Lead Optimization of MTL1-44 for Antibacterial Efficacy Against *Staphylococcus aureus*

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Abstract

Antimicrobial-resistant strains of bacterial pathogens like *Staphylococcus aureus* are becoming more prominent each year, highlighting the need for alternative antibacterial agents. Peptoids, peptide mimics with side chains on the amide nitrogen (N-substituted oligoglycines), are one class of compounds with growing interest due to improved stability and bioavailability compared to that of peptides. MTL1-44, a peptoid discovered in the Bicker Lab, has shown promising levels of antibacterial activity against *S. aureus* while retaining low cytotoxicity. The focus of this study is on performing lead optimization of MTL1-44 via structural modifications to improve its antibacterial efficacy. Utilizing solid-phase synthesis, a sarcosine scan was done to better understand the structure-activity relationship (SAR) within the peptoid. Antibacterial activity will be evaluated through minimum inhibitory concentration (MIC) testing.

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Terms and Definitions

1. **Peptide**: a molecule with a backbone consisting of a chain of amino acids, each with distinctive side chains branching off an alpha-carbon
2. **Peptoid**: peptide-like compound, with side chains branching off the amide nitrogen rather than the alpha carbon
3. **Solid-Phase Synthesis**: a synthetic technique in which a compound is synthesized off a resin (polystyrene) bead with little to no intermediate purification
4. **Minimum Inhibitory Concentration (MIC_{90})**: The lowest concentration of compound required to inhibit 90% growth of a microbial pathogen
5. **Sarcosine Derivatives**: derivatives of a lead compound with a deletion at each side chain position that is replaced by a methyl group

Abbreviations of Chemical Names

1. **DMF**: dimethylformamide
2. **DIC**: diisopropylcarbodiimide
3. **TFA**: trifluoracetic acid
4. **MIC:** minimum inhibitory concentration
5. **HBTU**: N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
6. **Sar**: Fmoc-*N*-methylglycine

Introduction

Bacterial infections are becoming increasingly prevalent every year. The leading cause of such infections comes from *Staphylococcus aureus*,[1] the bacterial pathogen this project is focused on. *S. aureus* infections occur in about a third of the population, and methicillin-resistant *S. aureus* (MRSA) is responsible for roughly 80% of those infections.[2] The pathogen causes a variety of invasive effects from respiratory infections to bloodstream infections.[3] As a result of the increased use of antibiotics and the ability of bacteria to rapidly evolve or transfer information, antibacterial resistance has also increased.[4] While some countries boast a methicillin-resistance rate in the single digits, the US reports a MRSA rate greater than 50%,[3] an alarming number for such a developed country. Not only is there high antibiotic resistance in the US, but MRSA is also the pathogen with the highest mortality rate among those displaying antimicrobial resistance (AMR).[3] *S. aureus* has shown an astonishing ability to develop resistance in the era of antibiotics as the pathogen has developed resistance mechanisms to early antibiotics like penicillin and methicillin, as well as more recent ones like linezolid and daptomycin.[5] The versatility and rapid adaptability of *S. aureus* have caused the emergence of many strains that display antibiotic resistance, including multidrug-resistant (MDR) strains that are resistant to all forms of treatment.[3] Due to this high resistance among multiple strains of *S. aureus*, new therapeutic agents are critical.

A potential novel agent with growing interest to this problem is peptoids. Antimicrobial peptoids are mimics of naturally occurring antimicrobial peptides, which are part of the innate immune system.[6] The difference between peptides and peptoids lies in their structure. The naturally occurring peptides in our body have the side chain on the α-carbon of the amide backbone, while peptoids have the side chain on the amide nitrogen (**Figure 1)**. The challenge with peptides is that they are recognized in our bodies by proteolytic enzymes, causing them to be readily broken down and have a short half-life which lowers their bioavailability and prevents them from interacting with the desired target.[7] In contrast, due to the aforementioned structural difference, peptoids are not recognized by enzymes, giving them greater bioavailability and improved stability.[7] Coupled with these benefits is decreased cytotoxicity,[8] giving peptoids a therapeutic advantage over peptides. In addition, the positional difference of the R-group increases the permeability of the bacterial membrane to peptoids.[9]

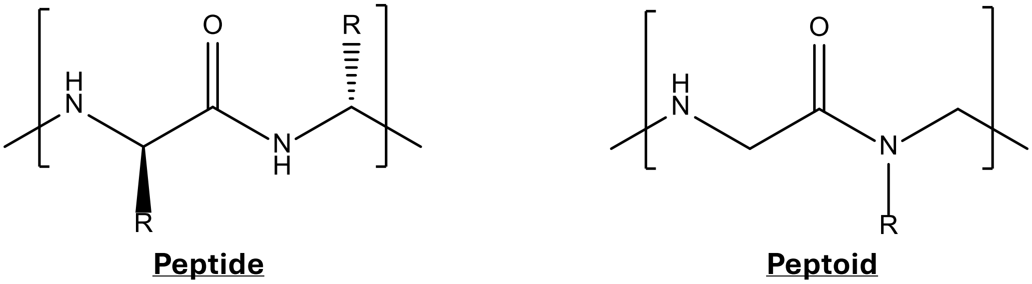

Figure 1. Structural comparison of a general peptide and peptoid backbone

Aside from biological advantages, peptoids are also cost-effective to synthesize. Peptoids are synthesized via solid-phase synthesis, a method in which monomers are added one at a time on polystyrene Rink Amide resin.[10] This method is also time efficient as it allows for the synthesis of different combinations of monomers in a relatively short timeframe.

Under the guidance of Dr. Bicker, a specific peptoid known as MTL1-44 was discovered using the Peptoid Library Agar Diffusion (PLAD) assay,[6] a high-throughput method to quickly screen large libraries of antimicrobial peptoids against *S. aureus*. This peptoid was shown to have good antibacterial activity and low cytotoxicity, making for a promising lead that could be developed into a treatment against *S. aureus* infections. This project seeks to perform lead optimization on MTL1-44 to improve its antibacterial activity and mammalian cytotoxicity. Lead optimization is a process where the structure is modified to enhance biological activity, a process our lab has done previously with antifungal peptoids.[11] By modifying the structure of MTL1-44 and identifying the side chains that are the most important, we will better understand the structure-activity relationship (SAR), giving us a better idea of the biological activity of our lead peptoid.[11]

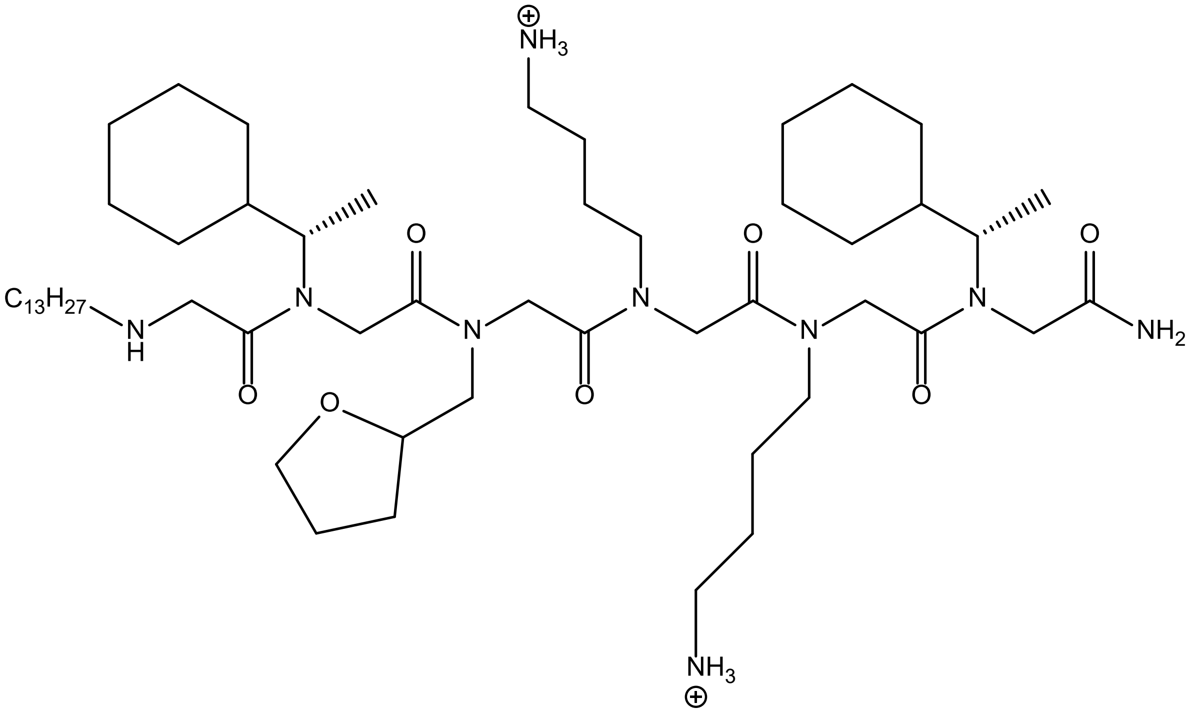

Figure 2. Structure of antibacterial peptoid MTL1-44

Thesis Statement

The goal of this project is to optimize MTL1-44's killing capacity against bacterial infections, specifically, *S. aureus* by performing lead optimization to see which amines are most important in its antibacterial activity while retaining low cytotoxicity.

Purpose

The overall goal of this project is to successfully perform lead optimization on MTL1-44 and test the resulting peptoids effects on bacterial infections like *S. aureus* in the hopes of one day utilizing them clinically. We are pursuing this goal because the peptoid has shown promising activity in dealing with *S. aureus*, thus we hope to increase its efficacy and reduce its toxicity to help those dealing with such infections. Specifically, we will do this through the design and synthesis of MTL1-44 derivatives using standard procedures in our lab. The resulting peptoids will then be characterized to understand the side chains most involved in antibacterial activity against *S. aureus* and their cytotoxicity against HepG2 cells. This project aims to provide a new treatment for infections caused by *S. aureus*.

Methodology

Phase I: Synthesis and Confirmation of MTL-43 and MTL1-44 Sarcosine Derivatives

To train in synthetic techniques, a peptoid similar to MTL1-44, known as MTL1-43, was synthesized using solid-phase peptoid synthesis.[12] This process involved using polystyrene resin with a Rink Amide linker. The resin was placed in a fritted column and was then swelled with reagent-grade dimethylformamide (DMF) for 30 minutes. After this, the resin was drained in an aspirator, followed by the addition of 20% piperidine twice, then placement on the rocker for 10 minutes to remove the Fmoc protecting group, a process known as Fmoc deprotection. Removal of the Fmoc protecting group is the first step which allows for exposure of a free amine onto which the individual monomers can be added. Confirmation of the removal of the Fmoc protecting group was done through a Kaiser/Ninhydrin test which was visualized as a purple color (positive Kaiser test). Upon successful removal of the protecting group, the resin was washed with reagent-grade DMF three times. Following this, the synthesis of each individual building block was done through two steps: bromoacylation followed by amination. Beginning with bromoacylation, 2M bromoacetic acid was added to anhydrous DMF (about 1.5 mL) in one vial and 3.2M diisopropylcarbodiimide (DIC) to anhydrous DMF (about 1.5 mL) in another vial. The resulting solutions were then added to the resin, which was microwaved at 10% power for 30 seconds, pausing at the halfway mark to shake up the reaction tube, and then placed on the rocker for 15 minutes. The solution was then drained in an aspirator, washed with reagent-grade DMF three times, and checked with a Kaiser test to confirm the reaction was successful. Successful acylation was seen by a yellow color (negative Kaiser test), indicating the absence of free amines. The overall purpose of bromoacylation is to make the OH of the carboxylic acid a good leaving group. Now that the carboxylic acid is activated, amination was done — reacting the carboxylic acid with an amine to form an amide via nucleophilic acyl substitution. In this step, we added 2M of the desired amine (3 mL) to the resin, microwaved it at 10% power for 30 seconds like before, placed it on the rocker for 30 minutes, drained it in an aspirator, then checked for successful amination using the Kaiser test, expected by a purple color, with the exception of branched amines like cyclohexylethylamine. This process of bromoacylation and amination, coupled with the colorimetric Kaiser test after each step was repeated for each building block of MTL1-43. We focused on MTL1-43 before pivoting our focus to MTL1-44. The overall synthesis process is the same. Each amine used in the synthesis of MTL1-44 is listed below in (**Table 1).** After the final amination, we cleaved the peptoid from the resin using a mixture of 95% TFA; 2.5% triisopropylsilane; 2.5% water and rocked for an hour. Next, Reverse-Phase High-performance liquid chromatography (RP-HPLC) was then used to purify the peptoid. **(Figure 3)** outlines the overall synthetic scheme of MTL1-44. Following purification, mass spectrometry was used to analyze the mass of our peptoids to confirm it was synthesized correctly. The peptoid was then dried down into a solid through the use of a rotary evaporator and lyophilizer. MTL1-43 served as training in the technique of solid-phase synthesis.

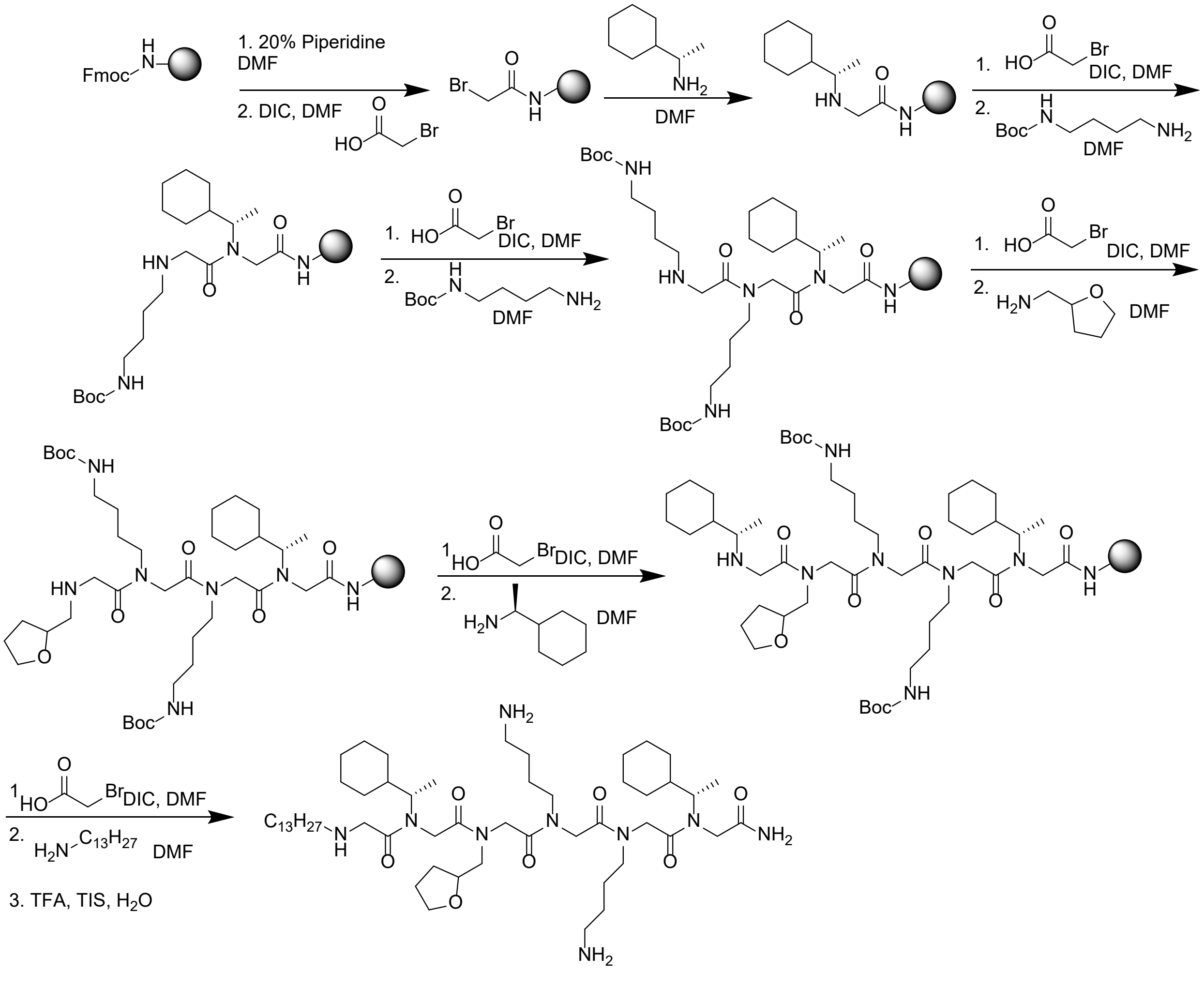

Figure 3. Solid-phase synthetic scheme of MTL1-44.

Table 1. Amine submonomer positions in MTL1-44 synthesis

Position	**Peptoid**
	MTL1-44
1	Tridecylamine
2	S-Cyclohexylethylamine
3	Tetrahydrofurfylamine
4	Boc-1,4-butanediamine
5	Boc-1,4-butanediamine
6	S-Cyclohexylethylamine

We focused on conducting a sarcosine scan of MTL1-44. Similar to an alanine scan in peptides, a sarcosine scan is utilized to determine the role of each individual monomer of the peptoid.[11] Sarcosine, also known as N-methylglycine, was used to replace the individual monomers of MTL1-44 one at a time. The synthesis of the sarcosine derivatives largely follows the same process explained above for MTL1-44. After performing Fmoc deprotection, at the position of deletion, 148 mg of N,N,N',N'-tetramethyl-O-(1Hbenzotriazol-1-yl)uronium hexafluorophosphate (HBTU) along with 121 mg Fmoc-*N*-methylglycine (Sar) were weighed out and placed in a small glass vial with 7 mL of 5% *N*-methylmorphine (NMM) in DMF. The vial was left to sit out for 10 minutes, then was poured into the tube with the beads for whichever derivative was receiving sarcosine for that round of synthesis. Synthesis tubes were then rocked for 45 minutes, followed by a drain and wash with DMF in an aspirator. This resulted in the synthesis of 6 sarcosine derivatives **(Figure 4)** since there are 6 different amines in MTL1-44. Testing of these derivatives will provide a better understanding of the importance of each side chain on the pharmacological activity of MTL1-44.[11]

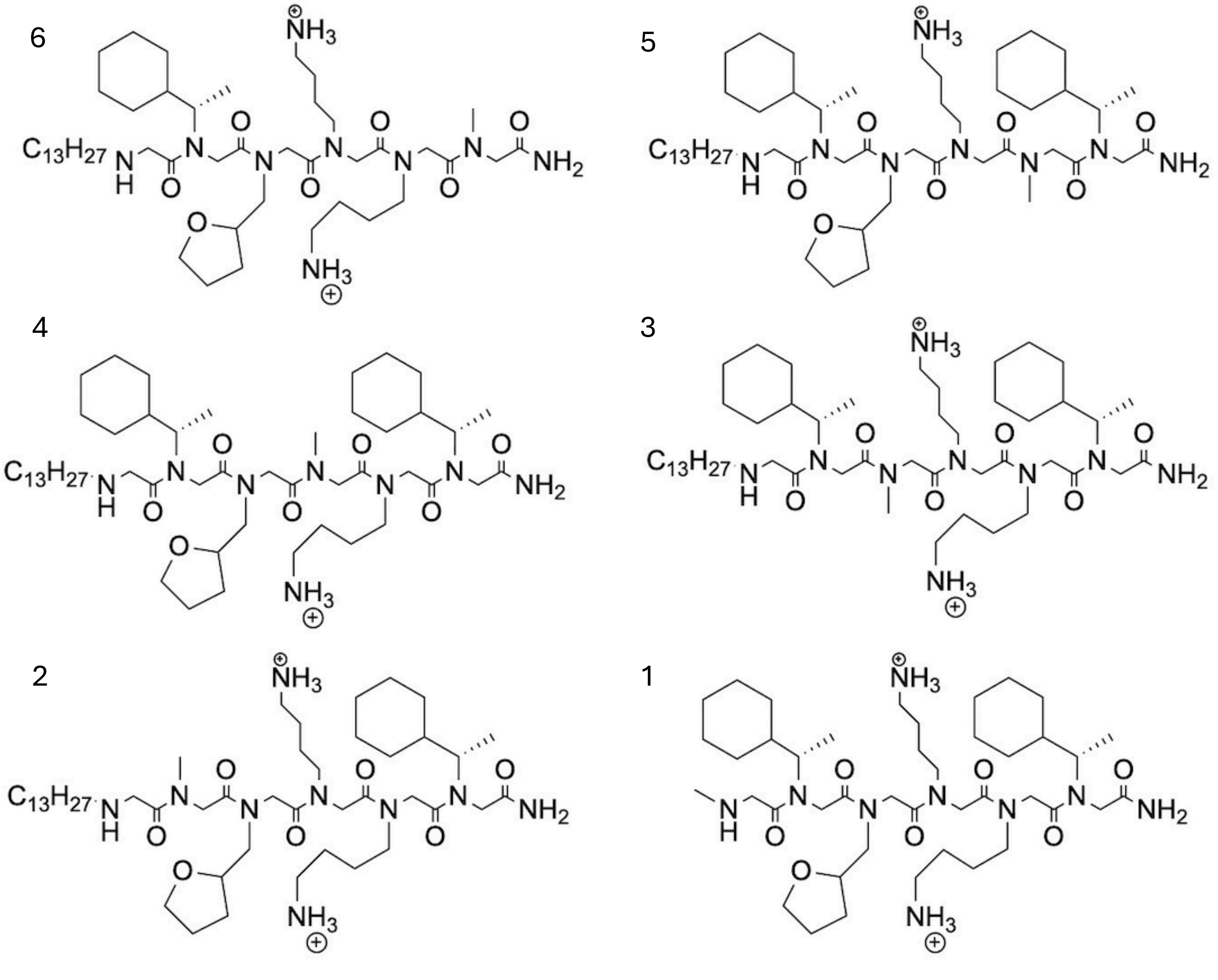

Figure 4. Sarcosine derivatives of MTL1-44.

The expected molecular weights for all 6 sarcosine derivatives were calculated and are shown in **(Table 2)**, as well as observed molecular weights which were obtained through mass spectrometry. Analysis of mass spectra involved observing and recording mass/charge (m/z) values. Since the m/1 of some of the sarcosine derivatives are greater than 900 g/mol (the cutoff of the mass spectrometer, the observed molecular weight was calculated from the m/2 value [(m/2 value multiplied by 2 minus 1).

Table 2. Expected molecular weights obtained from ChemDraw compared to observed molecular weights obtained from mass spectra.

Compound	Expected Molecular Weight (g/mol)	Observed Molecular Weight (g/mol)
SAR 1	821.1	820.5
SAR 2	893.3	892.7
SAR 3	919.4	918.8
SAR 4	931.4	931.8
SAR 5	931.4	931.8
SAR 6	893.3	892.7

Phase II: Characterization of Sarcosine Derivatives

To analyze the sarcosine derivatives, all peptoids were evaluated for antibacterial activity against *S. aureus*. The antibacterial activity of peptoid derivatives was determined as has been done previously in Dr. Bicker's lab.[13] This involved combining *S. aureus* with varying concentrations of MLT1-44 derivatives and determining the lowest concentration that prevents 90% of growth, defined as the minimum inhibitory concentration (MIC_{90}).

To determine the MIC_{90} using the broth microdilution method involved streaking a Lysogeny Broth (LB) plate with *S. aureus,* which was incubated overnight at 37°C. A 20 mg/mL stock solution was made for each sarcosine derivative in sterile water. Next, 10x stocks of each concentration of MTL1-44 derivatives were prepared in two-fold serial dilutions in water. Dilutions started at 100 μg/mL and ended at 1.56 μg/mL. Bacterial colonies were then transferred to Tryptic Soy Broth (TSB) to an OD_{600} between 0.08-0.15. This inoculant was diluted 1:200 in Cation Adjust Muller-Hinton broth (CAMHB) for a final concentration of ~$5x10^5$ CFU/mL. In a 96-well plate were added 90 μL of 1:200 cell inoculant or 100 μL of media as a control. Following this, 10 μL of the 10x compound stocks were added to the appropriate wells (diluted to 1x in the well; final well volume 100 μL). A negative (vehicle) control (vehicle = water) and a positive control (20 μg/mL tetracycline) were also added. These plates were incubated overnight at 37°C. Next, 10 μL of Presto Blue was added and incubated at 37°C for 1-2 hours. Finally, fluorescence was measured on a SpectraMax M5 Plate Reader (excitation 555 nm; emission 585 nm) to determine the metabolic activity of the bacteria in each well. The general setup of the 96-well plate is outlined in **(Figure 5).** These assays were run in technical and biological triplicates on three different days.

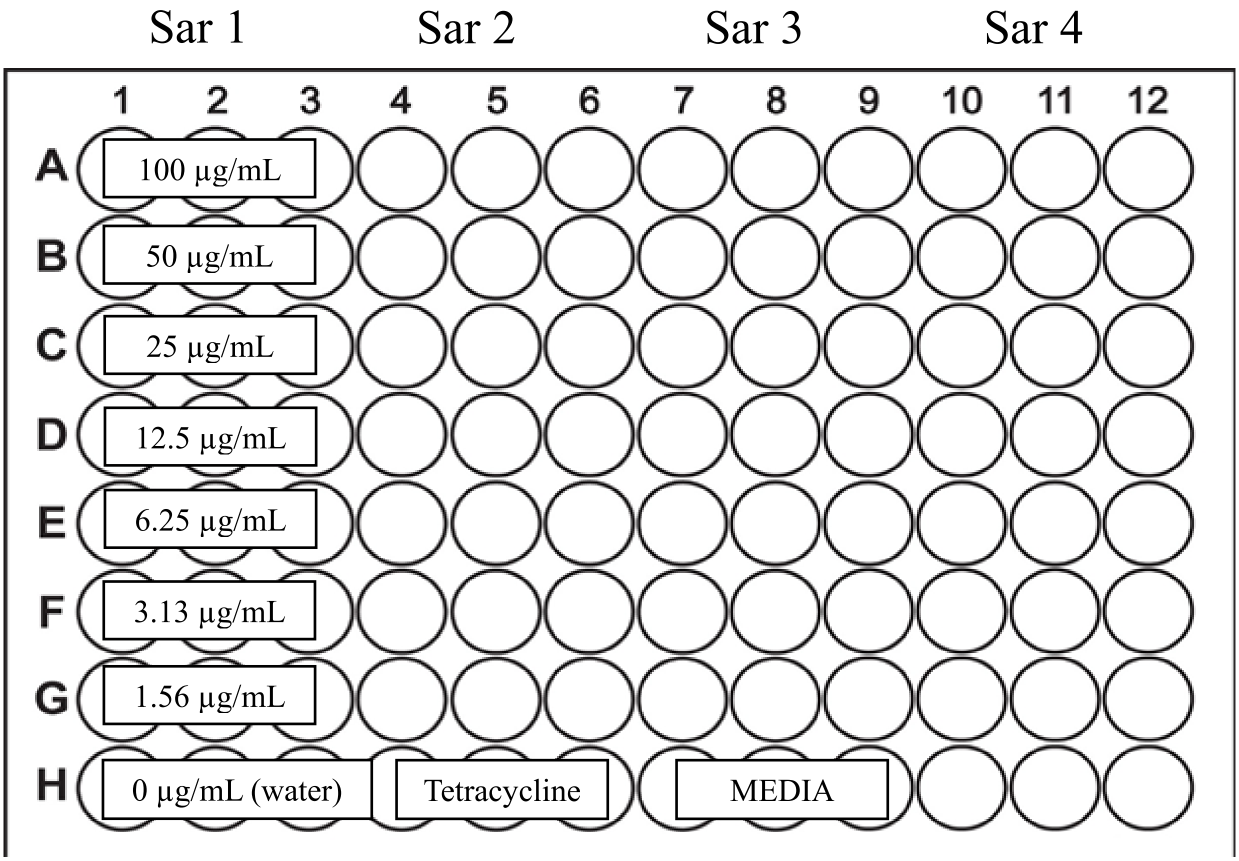

Figure 5. 96-well plate MIC_{90} for *Staphylococcus aureus*

Results & Discussion

I. Peptoid Synthesis and Confirmation

Utilizing solid-phase synthesis, MTL 1-43 and MTL1-44 sarcosine derivatives were successfully synthesized and confirmed through mass spectrometry in **(Figure 6)** and **(Figures 7-12),** respectively. MTL1-43 served as training in solid-phase synthesis techniques, allowing for the preparation of synthesis of MTL1-44 derivatives. With expected molecular weights of 821.1, 893.3, 919.4, 931.4, 931.4, and 893.3 Da for

SAR1-6, respectively, mass spectrometry (MS) confirmed the successful synthesis of each derivative, displaying m/1 peaks of 820.5, 892.7, 918.8, 931.8, 931.8, and 892.7 Da, as well as m/2 peaks of 410.7, 446.9, 459.9, 466.4, 466.4, and 446.8 Da. SAR1-6 had percent yields of 6.11%, 22.82%, 8.58%, 38.94%, 5.17%, and 8.89% (**Table 3**). The wide range in percent yields is due to difficulties during the purification process.

Figure 6. Mass spectra for MTL1-43, a model peptoid for training in solid-phase peptoid synthesis. The spectrum displays the expected m/1 peak of 988 Da and the expected m/2 peak of 494 Da.

Figure 7. Mass spectra for MTL1-44-SAR1, showing the expected m/1 peak of 820.5 Da and the expected m/2 peak of 410.7 Da.

Figure 8. Mass spectra for MTL1-44-SAR2, showing the expected m/1 peak of 892.7 Da and the expected m/2 peak of 446.9 Da.

Figure 9. Mass spectra for MTL1-44-SAR3, showing the expected m/1 peak of 918.8 Da and the expected m/2 peak of 459.9 Da.

Figure 10. Mass spectra for MTL1-44-SAR4, showing the expected m/1 peak of 931.8 Da and the expected m/2 peak of 466.4 Da.

Figure 11. Mass spectra for MTL1-44-SAR5, showing the expected m/1 peak of 931.8 Da and the expected m/2 peak of 466.4 Da.

Figure 12. Mass spectra for MTL1-44-SAR6, showing the expected m/1 peak of 892.7 Da and the expected m/2 peak of 446.8 Da.

II. Minimum Inhibitory Concentration

Following synthesis and confirmation of the peptoids, antibacterial activity was determined against *S. aureus* through standard broth microdilution techniques. The purpose of this test is to measure the lowest concentration of the test compounds that inhibit at least 90% of the growth of the pathogen consistently. The study is still being replicated, but two rounds of MIC have given the results below **(Table 3).** The data from MIC indicates that SAR 1, the derivative with a deletion of the tridecylamine, has significant reduction (MIC >100 μg/mL) in antibacterial activity compared to the parent compound, MTL1-44, which has an MIC value of 6.25 μg/mL. Among gram-positive bacteria, like *S. aureus*, the tridecylamine alkyl tail has shown the most antibacterial activity.[14] Naturally, a loss of this important side chain will hinder antibacterial activity. SAR 2, the derivative with a deletion of the cyclohexylethylamine group at position 2, also had a significant decrease in antibacterial activity compared to MTL1-44 (MIC 50 μg/mL). SAR 3, a loss of tetrahydrofurfylamine, does not result in a change of antibacterial activity (MIC 6.25 μg/mL). SAR 4 has better antibacterial activity compared to MTL1-44 as it shows MIC data of 3.13 μg/mL. The structural difference of SAR 4 compared to MTL1-44 is a deletion of the butanediamine at position 4. Given its improved antibacterial activity, this indicates that the diamine is not optimal for antibacterial activity in that position. SAR 5 also has a deletion of butanediamine, but this time at position 5. This causes a 2-fold difference in MIC compared to SAR 4 (MIC 6.25 μg/mL). It is unclear at the moment why, while both SAR 4 and SAR 5 are similar structurally, the MIC values differ between the two. Similarly, SAR 6 shows a 2-fold difference in MIC compared to SAR 2 (MIC 100 μg/mL), though both have a deletion of cyclohexylethylamine.

Table 3. Expected and observed molecular weights, percent yields, and MIC_{90} data for sarcosine derivatives 1-6 against *Staphylococcus aureus*

Compound	Expected MW (Da)	Observed MW (Da)	% Yield	MIC (µg/mL)
SAR 1	821.1	820.5	6.11%	>100
SAR 2	893.3	892.7	22.82%	50
SAR 3	919.4	918.8	8.58%	6.25
SAR 4	931.4	931.8	38.94%	3.13
SAR 5	931.4	931.8	5.17%	6.25
SAR 6	893.3	892.7	8.89%	>100

Conclusions & Future Plans

The goal of this project was to optimize the performance of a lead compound, MTL1-44, through structural modifications to increase antibacterial efficacy while maintaining or even improving upon cytotoxicity. Through these structural modifications, namely, a sarcosine scan, we are better able to understand the importance of each side chain as well as the positional importance of the same side chains on antibacterial activity. Preliminary data suggests that SAR 4, through the deletion of butanediamine at position 4, has greater efficacy compared to its parent compound, MTL1-44. Though a difference was seen, this difference is marginal, showing no significant improvements in the antibacterial activity of the parent compound MTL1-44. Additionally, prior SAR studies by our group[13] have shown that cationic side chains help to mitigate cytotoxicity, so deletion of the side chain in this position may not be a viable option for improving antibacterial activity.[15]

Despite a significant difference not being seen, the groundwork for future plans has been set. Moving forward, cytotoxicity testing will be done on Hepatocellular carcinoma (HepG2) cells originating from the liver. Further optimization of sarcosine derivatives will be carried out after analyzing data from both antibacterial and cytotoxicity testing. This will involve a process much like the one used to develop, purify, and test the sarcosine derivatives. With a better understanding of side chain importance, round 1 derivatives will be synthesized and tested. Synthesis of these derivatives will involve changing the side chains deemed to be most important (likely positions 1, 2, or 6) to see if there are improvements in antibacterial activity, as well as changing those that are deemed to have no effect. For example, SAR 6 has a deletion of cyclohexylethylamine. Instead of getting rid of it completely, different amines with different properties (hydrophilic, hydrophobic, cationic, etc.) could be tested to see if there is an improvement in biological activity. The same process will be done for round 2 derivatives after analyzing results from round 1 derivatives to create as refined a peptoid as possible.

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