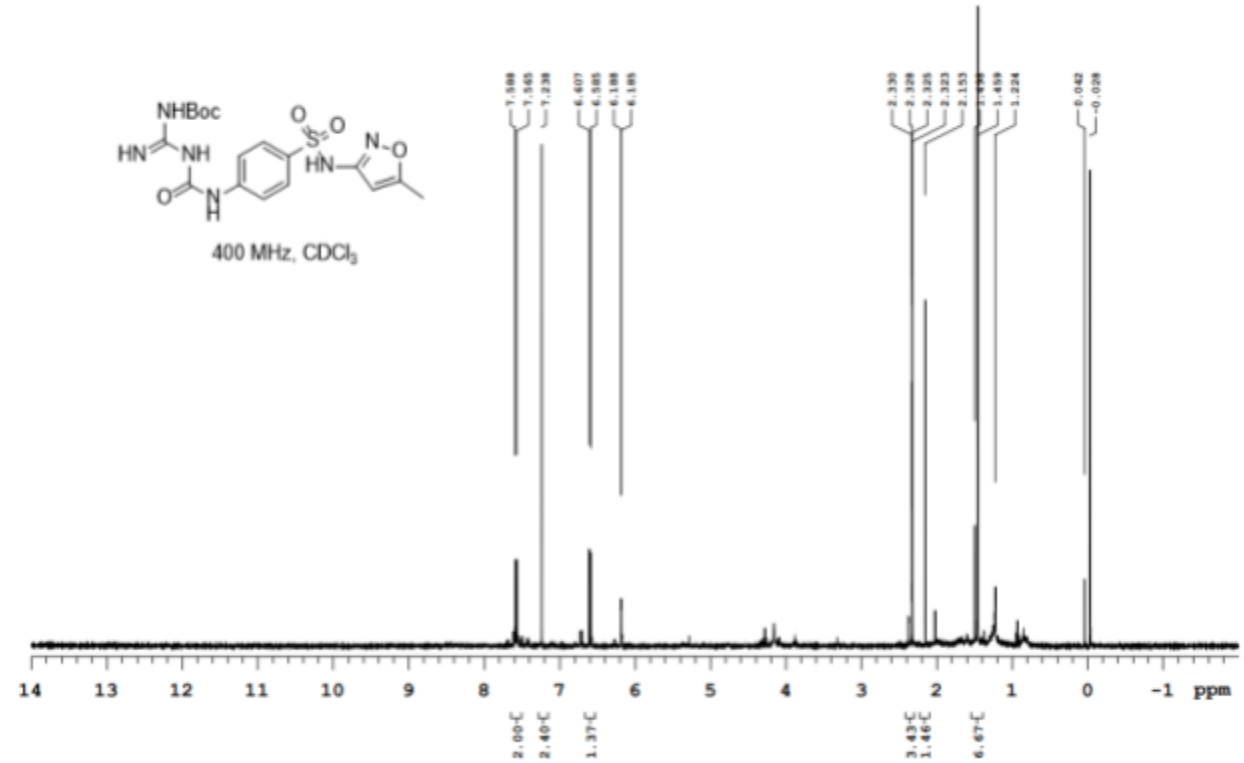
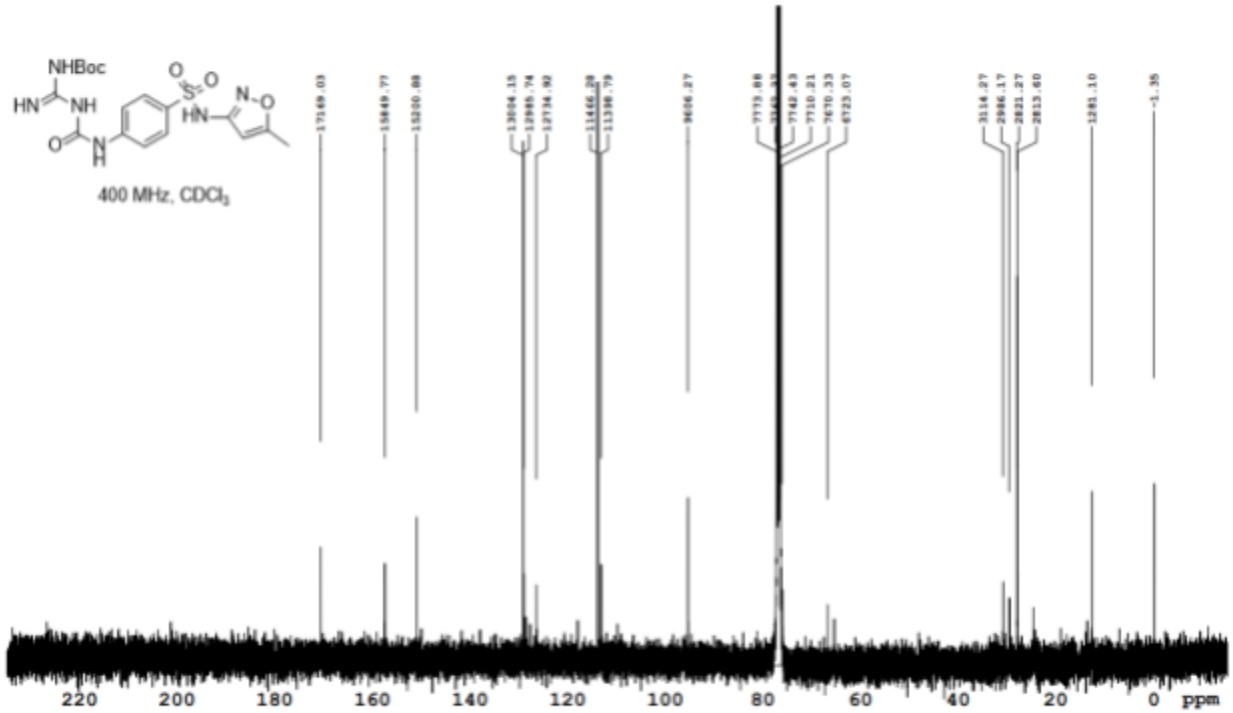
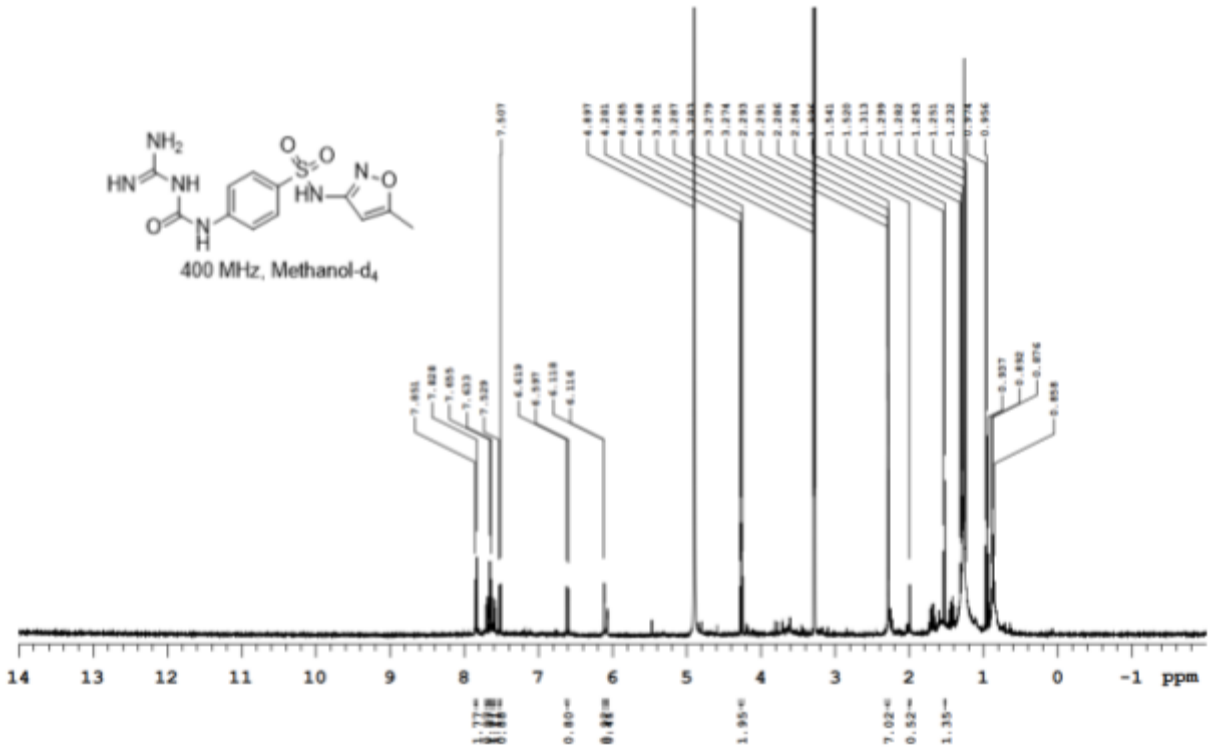
Supplemental



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