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Development of amine-containing cleavable antibiotic-adjuvant linkers for the potentiation of antibiotics in Gram-negative bacteria

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Abstract

Antibiotic resistance is a growing health concern and can be deemed a global threat; therefore, the need to develop new novel drugs is essential in combating this problem. In addition to the development of novel drugs, on the market drugs can be reactivated and used to overcome resistance mechanisms that are present in Gram-negative bacteria. One resistance mechanism is the inability for antibiotics to permeate the outer membrane (OM), constructed of negatively charged lipopolysaccharides, leading to reduced drug accumulation within the cell. One solution to combat this is using permeating adjuvants which can passively diffuse the OM and effectively accumulate an antibiotic within the cell, leading to cell death. Although adjuvants cannot kill bacteria themselves, the potential co-dosing mechanisms with previously synthesized antibiotics can be utilized to overcome resistance and reactivate these drugs. Through the evaluation of previous work on pentamidine and the eNTRy rules developed by Hergenrother et al., there is potential for the formation of permeating adjuvants containing nitrogenous groups to be evaluated. From here, a series of adjuvants were developed which retained the same structural backbone of diphenylsuccinamide with alterations to the nitrogenous groups attached. These poly-nitrogenous compounds, when co-dosed in the presence of an antibiotic, had the ability to cross the OM and accumulate within the cell. Further evaluation regarding the ability for a cleavable antibiotic-bisamine adjuvant hybrid is ongoing. Through a carbamate series linker, antibiotic-adjuvant hybrid molecules can be evaluated for increased effectiveness in comparison to co-dosed permeating adjuvants. In addition to a cell death assay,

cleavage studies will be implemented to evaluate the stability and cleavage rates of the synthesized antibiotic-bisamine adjuvant hybrid molecules. The results of this study have the potential to provide a new route to synthesizing molecules by overcoming resistance mechanisms through the reactivation of antibiotics.

1. Introduction

1.1 Background: Antibiotic Resistance

Antibiotic resistance is continuing to grow and is posing a global threat to human health. According to the Centers for Disease Control, more than 2.8 million people suffer from an antibiotic-resistant infection and over 35,000 people die yearly in the United States.[1] If no changes are made, deaths due to antibiotic resistance by the year 2050 are estimated to surpass ten million a year worldwide.[2] In order to combat this, immediate action needs to be taken in order to stop this rise in antibiotic resistant infections.

Various factors have led to the increase in antibiotic resistant bacteria, including the misuse, overuse, and lack of novel antibiotics being synthesized. It has been estimated that approximately 50% of prescribed antibiotics are deemed to be unnecessary or avoidable.[1] As physicians overprescribe antibiotics, the selective pressure produces resistance mechanisms that allow bacteria to multiply and spread. This can then lead to the presence of resistance genes within bacteria, ultimately leading to the spread through the contamination of food, water, and the environment.[4] This contamination can lead to bacteria entering the body through drinking or eating, leading to these resistant bacteria being present within the human body. The most common place that bacteria reside is within the gut of humans and animals, therefore, leading to the resistance genes that are present being transferred between phyla.[4] Once the resistance mechanism has reached the microbiomes of the majority of the human population, it will be necessary to create a new antibiotic to treat resistant infections. The most common resistance mechanisms are the inability to permeate the membrane, the presence of efflux pumps, a genetic mutation, and inactivation of the drug.[5]

As new antibiotics are synthesized and used as treatment methods, bacteria evolve to create resistance mechanisms. Specifically, Gram-negative bacteria have evolved to contain an outer membrane (OM) containing negatively charged lipopolysaccharides as well as efflux pumps (**Figure 1**) making them increasingly difficult to treat. Due to the presence of this OM, it is difficult for antibiotics to cross the membrane and enter the cell. Many academic and commercial labs are working towards synthesizing molecules that target Gram-negative bacteria through an alternative mechanism of action. Gram-negative bacterium, specifically *Pseudomonas aeruginosa* (PA), are extremely difficult to treat due to the inability of known drugs to successfully cross the outer membrane. PA is extremely resistant to treatment and often develops resistance during treatment.

Antibiotics are used as a last resort and can include a combination of antipseudomonal beta-lactam (e.g., penicillin) and an aminoglycoside.[9] This combination from two different drug classes is used to limit the likelihood of a resistance mechanism arising during or after treatment.

The OM that Gram-negative bacteria possess limit the uptake and accumulation of antibiotics within the cell. Once a potential drug crosses the membrane, efflux pumps have the potential to eject small molecules from the cell. In order for a small molecule to be effective in killing the bacteria, there has to be enough accumulated within. Not enough antibiotic being accumulated within the cell becomes a problem when more of the antibiotic is pumped out than the amount entering through the porins or through passive diffusion. Hergenrother et al. experimentally evaluated various compounds to conclude rules of entry and accumulation within Gram-negative bacteria. These "eNTRy" rules are: 1) Nitrogen containing groups (primary amine > secondary amine > tertiary amine), 2) Less three dimensional, 3) Rotatable bonds (< or equal to 5).[6] One medical research group, the Wolfe Laboratory at the University of North Carolina at Asheville, used the previously evaluated and concluded "eNTRy" rules to synthesize adjuvants that have the ability to cross the outer membrane of PA. In addition to this, the the use of covalently attaching antibiotics to nitrogen-containing adjuvants through a carbamate linker is being evaluated for increased efficacy and accumulation within the cell.

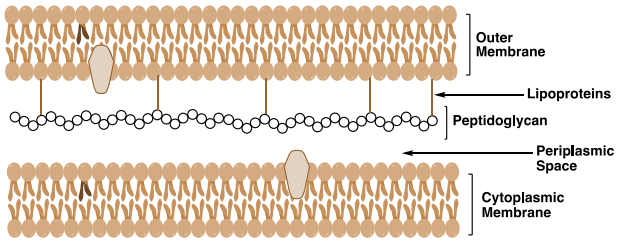


Figure 1. Structure of Gram-negative bacteria.

1.2 Background: Adjuvants

Adjuvant molecules do not have the ability to kill bacteria on their own but use a variety of mechanisms to increase efficacy of varying molecules, specifically antibiotics, through the enhancement of accumulation activity.[8] Adjuvants can be specifically described as any molecule that has the ability to overcome a resistance mechanism and improve drug activity, which can be done through various mechanisms. A way an adjuvant can do this is this through promoting passive diffusion across the membrane in the presence of already known antibiotics. This alternative mechanism allows for the use of previously synthesized and on the market drugs to be used to treat bacterial infections and decrease the rise in antibiotic resistance.

The overall problem of accumulation is due to both the inability to penetrate the membrane and the role of efflux pumps. Specifically, the ability to effectively cross the outer membrane of Gram-negative bacteria is going to be targeted here using protonatable

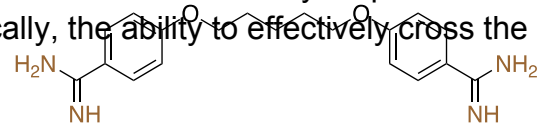
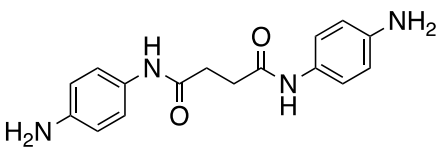


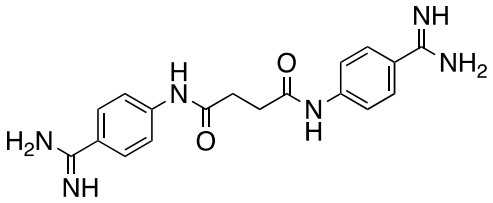
Figure 2. Structure of Pentamidine.

groups present within the adjuvants structure. Previous work done by Stokes et al. proved that when an antibiotic was co-dosed in the presence of a pentamidine molecule, it had the ability to effectively cross the outer membrane.[10] Pentamidine (**Figure 2**), a bisbenzamidine that has a 5 carbons linker attached, has been concluded to act as an adjuvant specifically capable of crossing the outer membrane layer of Gram-negative bacteria.[10] These previous findings can be used to design a molecule that retains the same ability to cross the outer membrane but has an alternative linker to increase accumulation into Gram-negative bacteria, specifically PA.

Previous work done by the Wolfe Laboratory has been in synthesizing and evaluating pentamidine containing poly-nitrogenous compounds for antibacterial properties (**Figure 3**). Through the use of "eNTRy" rules and previous conclusions about adjuvant activity on pentamidine, four molecules were synthesized and evaluated against *Escherichia coli* (EC) and PA.[7] The addition of various nitrogen containing groups (amine vs. amidine vs. guanidine), all four synthesized molecules had the ability to penetrate the membrane of PA (**Table 1** and **2**). All values that are purple in color show a 2-fold increase in efficacy, the values that are blue in color show a reduced MIC value but do not have a significant increase in efficacy. Specifically, a 2-fold increase was seen with co-treatment of erythromycin and all four adjuvants against PA. All four adjuvants contained a nitrogen group, allowing for protonation. The interaction between



A



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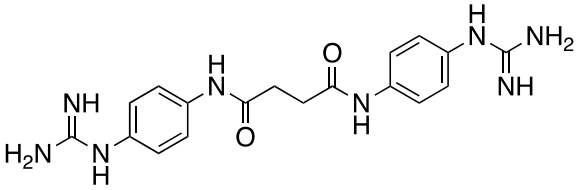

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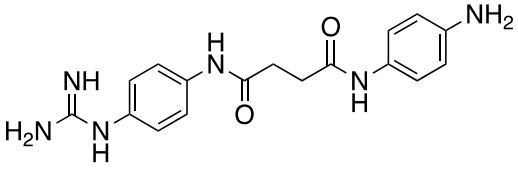


D

the negatively charged lipopolysaccharide OM and the positively charged nitrogenous groups on the adjuvants allowed for the membrane to be permeated.

In addition to this, the symmetry within the molecule was also evaluated (monoguanidine vs. bisguanidine) and proven to have altering effects on penetration and accumulation. When comparing the monoguanidine and bisguanidine in symmetrical structure, the monoguanidine has one guanidine group and one amine group at the para positions while the bisguanidine group has one guanidine group present at each of the para positions. This difference in symmetry allowed for

conclusions to be drawn regarding the impact of symmetry on efficacy. Further, future work can be done to increase the efficacy of these synthesized adjuvant molecules to permeate the membrane and kill Gram-negative bacteria.

To conclude the effective OM penetration, each adjuvant was tested individually to define the ability for accumulation within WT EC and PA (Table 3). This was tested through comparison to ciprofloxacin, a high accumulator, and ampicillin, a low accumulator, through the modification of a method created by Hergonrother et al. Through this method, it was found that compounds **A**, **B**, **C**, and **D** all accumulated 5-50 times higher for both EC and PA in comparison to ampicillin. Specifically, in WT EC all the adjuvants and ciprofloxacin accumulated between 1–2 nmol per 10^{12} CFU. But, in PA, compound C showed a slightly reduced accumulation in comparison to all of the tested adjuvants and ciprofloxacin.[14] Through these findings, (**Table 2**) it can be concluded that symmetrical adjuvant molecules, like bisguanidine (**C**), had an increased accumulation in comparison to non-symmetrical molecules, like monoguanidine (**D**).[14] Four compounds were analyzed for their adjuvant activity, bisamidine, bisamine, monoguanidine, and bisguanidine. The adjuvants were evaluated in the presence of already known antibiotics which included, penicillin G, ampicillin, erythromycin, novobiocin, rifampicin and kanamycin against wild-type EC and PA using a standard MIC adjuvant assay. Using these antibiotics, Pugh et al. effectively analyzed the adjuvant activities of these compounds and whether their activities were specific to the antibiotic class or if they could be broadly used (**Table 1**).[14]

Table 1. Adjuvant activity of compounds **A, B, C, and D** (100 mM) with known antibiotics against *E. coli* and *P. aeruginosa*. (n = 3)

Adjuvant MIC (mg/mL)[a,b]					
Antibiotic	***E. coli***				
	Antibiotic Only	Antibiotic + **A**	Antibiotic + **D**	Antibiotic + **B**	Antibiotic + **C**
Penicillin G	64	64[0]	64[0]	64[0]	64[0]
Ampicillin	16-32	16-32[0]	16-32[0]	16-32[0]	16-32[0]
Rifampicin	8	8[0]	4-8[0]	8[0]	8[0]

Erythromycin	128	128[0]	128[0]	64[2]	64[2]
Kanamycin	64	64[0]	64[0]	64[0]	64[0]
Novobiocin	128	128[0]	64-128[0]	64-128[0]	64[2]
	P. aeruginosa				
Penicillin G	>256	>256 [0]	>256 [0]	>256 [0]	>256 [0]
Ampicillin	128	128[0]	128[0]	128[0]	128[0]
Rifampicin	16	16[0]	16[0]	16[0]	16[0]
Erythromycin	128	64[2]	64[2]	64[2]	64[2]
Kanamycin	>64	>64[0]	>64[0]	>64[0]	>64[0]
Novobiocin	256	256 [0]	256 [0]	256 [0]	256 [0]

[a]MIC = minimum inhibitory concentration of >90% pathogen growth inhibition of at OD = 590nm compared to (-)-control (DMSO + pathogen); [b]Fold reduction of MIC in brackets

Table 2. Accumulation of compounds **A**, **B**, **C**, and **D** in EC and PA compared to ciprofloxacin (high accumulator) and ampicillin (low accumulator).

Accumulation Results				
Compound	**SIM m/z Monitored**	**Ionization Mode**	**EC Accumulation (nmol /10^{12} CFU)**	**PA Accumulation (nmol /10^{12} CFU)**
A	299	$[M+H]^+$	3.3 ± 1.9	2.0 ± 1.1
B	171	$[M+2H]^{2+}$	1.3 ± 0.6	0.3 ± 0.06
C	177	$[M+2H]^{2+}$	1.0 ± 0.2	0.9 ± 0.2
D	192	$[M+2H]^{2+}$	1.1 ± 0.1	1.1 ± 0.1
Ciprofloxacin	332	$[M+H]^+$	1.4 ± 0.8	1.8 ± 0.2
Ampicillin	348	$[M-H]^-$	< 0.06[c]	< 0.06[c]

Through these findings from Pugh et al., further work can be done to increase the efficacy of these molecules against Gram-negative bacteria. Specifically, synthesizing an antibiotic-adjuvant molecule with the use of cleavable carbamate linkers can be utilized to reactivate antibiotics that might have resistance mechanisms against them. An antibiotic-bisamine adjuvant linker (**Figure 4**) is being evaluated for its antibacterial activity and accumulation ability with the hopes of developing a new approach to overcome the resistance mechanisms that Gram-negative bacteria have acquired over time.

Based off previous work done by the Wolfe Laboratory and various academic labs, exploring the ability of a linked antibiotic to an adjuvant to promote diffusion and increase accumulation is being explored. A cleavable antibiotic-adjuvant hybrid (**Figure 4**) is being targeted to evaluate the accumulation ability in addition to the ability to successfully kill Gram-negative bacteria. One specific way of improving the uptake of antibiotics is through the use of covalent adjuvant-antibiotic hybrids. These hybrids rely on the linked adjuvant to carry the antibiotic into the cell. Cleavable adjuvant-antibiotic linkers provide an alternative way do deliver antibiotics to resistant bacteria without tampering molecular make-up of the antibiotic. In order to ensure the molecule is properly cleaved and the antibiotics structure is not affected, the molecular design must be specific to the chemical environment or to the enzymes that are present. Specifically, the chosen cleavable linker needs to be designed such that it is only cleaved in the presence of a specific enzyme or chemical environment. Probing a specific enzyme through molecular design can lead to increased specificity and efficacy.

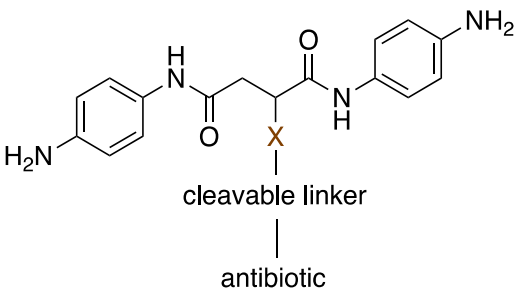


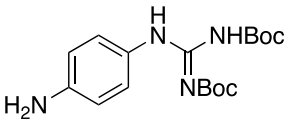
Figure 4. Proposed antibiotic-adjuvant cleavable linker.

There are a variety of enzymes that can be used as a target for cleavable linker development, but the use of hydrolytic esterases/peptidases and nitrite peptidases can be successfully utilized. Through the development of two classes of adjuvant-antibiotic cleavable linkers, carbamate-type linkers and reductive N-O linkers, there is anticipated to be an increased selectivity and efficacy. By increasing the selectivity and efficacy of antibiotic activity, the results of this study can influence the field of medicinal chemistry, specifically in overcoming antibiotic resistance through a different mechanism.

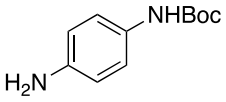
2. Experimental

2.1 Synthetic Strategies

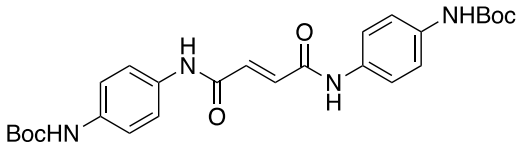
General: All reagents and solvents were purchased reagent-grade, further purification was not required. TLC was conducted using precoated SiO_2 60 F254 glass plates from EMD with visualization by UV light (254 or 366 nm). An Oxford Varian-400 spectrophotometer at 298K was used for NMR visualization (1H or 13C). All IR spectra were recorded on a Thermo Scientific Nicolet iS10 FT-IR spectrophotometer. Shimadzu single quadrupole LCMS-2020 was used to collect all Low-resolution mass spectral data.



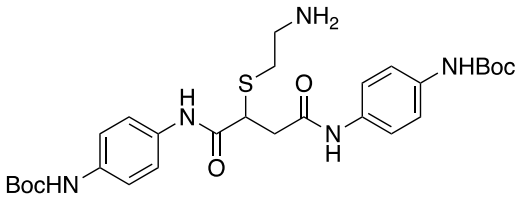
Compound **E**: **4-[2,3-di(tert-butoxycarbonyl)guanidino]aniline:** p-phenylenediamine dihydrochloride (0.500 mg, 0.842 mmol, 1 equiv.) and triethylamine (1.92 mL, 3.37 mmol, 4 equiv.) were combined in glassware and dissolved in DCM (0.5 M). 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (1.35 mg, 0.842 mmol, 1 equiv.) was added to the flask and the reaction was stirred overnight. A silica plug was used for purification and eluted with DCM to remove any remaining starting material. A 5% MeOH/DCM flush was used to yield compound **E** as an off-white solid.



Intermediate: **Boc-protected bisamine** was synthesized using **1,4-phenylenediamine dihydrochloride** (5 g, 28 mmol, 1 equiv.). The starting material was dissolved in 2:1 dioxane/H_2O (45 mL/28 mL) with the addition of potassium carbonate (8 g, 58 mmol, 2.07 equiv.). Boc anhydride (6.2 g, 28 mmol, 1 equiv.) was added to the flask. The reaction was placed at room temperature and stirred overnight. The crude product was concentrated under vacuo. 100 mL of H2O was added. The reaction was washed with H2O three time to yield a white powder.



Intermediate: **2-((2-aminoethyl)thio)-N^1,N^4-bis(4-carbamimidoylphenyl)succinimide** was synthesized using **Boc-protected bisamine** (1 g, 2.5 mmol, 2.5 equiv.). The starting material was dissolved in 6 mL of 1,4-dioxane (0.33 M). Fumoryl chloride (0.21 mL, 1.92 mmol, 1 equiv.) was added dropwise to the reaction. This reaction was placed at room temperature and stirred for 16 hours. The crude product was concentrated in vacuo and sodium bicarbonate was added. Product was washed three times with H2O to yield a white powder.

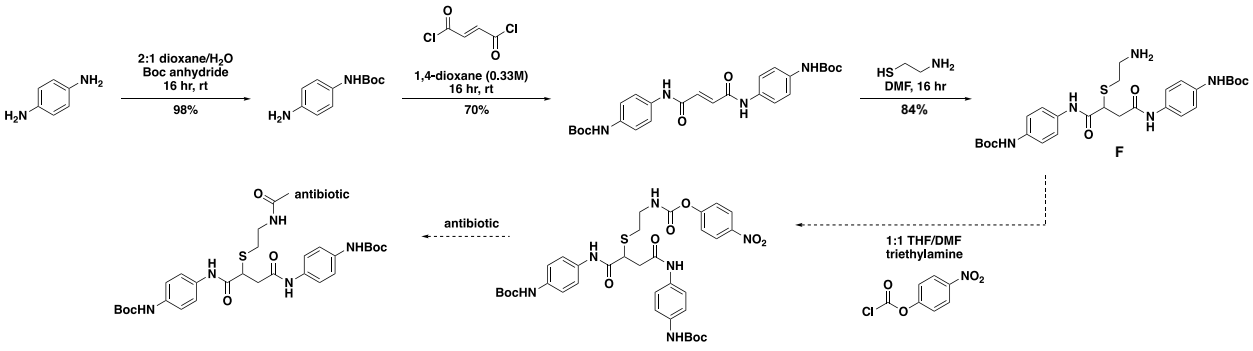


Compound **F**: **tert-butyl (4-(2-((2aminoethyl)thio)-4-((4-((tert-butoxycarbonyl)amino)phenyl)amino)-4-oxobutanamido)phenyl)carbamate: 2-((2-aminoethyl)thio)-N^1,N^4-bis(4-carbamimidoylphenyl)succinimide** (300 mg, 0.604 mmol, 1 equiv.) and 2-mercoptethylamine (0.606 mg, 5.53 mmol, 8.83 equiv.) were dissolved in DMF and heated to 70°C. 0.5 mL of pyridine was added to the reaction after 2 hours of heating. The reaction was refluxed at 70°C overnight. The crude product was filtered with water three times and dried under vacuum to yield a light yellow powder.

3. Results and Discussion

3.1 Synthetic Strategies and Outcomes

To begin the synthesis of antibiotic-adjuvant hybrid molecules a series of reactions were utilized to yield a Boc-protected bisamine starting material (**Scheme 1).** This series of reactions aimed to find ideal reagents and conditions to synthesize 2-((2-aminoethyl)thio)-N^1,N^4-bis(4-carbamimidoylphenyl)succinimide, the linker molecule that will be used to attach various antibiotics for improved potency. **Table 3** shows all the reagents and conditions analyzed to produce the desired product.

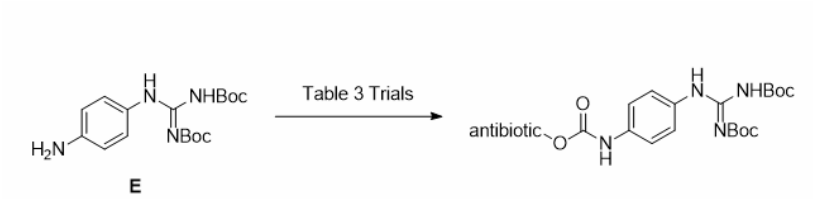


Scheme 1. Proposed synthetic scheme for the formation of antibiotic-adjuvant hybrid molecules.

Table 3. Synthetic trials for adjuvant-antibiotic hybrid molecules to develop antibiotic-adjuvant hybrid containing amidine groups using 4-nitrophenylchloroformate at the coupling reagent (linker).

Trial Number	Adjuvant (eq.)	Base	Linker (eq.)	Solvent	Temperature	Time	Result
1	1	-	8.83	DMF	70 ºC	36h	-
2	1	-	8.83	DMF	150 ºC	16h	-
3	1	-	8.83	MeOH	80 ºC	16h	-
4	1	-	8.83	H_2O	80 ºC	16h	-
5	1	-	8.83	H_2O	50 ºC	16h	-
6	1	Pyridine (9 eq.)	8.83	H_2O	50 ºC	16h	-
7	1	Pyridine (9 eq.)	8.83	1,4-dioxane	100 ºC	16h	-
8	1	Pyridine (9 eq.)	2	DMF	150 ºC	16h	-
9	1	Pyridine (9 eq.)	8.83	DMF	160 ºC	16h	-

After nine trials in attempt to synthesize the desired product with no yield, a new approach was designed evaluate the ideal conditions for the synthesis of the antibiotic-bisamine hybrid molecule. Compound **E** was utilized to evaluate conditions, starting materials, and solvents for a successful linking of the Boc protected starting material and antibiotic. **Scheme 2** shows the predicted synthetic scheme and **Table 4** includes the tested reagents and conditions to produce the desired product. Compound **F**, tert-butyl (4-(2-((2aminoethyl)thio)-4-((4-((tert-butoxycarbonyl)amino)phenyl)amino)-4-oxobutanamido)phenyl)carbamate, shown in **Scheme 1** (Product 4) was utilized in attempted syntheses as well.



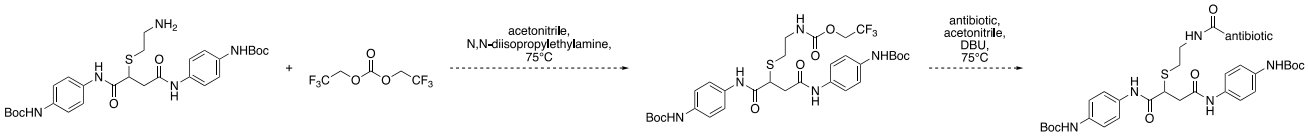
E

Scheme 2. Synthetic Scheme for Successful Synthesis of Antibiotic-Adjuvant Hybrid Molecules

Table 4. Synthetic Trials for Attempted Synthesis of Antibiotic-Adjuvant Hybrid Molecules

Trial Number	Compound (1 eq.)	Base	Linker (1 eq.)	Antibiotic	Solvent	Temp.	Time	Result
1	Compound F	-	4-nitrophenylchloroformate	-	THF/ DCM	0 ºC to rt	2.5hr	-
2	Compound E	K_2CO_3	4-nitrophenylchloroformate	Ampicillin	EtOAc/ H_2O	0 ºC to rt	16hr	-
3	Compound E	DIPEA	4-nitrophenylchloroformate	Ampicillin	DCM	rt	16hr	-
4	Compound E	DMAP	4-nitrophenylchloroformate	Ampicillin	H_2O	rt to 0 ºC to rt	31hr	-
5	Compound F	-	Triethylamine	Ampicillin	THF/ DCM	0 ºC to rt	16hr	-
6	Compound E	-	Triethylamine	Ampicillin	THF/ DCM	0 ºC to rt	16hr	-
7	Compound E	-	Triphosgene	Ampicillin	THF/ DCM	0 ºC to rt	16hr	-
8	Compound E	-	Triphosgene	Ampicillin	THF	70 ºC	16hr	-
9	Compound E	-	Triethylamine	Ampicillin	DMA	0 ºC to rt	36hr	-
10	Compound E	-	Triethylamine	Ampicillin	DMF	70 ºC	36hr	-
11	Compound E	Pyridine	4-nitrophenylchloroformate	Ampicillin	H_2O	rt to 0 ºC to rt	31hr	-
12	Compound F	Pyridine	Triphosgene	Ampicillin	DCM	0 ºC to rt	16hr	-

After numerous attempts to synthesize the desired product, another new approach was designed to try and successfully synthesize the antibiotic-adjuvant hybrid molecule. Again, compound **E** was utilized to determine ideal conditions, equivalents, and solvents for a successful linkage between the Boc protected starting material and chosen antibiotic. **Scheme 3** shows the predicted synthetic scheme and **Table 5** includes the tested reagents and conditions to produce the desired product.



Scheme 3. New Synthetic Scheme for Synthesis of Antibiotic-Adjuvant Hybrid Molecules using a new reagent.

Table 5. Synthetic Trials for New Synthetic Pathway to form Antibiotic-Adjuvant hybrid molecule.

Trial Number	Compound (1 eq.)	Base	Linker (1 eq.)	Antibiotic	Solvent	Temp.	Time	Result
1	Compound E	N,N-diisopropylethylamine	bis(2,2,2-trifluoroethyl) carbonate	Ampicillin	Acetonitrile	75 ºC	8hr	-
2	Compound E	N,N-diisopropylethylamine	bis(2,2,2-trifluoroethyl) carbonate	Ampicillin	Acetonitrile	75 ºC	8hr	-

3.2 Antibacterial Activity

Upon purification of 2-((2-aminoethyl)thio)-N^1,N^4-bis(4-carbamimidoylphenyl)succinimide, an antibacterial assay will be used to conclude MIC values. These synthesized compounds will be tested against penicillin, ampicillin, rifampicin, erythromycin, kanamycin, and novobiocin.

4. Conclusion

While there is still a prevalent need for the development of new and novel antibiotics, it is also essential to use different mechanisms to overcome antibiotic resistance. Through a different approach, current on the market antibiotics can be used with the help of a permeating adjuvant to overcome antibiotic resistant bacteria. Synthesis and evaluation of poly-nitrogenous compounds found that there was an increased ability to overcome the OM of Gram-negative bacteria through improved diffusion across the membrane.[14] Compounds **A**, **B**, **C** and **D** showed an increase in inhibition when the antibiotic and adjuvant were co-dosed. Currently, synthesis of antibiotic-bisamine adjuvant hybrids with covalent cleavable linkers is being targeted with the goal of increasing diffusion and accumulation within Gram-negative bacteria. Antibiotic-adjuvant hybrid molecules have the ability to provide a new approach to overcoming the global issue of antibiotic resistance. While there is no found synthetic pathway for the formation of bisamine adjuvant-antibiotic hybrid molecules as of now, further work can be done to find a successful pathway that yields the targeted product. Once this pathway is found, further purification and optimization can be done to yield pure bisamine adjuvant-antibiotic hybrid molecules that can be biologically evaluated for their efficacy against Gram-negative bacteria.

5. Acknowledgements

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6. References

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AR_SH_product

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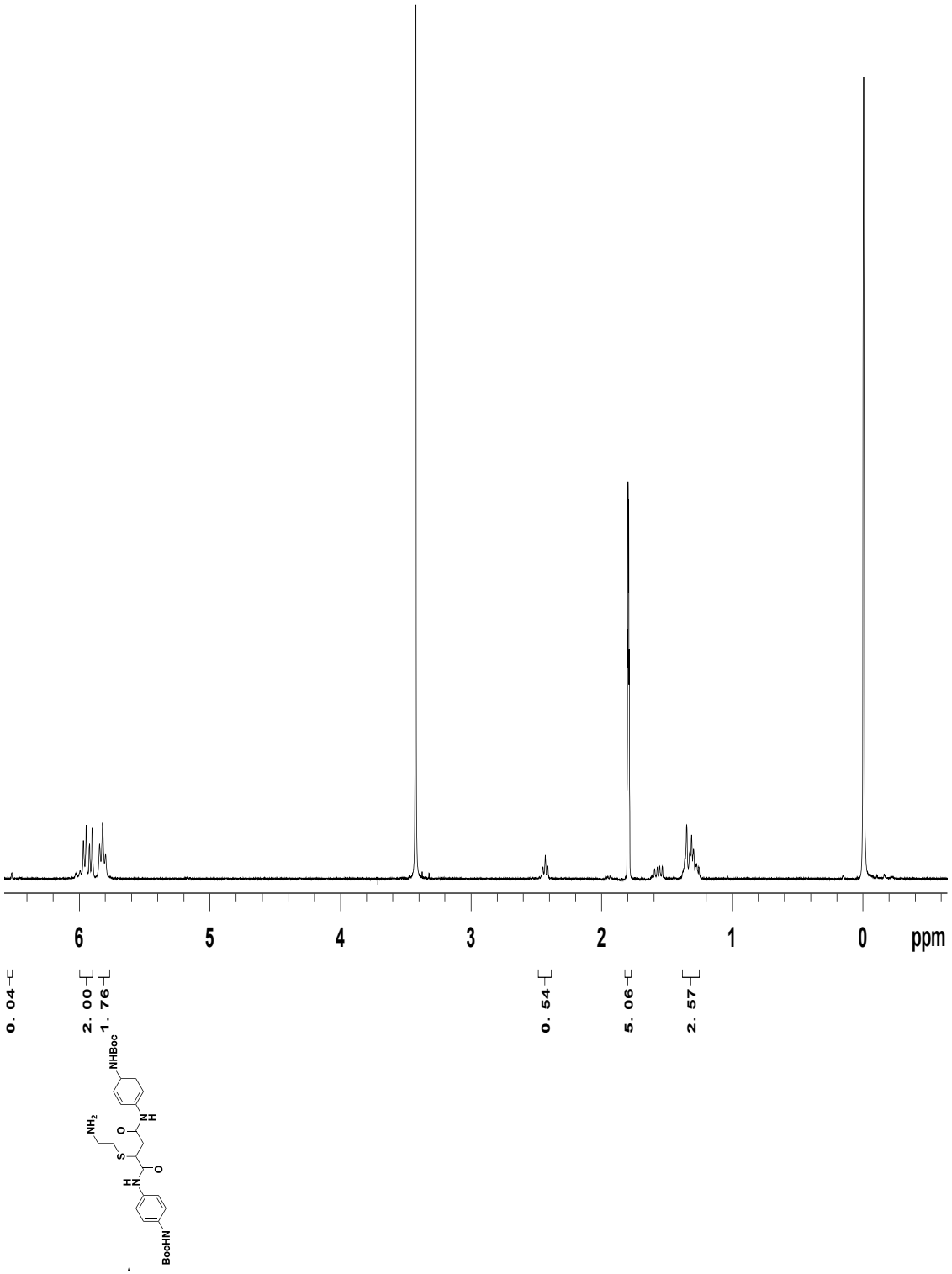

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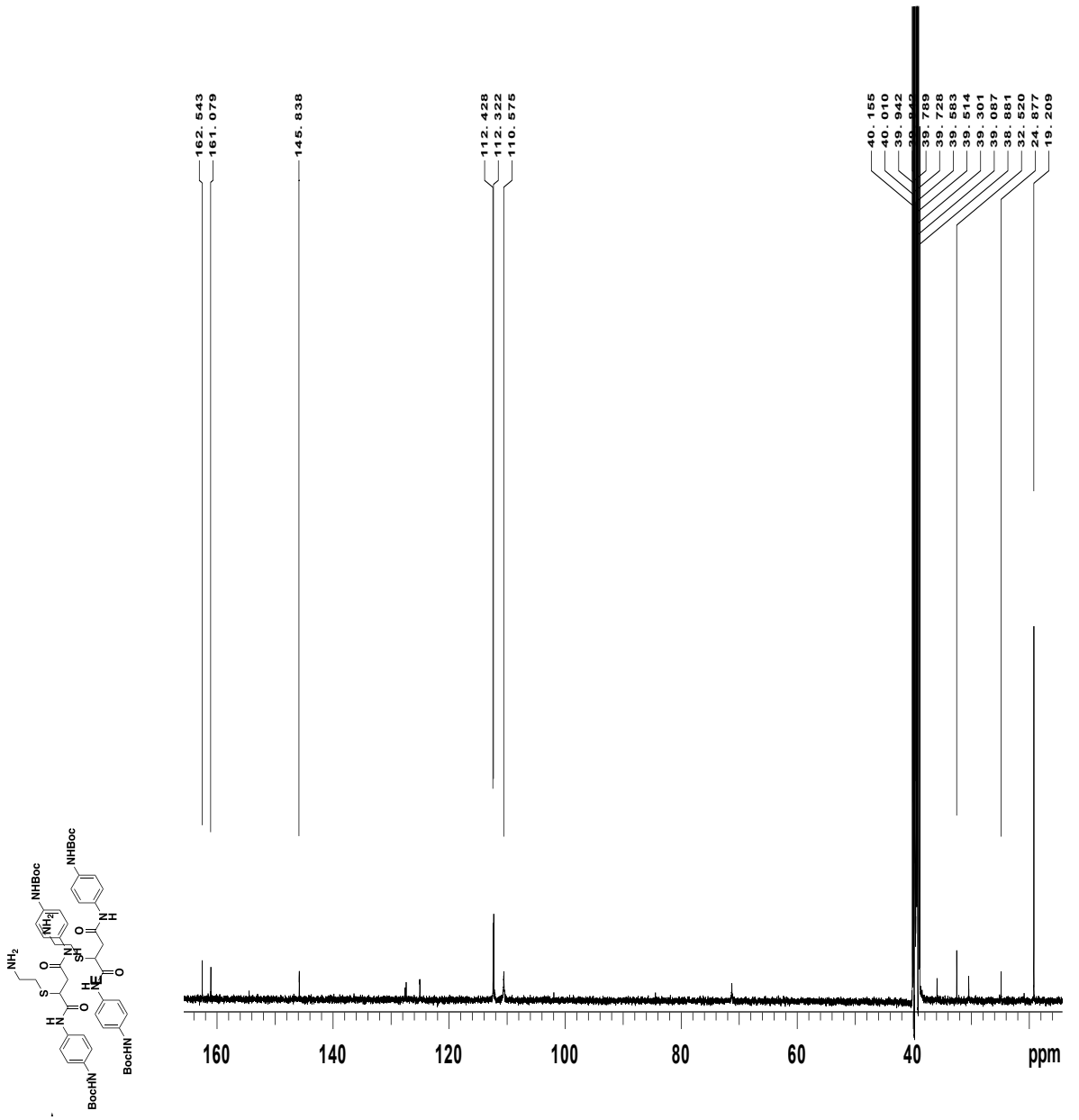

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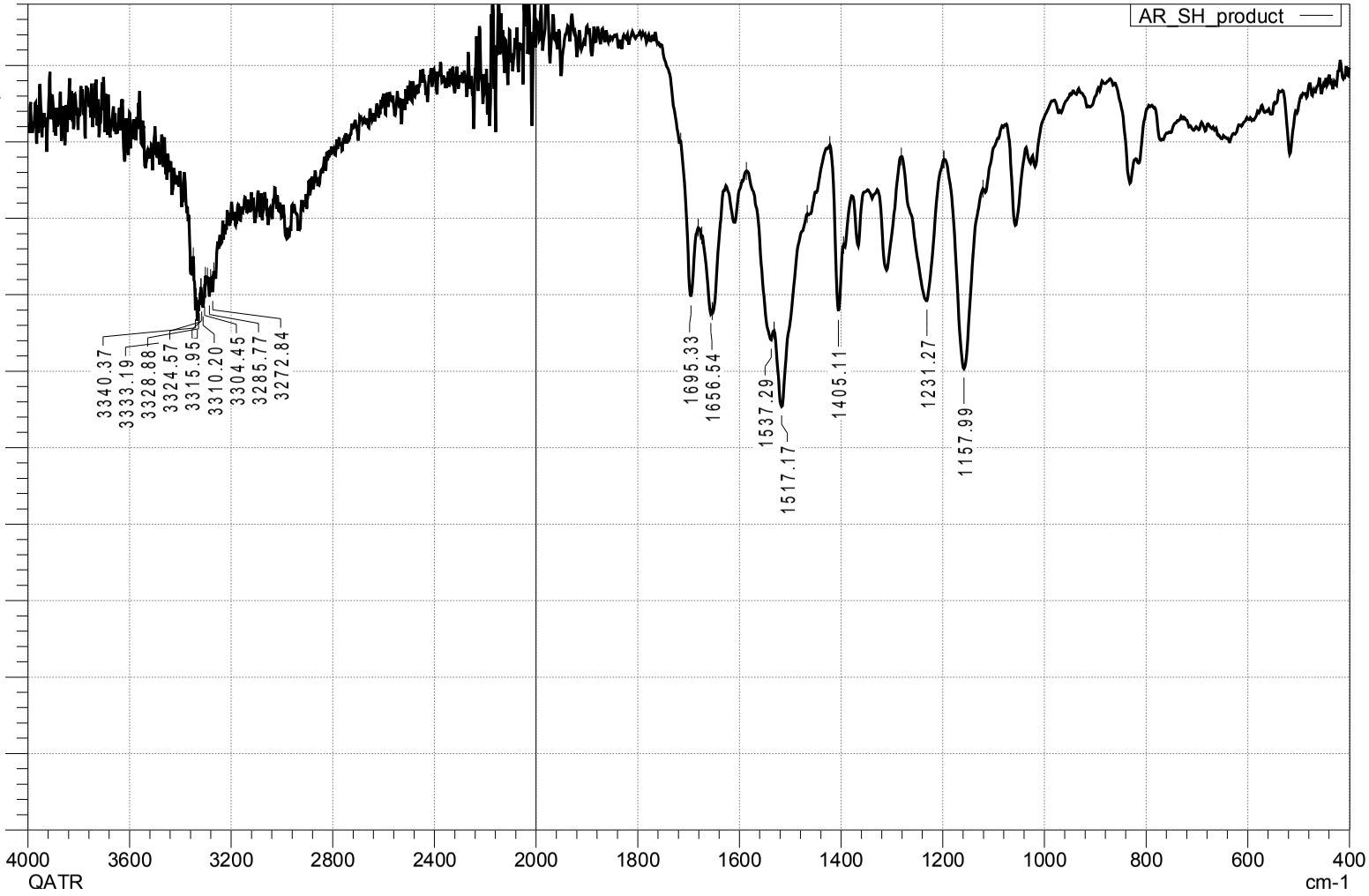
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BAP-2-80-f-dry-f

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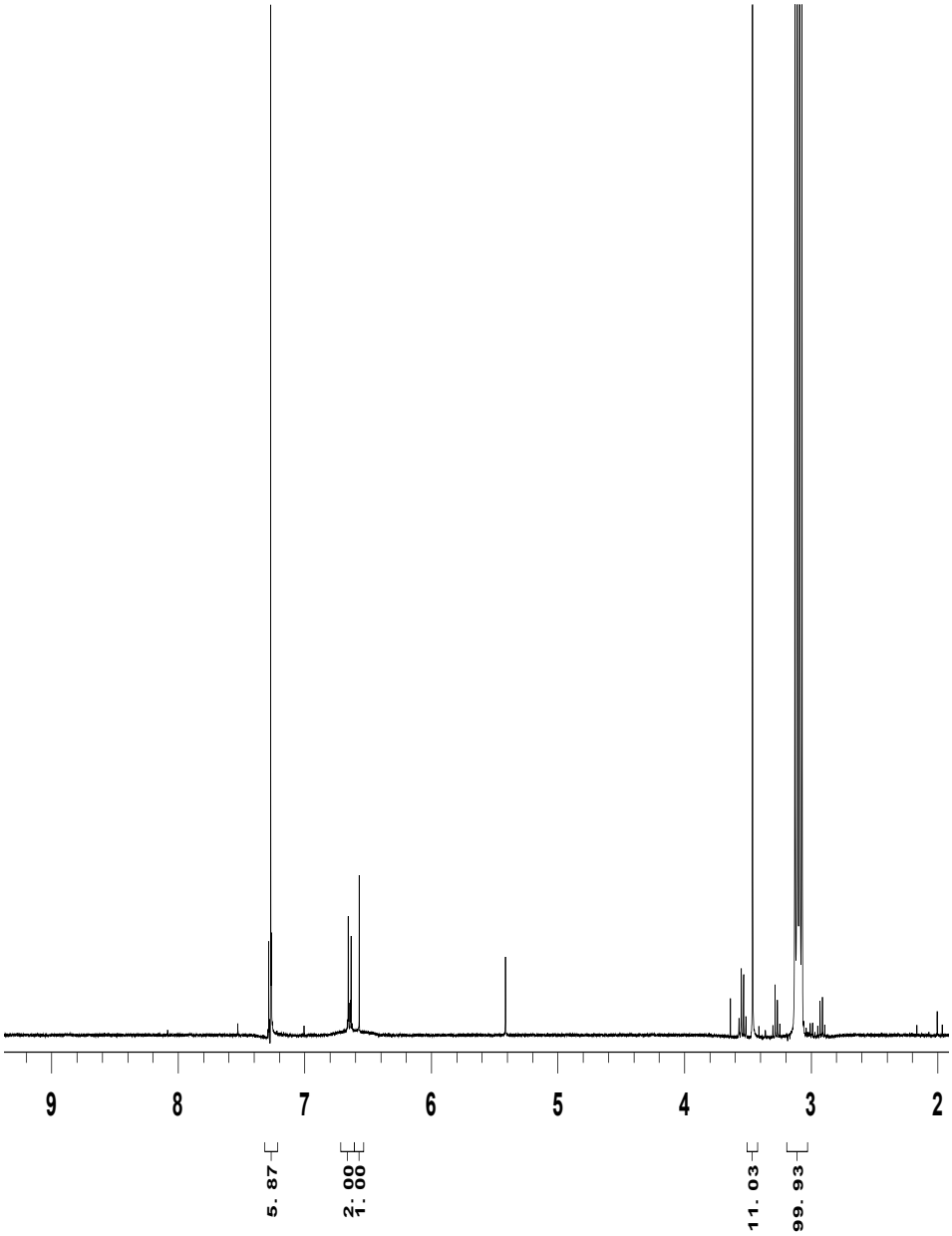

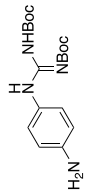

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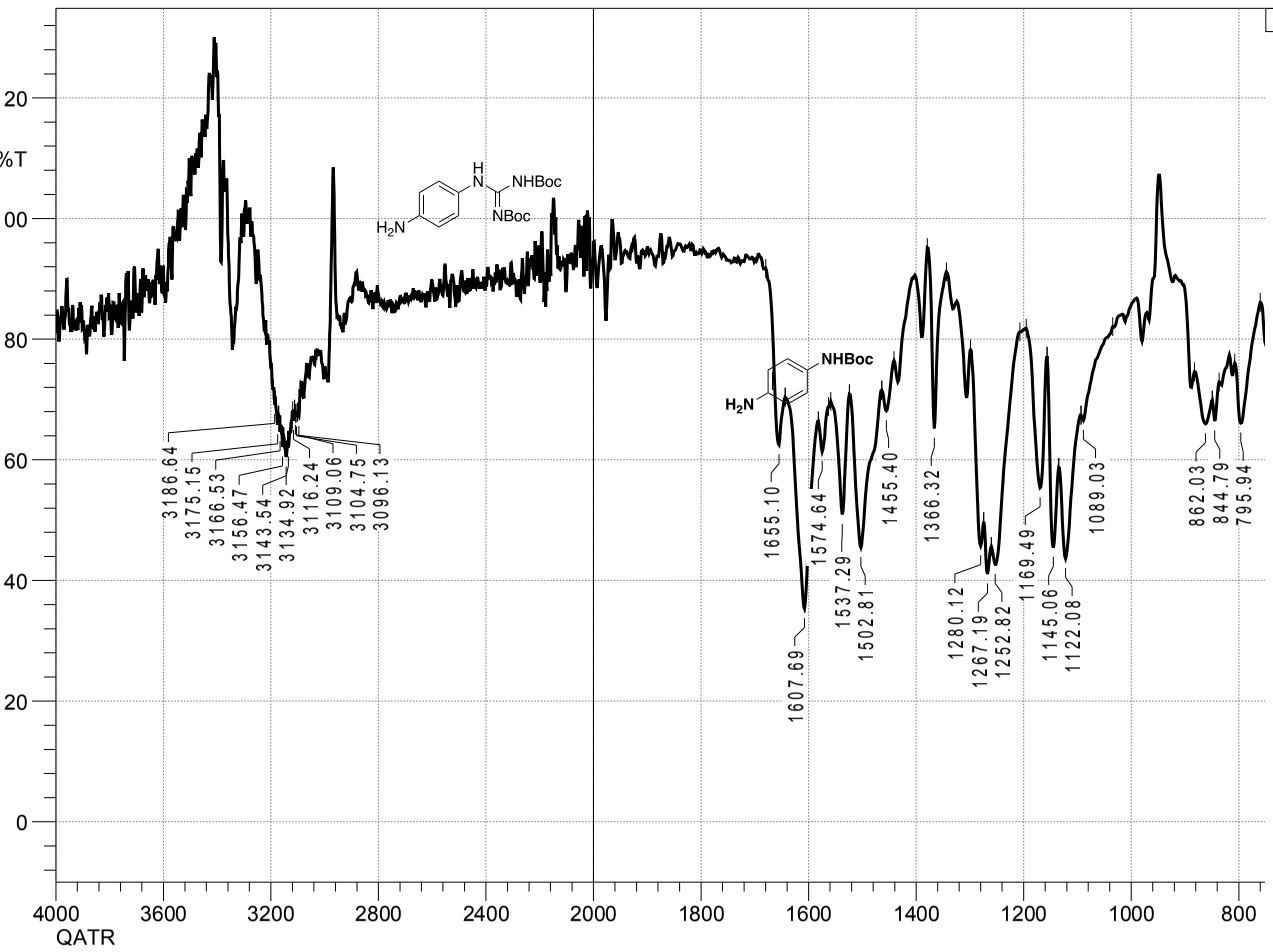
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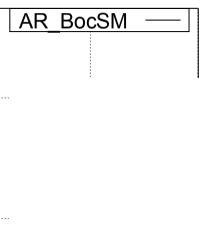
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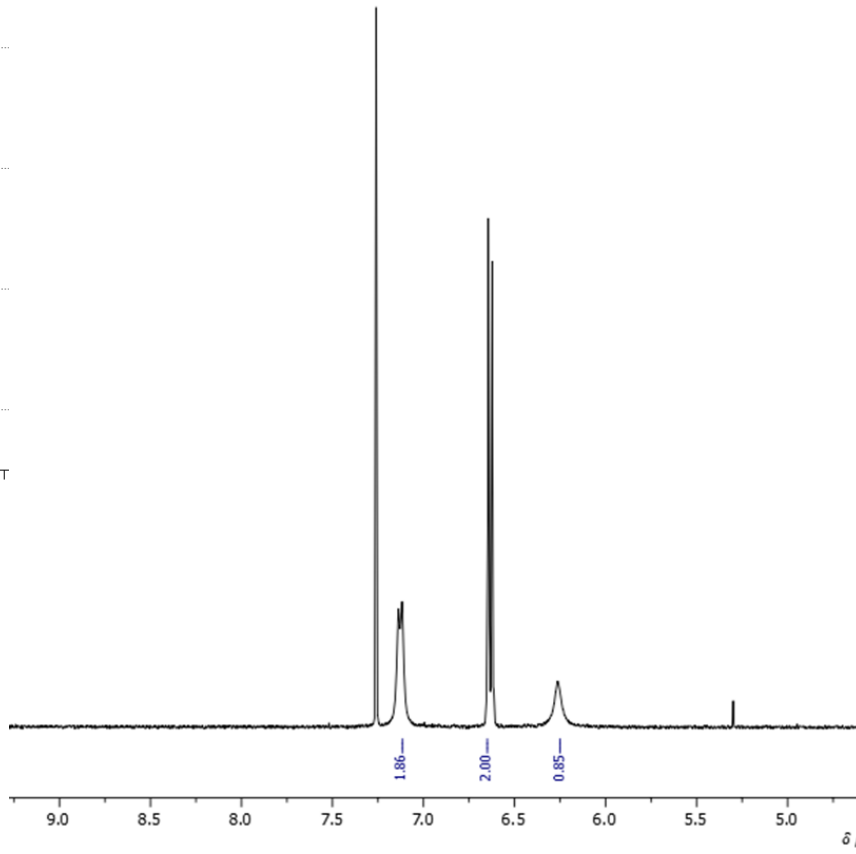
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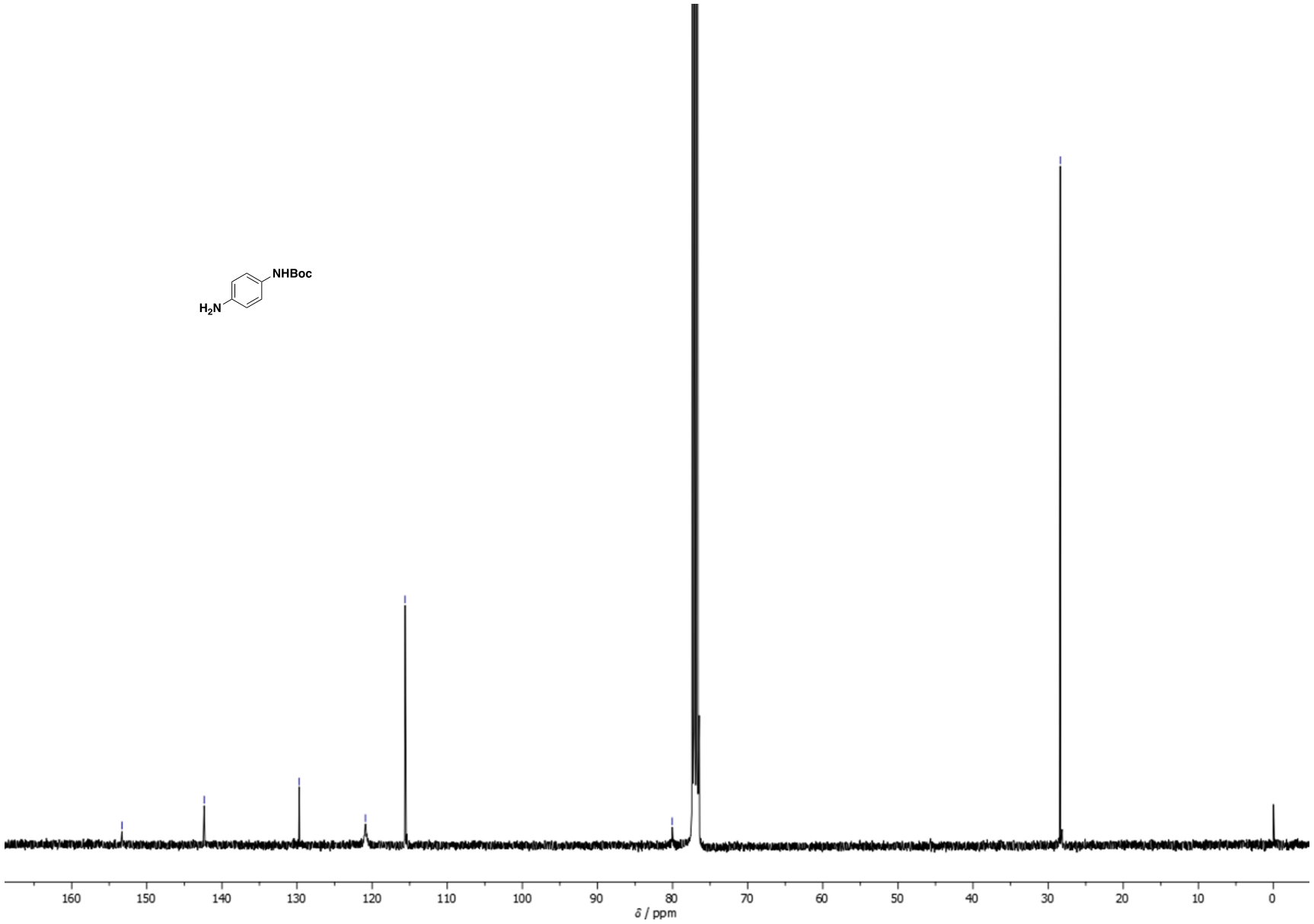
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