University of Nevada, Reno

An Investigation into the Modulation of the Quorum Sensing Circuitry of *Enterococcus faecalis* with Rationally Designed Peptide Analogs of Native Signaling Molecule GBAP

A thesis submitted in partial fulfillment of the
requirements for the degree of Bachelor of Science in
Chemistry and the Honors Program

by

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THE HONORS PROGRAM

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ABSTRACT

Rising antibiotic resistance over the past several decades calls for more effective antibiotics or a sustainable alternative. In the bacterial species *Enterococcus faecalis,* this can be accomplished through targeted attacks on the communication pathway controlled by quorum sensing: the ability of the bacteria to act as a group, sometimes leading to pathogenicity. In this study, a structure activity relationship was established between the quorum sensing circuitry in *E. faecalis* and the native signaling peptide. These results were then used to design other peptide modulators aimed to better activate the pathway. Using this method of rational design, the most potent peptide modulator known to date was discovered.

TABLE OF CONTENTS

INTRODUCTION

PROBLEM OF ANTIBIOTIC RESISTANCE

After the introduction of antibiotics in the 1940s, this class of prescription pharmaceuticals has become a popular form of treatment for bacterial infections (Ventola, 2015). Antibiotics like penicillin successfully treated bacterial infections contracted by soldiers during the second World War (Ventola, 2015). Antibiotics have also served as a way to protect immunocompromised patients from contracting bacterial infections in the clinical setting (Ventola, 2015). The introduction of antibiotics and other advances in western medicine contributed to a drastic increase in life expectancy from approximately 56 years in the 1920s to approximately 80 years in modern time (Ventola, 2015). Overall, the introduction of antibiotics was an instrumental advancement in modern medicine and has allowed for the treatment of common bacterial infections that were once untreatable.

Despite the major advances that antibiotics have facilitated in the medical industry, overuse of antibiotics in the clinical setting has led to the development of resistance in certain bacterial strains. Due to the nature of antibiotics to kill susceptible bacteria, a selective pressure is placed on the bacteria, allowing for the development and spread of antibiotic resistance genes throughout a bacterial population. Random chromosomal mutations can sometimes lead to phenotypic traits like resistance to the mechanism that antibiotics use to kill susceptible bacteria. These resistant bacteria survive treatment with antibiotics and proliferate to colonize the site of infection. This development of antibiotic resistant strains of bacteria is common in the clinical setting because many bacteria are present and treatment with antibiotics is commonly coupled with treatments for other diseases.

Quickly after the introduction of vancomycin in 1972 as a solution to the development of methicillin resistant *Staphylococcus aureus* (MRSA) identified in 1962, resistance developed in the clinical setting despite strong evidence that vancomycin resistance in a laboratory setting was unlikely (Ventola, 2015). Since then, several other antibiotics of last resort have been developed and released to the antibiotic market, though vancomycin remains an important antibiotic of last resort in the clinal setting (Ventola, 2015). Vancomycin resistant *Enterococci* (VRE) have been identified as a serious threat by the Center for Disease Control (CDC), laying the groundwork for this project.

ENTEROCOCCUS FAECALIS

OVERVIEW *Enterococcus faecalis* is a Gram-positive bacterium that naturally resides in the human gut biome, but is also an opportunistic pathogen, causing the majority of surgical wound and urinary tract infections (Huycke 1998; Arias, 2012). All strains of *E. faecalis* have intrinsic resistance to some antibiotics and may have acquired resistance to additional antibiotics, including to the last resort antibiotic vancomycin, making it an important bacterial species to study (Huycke 1998; Arias, 2012). Additionally, *E. faecalis* has the ability to share resistance genes with other species of bacteria, including MRSA. The ability of *E. faecalis* to develop and share resistance to a common method of treatment, vancomycin, elicits the need for an alternative to antibiotics to stop the infectious nature of this bacteria.

QUORUM SENSING Quorum sensing (QS) is the process of regulating bacterial group behaviors, both symbiotic and pathogenic, through the detection of small signaling molecules in the extracellular environment. Utilizing the fsr QS circuitry, *E. faecalis* can assess cell density by evaluating the concentration of a signaling peptide, termed gelatinase

biosynthesis-activating pheromone (GBAP) (Nakayama, 2001; McBrayer, 2017). When a sufficiently high cell density is detected, a signaling cascade is activated, resulting in the transcription of genes involved in virulence factor production (Figure 1) and the formation of biofilms, allowing the bacteria to establish an infection and attack their host (Nakayama, 2001; McBrayer, 2017; Carniol, 2004; Sifri, 2002).

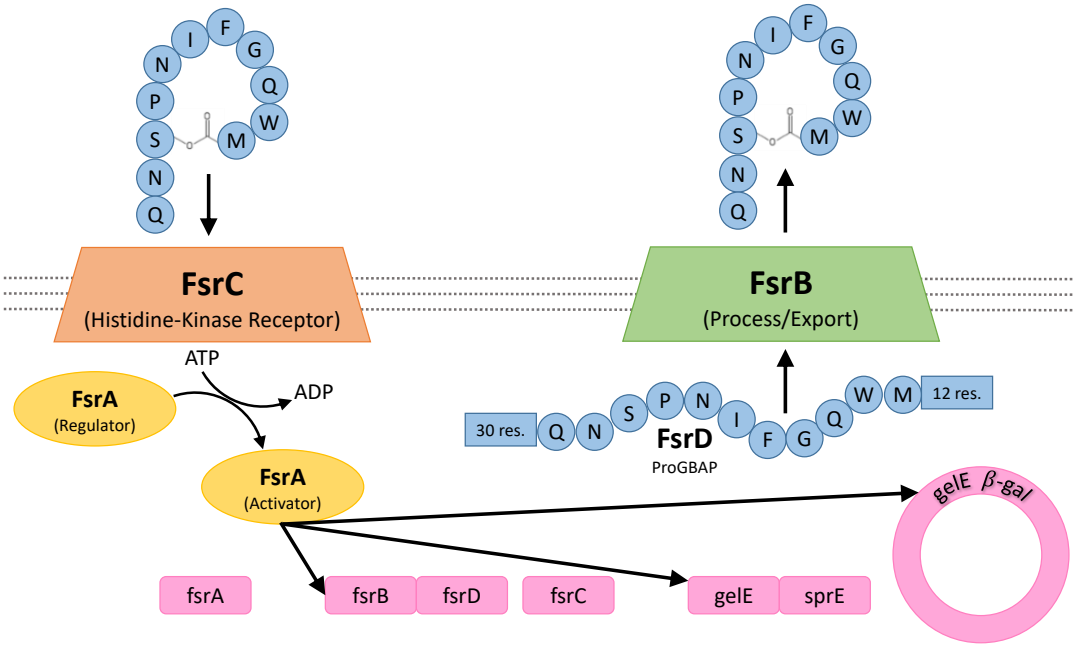

Figure 1: *The fsr QS circuit in* E. faecalis. *Cell density is assessed through the binding of GBAP to the FsrC receptor and the transcription of the genes activated by the phosphorylated FsrA. FsrD is processed and exported out of the cell by FsrB to contribute to the signal for cell density. The plasmid hosting the PgelE and β-gal gene is included in the reporter strain of* E. faecalis *to allow for the completion of a β-gal assay to quantify QS activation.*

The fsr QS circuit is activated by GBAP, an eleven amino acid cyclic peptide with an ester linkage between the side chain of serine at position 3 and the *C*-terminus of methionine (Nakayama, 2001; McBrayer 2017). GBAP binds the transmembrane receptor, FsrC, leading to FsrA phosphorylation, which, in turn, results in the transcription of the

fsrBDC (produces more QS machinery to maintain cell coordination), and the *gelE* and *sprE* genes (responsible for triggering production of virulence factors and formation of biofilms) (Nakayama, 2001; McBrayer 2017). The 53 residue ProGBAP is produced by the *fsrD* gene and is then processed into its final product, GBAP, and exported outside the cell by FsrB; cells can then assess cell density by detecting the extracellular concentration of GBAP (Nakayama, 2001; McBrayer 2017).

RESEARCH PURPOSE

The goal of this research is to establish a structure-activity relationship (SAR) between the native signaling peptide, GBAP, and the QS circuitry under its control. This can be done by performing a series of scans of the native peptide where individual amino acids are replaced one at a time and the biological activity of those analogs tested and compared to the biological activity of GBAP. To establish an SAR, a d-amino acid scan and an alanine scan were conducted to gain insight into the effects of stereochemistry and side chain identity, respectively. Interpretation of these results led to the testing of the hypothesis that the tail residues were not important to activity in a library of tail modifications. Finally, a library of agonists was designed using the data produced in the SAR study that identified the most potent activator of the fsr QS pathway to date. Completion of this research has aided in the design of new compounds that can modulate QS and has advanced efforts to develop alternative therapies for *E. faecalis* infections.

MATERIALS AND METHODS

OVERVIEW

First, a library of peptide analogs was designed based on the research goal outlined in the introduction. Sequences of these peptides can be found in Tables 3-6 in the results.

Each of the peptides in the designed library was synthesized using the solid phase peptide synthesis (SPPS) method on resin support. Based on the overall structure of the intended peptide, either aspartic acid or glutamic acid was attached to the resin by the side chain, which yields the desired asparagine in the fifth position or glutamine in the ninth position upon cleavage from the resin (Figure 2). After the initial loading, a peptide chain is formed by coupling each desired amino acid one at a time until the *N*-terminus is reached. Selective deprotection of the trityl protecting group on the serine side chain is then used to facilitate the formation of the ester linkage between the serine side chain and the *C*-terminus of the peptide using a symmetrical anhydride of the eleventh position amino acid.

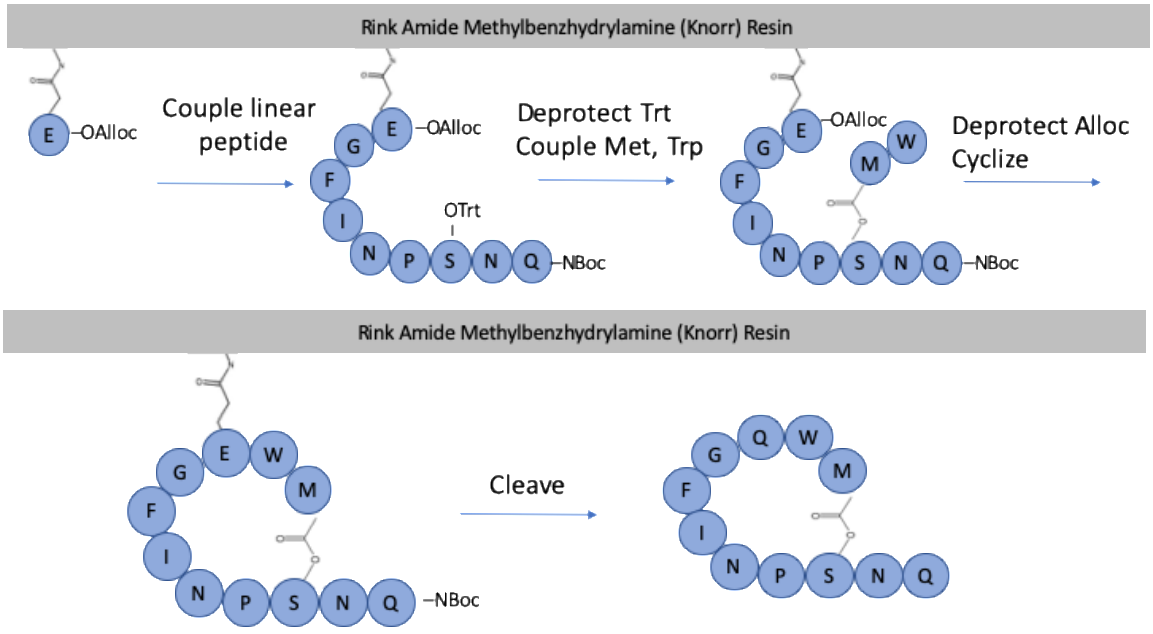

Figure 2 *Synthetic method for solid phase peptide synthesis beginning with glutamic acid*

After the successful synthesis of these peptide analogs, each was purified to greater than 95% using preparatory reverse phase high performance liquid chromatography (RP-HPLC) and a water: acetonitrile solvent system. Fractions were collected off the column and then each fraction was checked for the desired peptide mass using mass spectrometry. Purity of the desired fraction was determined using analytical RP-HPLC.

Peptides were then tested using a biological assay (bioassay) to assess their ability to activate\inhibit the QS pathway. A reporter strain of *E. faecalis* harboring a plasmid that codes for β-galactosidase (β-gal, *lacZ* gene) under the control of the fsr QS circuitry (*gelE* promoter) was used, allowing for the quantification of QS activation. When the *gelE* gene on the chromosomal DNA was transcribed, the *lacZ* gene on the plasmid was also transcribed, ensuring the amount of activation of the QS pathway was proportional to the amount of β-gal produced. During the bioassay, the bacteria were grown in the presence of different concentrations of the test peptide. The cells were then lysed with the detergent Triton X-100 to release the β-gal into solution and *ortho*-Nitrophenyl-β-galactosidase (ONPG) was added in excess. The β-gal then hydrolyzed the ONPG to galactose and *ortho*-nitrophenyl (ONP), the latter of which absorbs strongly at 420 nm. A stronger absorbance corresponds to a higher activation of QS. An EC_{50} was then obtained through a fitting *via* nonlinear regression of a plot of the signal *vs.* the different peptide concentrations, allowing identification of the half maximum effective concentration. If the analog was unable to activate the FsrC receptor, then an inhibition assay was conducted as described above, except that GBAP was added as a competitor, and the ability of the analog to inhibit activation by GBAP was measured by determining the IC_{50} through nonlinear regression fitting of a plot of signal *vs.* concentration of analog.

SOLID PHASE PEPTIDE SYNTHESIS

GENERAL All syntheses were conducted in 6 mL polypropylene reaction vessels fitted with porous frits at the bottom to allow for washing of resin using solvents and condensed air. References to equivalents are relative to the resin loading. After completion of synthesis, dried peptides were stored at -80 °C to prevent degradation.

CHEMICALS AND INSTRUMENTATION Reagents and solvents used in the synthesis of peptide analogs were purchased from Sigma-Aldrich or Chem-Impex and were used without further purification. Resin was purchased from Advanced ChemTech and the resin loading was confirmed with in-house testing. All dry solvents were dried over 3 Å molecular sieves. All reactions involving shaking used SHAKER SPECS shaking at 200 rpm.

INITIAL RESIN DEPROTECTION AND LOADING Synthesis was conducted using Rink Amide MBHA (Knorr) resin with a loading capacity of 0.89 mmol/g. All peptides were synthesized on a 100 mg scale of resin. Initial deprotection of the fluorenylmethyloxycarbonyl (Fmoc) protecting group was conducted with the Fmoc deprotection procedure described below with the following modifications: the deprotection solution was allowed to shake for 8 minutes for each deprotection. After deprotection of the resin, either aspartic acid or glutamic acid was loaded on the resin by a reaction between the amine group on the resin and the carboxylic acid group on the side chain of these specific amino acids. Specialized amino acids were used for this initial coupling that contain Fmoc protecting groups on the *N*-terminus and OAlloc protecting groups on the *C*-terminus to allow for the elongation of the peptide chain on the *N*-terminus and cyclization at the *C*-terminus upon completion of the synthesis of the peptide chain. Upon cleavage of

the side chain from the resin, an amide bond is formed, producing the desired asparagine in the fifth position (Asn5) or glutamine in the ninth position (Gln9), respectively. Initial loading of the resin was accomplished using the method for a standard amino acid coupling with either aspartic acid or glutamic acid with the following modifications: resin was allowed to react with the amino acid on an overnight time scale at room temperature.

FMOC DEPROTECTION In order to remove the base-labile Fmoc group from growing peptide chain, a 2 mL solution of either 20% piperidine or 2% piperidine, 2% 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) in dimethyl formamide (DMF) was added to reaction vessel and the container was shaken for seven minutes. This procedure was performed twice in order to increase the conversion of the Fmoc protected peptide chain to the Fmoc deprotected peptide chain. After the second deprotection, the resin was washed with 2 mL DMF three times.

QUANTIFICATION OF FMOC DEPROTECTION The progress of the synthetic process was tracked by following the amount of Fmoc that was removed from the resin during Fmoc deprotections. The deprotection solution was collected into disposable culture tubes and a solution of 3 µL deprotection solution in 1 mL DMF was prepared. After blanking with a 1 mL solution of 3 µL of either 20% piperidine or 2% piperidine, 2% DBU in DMF, the absorbance of the experimental solution was read at 290 nm. By comparing the absorbance value for each deprotection to the absorbance value for the initial deprotection of the resin, the percent yield for each reaction could be quantified.

STANDARD AMINO ACID COUPLING Most amino acid couplings were conducted using 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) as a coupling reagent and N,N-diisopropylethylamine

(DIPEA) as the base. Three equivalents of the desired Fmoc-protected amino acid and 2.85 equivalents of HATU were dissolved in 2 mL DMF. Then, 3 equivalents of DIPEA were added to the solution, shaken briefly, then added to the reaction vessel and shaken. After this time period, the resin was washed three times with DMF. Specific conditions for time scale and temperature for each amino acid can be found in Table 1.

Table 1 *Times and temperatures of standard amino acid couplings*

Amino Acid	**Time Shaking**	**Temperature**
Glutamine	2 hr or overnight	24 °C
Asparagine	2 hr or overnight	24 °C
Serine	Overnight-12 hr	50 °C
Proline	2 hr or overnight	24 °C
Isoleucine	2 hr or overnight	24 °C
Phenylalanine	2 hr or overnight	24 °C
Glycine	2 hr or overnight	24 °C
Alanine	2 hr or overnight	24 °C
Tryptophan	2 hr or overnight	24 °C
Boc-Glutamine	Overnight-12 hr	24 °C

SELECTIVE OTrt DEPROTECTION Before the formation of the ester linkage between the side chain of the Serine in the third position (Ser3) and the N-terminus of the peptide, the trityl protecting group must be removed from the Serine side chain. This was accomplished by first washing the resin three times with dichloromethane (DCM) for 1 minute and drying it under air. The resin was then treated with a 2 mL solution of 1% trifluoroacetic acid (TFA), 5% triisopropylsilane (TIPS) in DCM for 2 minutes. This

deprotection was conducted four times. Then, the resin was washed with DCM three times for 1 minute and then three times with DMF before the formation of the ester linkage.

FORMATION OF SYMMETRICAL ANHYDRIDE To a round bottom flask, 10 equivalents of the desired amino acid was added and dissolved in a minimal amount of dry DCM. To this solution, 5 equivalents of N,N'-diisopropylcarbodiimide (DIC) was added while stirring at 0 °C. The reaction was capped and allowed to proceed at 0 °C for 20 minutes. The reaction was then removed from the ice bath and allowed to warm to room temperature. Additional dry DCM was added to dissolve any precipitated product. The reaction was then capped again and allowed to react at room temperature for 20 minutes. The solvent was then removed using rotary evaporation. The resulting white solid was the symmetrical anhydride of the desire amino acid.

ESTER LINKAGE FORMATION ON RESIN The symmetrical anhydride formed in the previous step was dissolved in 2 mL or a minimal amount of dry DMF. This solution was added to the reaction vessel with the resin and was shaken for 30 minutes before the addition of 0.04 equivalents of 4-dimethylaminopyridine (DMAP) as a low concentration solution in DMF. This mixture was shaken for 3.5 hours at room temperature. Following the reaction, the resin was washed three times with DMF.

SELECTIVE O-ALLOC DEPROTECTION To prepare to cyclize the peptide, the allyloxycarbonyl (Alloc) protecting group must be removed from the amino acid attached to the resin by its side chain. This is accomplished by first washing the resin three times for 1 minute with DCM and drying the resin under air. Approximately 5 mL of dry DCM was placed in a 15 mL polypropylene centrifuge tube and sparged with argon for 3 minutes. Ten equivalents of phenyl silane was then added to the solvent and the solution was sparged

for another 3 minutes. Then, 0.5 equivalents of tetrakis(triphenylphosphine)palladium(0) was added to the solution and sparged for another 3 minutes. The resulting solution was added to the reaction vessel under argon. The cap of the reaction vessel was then sealed with parafilm and placed inside a 50 mL secondary container, which was also filled with argon and sealed with parafilm. This container was shaken for 2 hours at room temperature. After the specified reaction time, the resin was washed four times with 0.5% sodium diethyldithiocarbamate trihydrate in DMF for 2 minutes while shaking and then four times with DMF.

ON RESIN CYCLIZATION After the deprotection of the *C*-terminus of the initially loaded amino acid, the cyclization can occur between the *N*-terminus of the growing peptide chain and the *C*-terminus of the initially loaded amino acid. PyOxim in 1.25 equivalents was dissolved in 2 mL DMF. Then, 2.5 equivalents of DIPEA was added before the solution was added to the reaction vessel and shaken. Two cyclization reactions were conducted, one on an overnight timescale and the other on a two-hour timescale, with the two being exchangeable with respect to the order in which they were conducted. The resin was then washed three times with DMF, three times with DCM, and dried using air. In order to determine whether or not the cyclization was successful, the Kaiser test was performed prior to final cleavage from the resin.

KAISER TEST FOR PRIMARY AMINES A small amount of dried resin was removed from the reaction vessel and placed in a small disposable culture tube. Then, 2 drops of 5% (w/v) ninhydrin in ethanol, 2 drops of 80% (w/v) phenol in ethanol, and 2 drops of 20 µM potassium cyanide in pyridine were added to the resin. This mixture was then heated for five minutes at 120 °C. A lack of color change in the resin beads indicated

that there was no primary amine present, or that the cyclization was successful. In contrast, purple beads or solution indicated the presence of a primary amine, or that the cyclization was unsuccessful. If the Kaiser test suggested that the cyclization was unsuccessful, deprotection of the O-Alloc group was attempted again followed by the two cyclization reactions described previously. If the Kaiser test suggested that the cyclization was successful, the resin was washed and dried and final cleavage was conducted.

FINAL CLEAVAGE AND PRECIPITATION After the dried resin was transferred to a 15 mL centrifuge tube, the resin was treated with 3 mL of 2.5% TIPS, 2.5% water in TFA for 3 hours while shaking. The resin was then separated from the dissolved peptide by filtering the solution through a syringe fitted with a cotton plug into a 50 mL centrifuge tube. The final cleavage container was rinsed with a minimal amount of TFA to collect any residual peptide from the container. The crude peptide was then precipitated using approximately 45 mL of -20 °C 1:1 hexanes:ether solution. After cooling this solution for ten minutes in a -20 °C freezer, the tube was centrifuged, forming a pellet at the bottom of the container. The solution was decanted and discarded while the pellet was dissolved in 3-5 mL 1:1 acetonitrile:water, frozen in a dry ice/acetone bath, and lyophilized for at least 18 hours.

PURIFICATION OF PEPTIDE ANALOGS

PREPARATORY HPLC Peptides were purified by reverse phase preparatory HPLC fitted with a fraction collector. Crude peptide was dissolved in the minimal amount acetonitrile and diluted to 4 mL in water to make solutions of 3-36% acetonitrile depending on the solubility of the peptide and byproducts of synthesis. This peptide solution was filtered and injected onto a Phenomenex Kinetex 5 µm C18 semi-preparative column (10

mm x 250 mm, 100 Å). Gradient elution from 20% to 60% acetonitrile over 40 minutes was used to purify the peptide to approximately 80% purity. If necessary, a second preparatory run was conducted using gradient elution conditions based on the first run and the same injection conditions. The final purity of the isolated peptide was determined using analytical HPLC described below.

ANALYTICAL HPLC In order to determine the purity of the isolated peptide, a dilute solution of the peptide in 1:1 acetonitrile:water or a two-fold dilution of the fraction itself was prepared. 90 µL of this solution was injected onto a Phenomenex Kinetex 5 µm C18 analytical column (4.6 mm x 250 mm, 100 Å). Gradient elution from 5% to 95% acetonitrile over 24 minutes was used. The purity of the isolated peptide was determined by integrating the entire graph of time vs. absorbance. The percentage of integrated area due to the presence of the peptide was assumed to be equivalent to the purity of the peptide. All peptides were purified to greater than 95% purity before being assessed for biological activity.

VERIFICATION BY MASS SPECTROMETRY Peptide identity was confirmed using matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS). Matrix made of α-cyano-4-hydroxycinammic acid was dispensed onto a specialized plate in 7 µL portions and allowed to dry. Aliquots of the same volume either directly from the fractions collected during the preparatory purification or a solution of the fraction in 1:1 acetonitrile:water were then dispensed onto the dried matrix. These samples were then analyzed by MALDI-TOF MS, confirming the presence of the +1 charge species, also indicated by the presence of the mass + sodium and mass + potassium. After all purification steps were complete, the exact mass of the peptide was determined using high

resolution electrospray ionization (ESI) TOF mass spectrometry. Before analysis of the experimental peptide, the instrument was calibrated using an internal reference solution.

ANALYSIS OF BIOLOGICAL ACTIVITY

EC_{50} DETERMINATION A 2 mM in dimethyl sulfoxide (DMSO) stock solution of the test peptide was prepared and stored at -80 °C. This solution was only removed from the freezer at the time of bioassay preparation and was only thawed long enough to remove the required aliquot. *E. faecalis* bacteria were grown on Mueller-Hinton Broth 2 (MHB-2) plates overnight without the presence of the control antibiotic at 37 °C. A single colony was then removed from the plate and transferred to 5 mL of brain heart infusion (BHI) broth and shaken overnight at 37 °C. This broth contained the control antibiotic kanamycin to prevent the growth of other bacteria. The overnight culture was then diluted 50-fold into BHI broth containing 500 µg/mL kanamycin and this culture was shaken at 37 °C for 1 hour, determined by in-house experimentation with different reaction conditions. Aliquots of 198 µL were then transferred into 96-well plates containing either 2 µL DMSO (negative control), GBAP (positive control), or a dilution series of the test peptide. These plates were then incubated for 2 hours at 37 °C. The absorbance of these plates was then read at 600 nm (A600) to determine the relative concentration of bacteria in each well. The cells were then lysed using 20 µL 1% Triton X-100 in water for 30 minutes at 37 °C. Another 96-well plate was prepared with 100 µL of the substrate buffer made up of 0.8 mg/mL ortho-Nitrophenyl-β-galactoside (ONPG) and 2.7 µL/mL β-mercaptoethanol in Z-buffer (100 mM sodium phosphate buffer, 10 mM KCl, and 1 mM $MgSO_4$, pH 7.0). To this new plate, 100 µL of the lysed cell solution was added and incubated for 30 minutes at 37 °C. The reaction was then quenched with 20 µL 1 M sodium carbonate in water. The absorbance

of the wells was then read at 420 nm and 550 nm, which allowed for the activity to be determined with the use of Miller units, where *c* is a correction factor, *t* is the time of the experiment, and *v* is the volume of the original culture (mL) (Equation 1).

$$Miller\ Unit = 1000 * \frac{A420 - c * A550}{t * v * A600} \quad (1)$$

IC_{50} DETERMINATION Experiments to determine IC_{50} were conducted in the same manner as the EC_{50} experiments described above, though 50 nM GBAP was also included to act as a competitor.

RESULTS

SYNETHESIS AND PURIFICATION

The peptides detailed in Table 2 (McBrayer, 2018) were synthesized and purified by the author of this thesis with the goal of improving potency of known activators. Other peptide modulators that were synthesized and purified in conjunction with other contributing authors are included in subsequent tables and in biological activity results.

Table 2 *Synthesis and purification details of peptide modulators for improved potency*

Modifications	**Sequence**	**Yield**	**Purity**
DesQ1N2, S3s, lactam	(**s**PNIFGQWM)	4.1%	>= 99%
N2n, P4A	Q**n**(S**A**NIFGQWM)	0.2%	>= 97%
Q1q, N2n, P4A	**qn**(S**A**NIFGQWM)	1.0%	>= 95%
N-terminal Ac,N2n,P4A	**Ac**-Q**n**(S**A**NIFGQWM)	1.2%	>= 98%
N-terminal Ac, Q1q,N2n,P4A	**Ac**-**qn**(S**A**NIFGQWM)	0.4%	>= 98%
N-Terminal Z, Q1q, N2n	**Z**-**qn**(SPNIFGQWM)	2.1%	>= 98%
N-Terminal Z, N2n, P4A	**Z**-Q**n**(S**A**NIFGQWM)	0.3%	>= 95%
N-Terminal Ac, Q1q	**Ac**-**q**N(SPNIFGQWM)	2.1%	>= 99%

BIOLOGICAL ACTIVITY

In order to assess the biological activity, bioassays were conducted using the synthesized peptide analogs as detailed in the Materials and Methods. Table 3 (McBrayer, 2017) details the effects of inversion of chirality of the amino acids at the chiral center in the carbon backbone. Naturally occurring *L*-amino acids were replaced with their *D*-amino acid counterparts, resulting in a *D*-epimer scan of the native signaling peptide. Glycine in

the eighth position was not included in this scan because there is not a chiral center in this amino acid, and therefore, the chirality cannot be inverted.

Table 3 *Biological activity of D-epimer scan*

Modification	**Sequence**	**EC_{50} [95% CI] (nM)**
GBAP	QN(SPNIFGQWM)	1.15 [0.825-1.59]
GBAP-Q1q	**q**N(SPNIFGQWM)	0.211 [0.0798 - 0.559]
GBAP-N2n	Q**n**(SPNIFGQWM)	0.304 [0.168 - 0.552]
GBAP-S3s	QN(**s**PNIFGQWM)	45.1 [17.0 - 120]
GBAP-P4p	QN(S**p**NIFGQWM)	>1000
GBAP-N5n	QN(SP**n**IFGQWM)	>1000
GBAP-I6i	QN(SPN**i**FGQWM)	>1000
GBAP-F7f	QN(SPNI**f**GQWM)	4.97 [3.12 - 7.77]
GBAP-Q9q	QN(SPNIFG**q**WM)	>1000
GBAP-W10w	QN(SPNIFGQ**w**M)	>1000
GBAP-M11m	QN(SPNIFGQW**m**)	231 [129 – 414]

In order to gain insight into the importance of the amino acid side chain in binding to the receptor, each amino acid was individually replaced with the amino acid alanine, which has a methyl group side chain, resulting in an alanine scan of the native signaling peptide. Table 4 (McBrayer, 2017) details the results of this alanine scan. Because the side chain of serine participates in the ester linkage that forms the ring structure of GBAP, serine in the third position was not modified in the alanine scan.

Table 4 *Biological activity of alanine scan*

Modification	**Sequence**	**EC_{50} [95% CI] (nM)**
GBAP	QN(SPNIFGQWM)	1.15 [0.825-1.59]
GBAP-Q1A	**A**N(SPNIFGQWM)	0.549 [0.299 - 1.01]
GBAP-N2A	Q**A**(SPNIFGQWM)	0.704 [0.552 - 0.898]
GBAP-P4A	QN(S**A**NIFGQWM)	0.27 [0.202 - 0.360]
GBAP-N5A	QN(SP**A**IFGQWM)	341 [200 – 581]
GBAP-I6A	QN(SPN**A**FGQWM)	> 1000
GBAP-F7A	QN(SPNI**A**GQWM)	> 1000
GBAP-G8A	QN(SPNIF**A**QWM)	> 10,000
GBAP-Q9A	QN(SPNIFG**A**WM)	312 [222 – 437]
GBAP-W10A	QN(SPNIFGQ**A**M)	246 [117 – 520]
GBAP-M11A	QN(SPNIFGQW**A**)	164 [93.8 – 288]

From observations of the D-epimer scan and the alanine scan detailed in the discussion, the two amino acids that make up the tail region of the native signaling peptide, Q1 and N2, are modifiable. Several modifications to the tail were made and the biological activity of these analogs is detailed in Table 5 (McBrayer, 2017).

Table 5 *Biological activity of tail modifications*

Modification	**Sequence**	**EC_{50} [95% CI] (nM)**
GBAP	QN(SPNIFGQWM)	1.15 [0.825-1.59]
N-terminal Ac	**Ac-**QN(SPNIFGQWM)	1.5 [1.31 - 1.73]
DesQ1	N(SPNIFGQWM)	1.26 [1.15 - 1.39]
N-terminal Ac, DesQ1N2	**Ac-**(SPNIFGQWM)	1.01 [0.496 - 2.06]
DesQ1, N-terminal Ac	**Ac-**N(SPNIFGQWM)	1.44 [0.855 - 2.44]
DesQ1N2, lactam	(SPNIFGQWM)	> 10000

Based on the individual modifications that were observed to improve potency, a library of peptide analogs was designed by combining these individual modifications. The bioassay results of this improved potency library are detailed in Table 6 (McBrayer, 2018).

Table 6 *Biological activity of improved potency library*

Modification	**Sequence**	**EC_{50} [95% CI] (nM)**
GBAP	QN(SPNIFGQWM)	1.15 [0.825-1.59]
Q1q, N2n	**qn**(SPNIFGQWM)	0.103 [0.043 - 0.249]
Q1q, P4A	**q**N(S**A**NIFGQWM)	1.87 [1.24 - 2.81]
N2n, P4A	Q**n**(S**A**NIFGQWM)	0.046 [0.0300 - 0.0691]
Q1q, N2n, P4A	**qn**(S**A**NIFGQWM)	17.0 [10.3 - 28.0]
N-terminal Ac, Q1q, N2n	**Ac-qn**(SPNIFGQWM)	0.504 [0.220 - 1.16]
N-terminal Ac, Q1q, P4A	**Ac**-**q**N(S**A**NIFGQWM)	3.51 [1.16 - 10.6]
N-terminal Ac, N2n, P4A	**Ac**-Q**n**(S**A**NIFGQWM)	0.590 [0.257 - 1.35]
N-terminal Ac, Q1q, N2n, P4A	**Ac-qn**(S**A**NIFGQWM)	42.1 [29.9 - 59.3]
N-terminal Ac, P4A	**Ac**-QN(S**A**NIFGQWM)	1.35 [0.704 - 2.59]
N-Terminal Z, Q1q, N2n	**Z-qn**(SPNIFGQWM)	0.0623 [0.0269 - 0.144]
N-Terminal Z, N2n, P4A	**Z**-Q**n**(S**A**NIFGQWM)	0.169 [0.096 - 0.295]
N-Terminal Ac, Q1q	**Ac-q**N(SPNIFGQWM)	0.314 [0.112 - 0.880]
N-terminal Ac, N2n	**Ac**-Q**n**(SPNIFGQWM)	1.47 [0.737 - 2.93]

DISCUSSION

D-EPIMER SCAN

By modifying every amino acid residue to its *D*-epimer, the importance of the stereochemistry of the side chain can be determined. A reduction in potency indicates that the *L* stereochemistry is important to the interaction of the peptide with the receptor. Observing the results presented in Table 3, the peptides with tail residue modifications, Q1 and N2, showed increased potency when compared to the native signaling peptide. This indicates that an inversion of chirality in the tail region of the signaling peptide is tolerated, even preferred, when binding the receptor. Modifications to chirality at positions S3, F7, and M11 were tolerated and still resulted in the activation of the pathway with reduced potency, suggesting that the stereochemistry of these residues is important to receptor binding. In contrast, changes in chirality of all other residues in the macrocycle resulted in a loss of activity, suggesting that the stereochemistry of these side chains is vital to receptor binding and activation.

ALANINE SCAN

By replacing each amino acid side chain with alanine, a short, hydrophobic residue, the importance of the side chain can be determined. A reduction in potency indicates that the side chain and its properties are important to the interaction of the peptide with the receptor. Modifications to the tail region of the peptide again resulted in improved potency, indicating that the tail region is not important to the interaction with the receptor and is therefore dispensable. Peptide analogs with side chain modifications at positions N5, Q9, W10, and M11 showed moderate activation of the pathway with reduced potency, suggesting that these residues are important to the interaction with the receptor.

Modifications at positions I6, F7, and G8 resulted in complete loss of activity, suggesting that these three residues are vital to the binding of the receptor. The replacement of proline with alanine resulted in increased potency. This may be due to the removal of the restrictive steric properties associated with proline; the proline side chain is cyclic with the backbone of the amino acid participating in the ring. The proline structure is therefore sterically restricted, as opposed to linear side chains, such as in alanine, that allow free rotation around the single bonds. Additionally, the hydrogen bonding site usually present on the *N*-terminus of amino acids is unavailable in the proline structure. By replacing proline with alanine, the ability to hydrogen bond at this site was introduced, which could have changed the conformation of the peptide analog or allow for additional productive interactions with the receptor binding pocket.

TAIL MODIFICATIONS

Because results from the *D*-epimer scan and the alanine scan suggested that the tail residues were modifiable, a library was designed to test the importance of the tail residues by acetylating the tail and removing residues. It was observed that acetylation and removal of one or both tail residues were tolerated modifications, yielding analogs with EC_{50} values similar to that of the native signaling peptide. In contrast, removing both tail residues and performing a head to tail cyclization (lactam linkage) resulted in complete loss of activity. This loss of activity could be due to the ester linkage in the native signaling peptide participating in binding the receptor or a change in conformation from a change in linkage type.

IMPROVED POTENCY LIBRARY

Although the acetylation of the *N*-terminus appeared to be a tolerated modification in the tail modification library, it was shown in this library that the acetylation decreased potency of the analog compared to its non-acetylated counterpart. In addition, the triple modification analogs consisting of the changes Q1q, N2n, and P4A showed a significant reduction in potency compared to the native signaling peptide, indicating that these modifications are somehow incompatible. This library also details the most potent activator of the fsr QS pathway to date including the following modifications: *N*-Terminal Z, Q1q, N2n.

REFERENCES

Arias, C. A.; Murray, B. E., The rise of the Enterococcus: beyond vancomycin resistance. *Nat Rev Microbiol* **2012,** *10* (4), 266-78.

Carniol, K.; Gilmore, M., Signal transduction, quorum-sensing, and extracellular protease activity in Enterococcus faecalis biofilm formation. *J Bacteriol* **2004,** *186* (24), 8161-8163.

Huycke, M. M.; Sahm, D. F.; Gilmore, M. S., Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. *Emerg Infect Dis* **1998,** *4* (2), 239-49.

McBrayer, D. N.; Cameron, C. D.; Gantman, B. K.; Tal-Gan, Y. Rational Design of Potent Activators and Inhibitors of the *Enterococcus Faecalis* Fsr Quorum Sensing Circuit. *ACS Chem. Biol.* **2018**, *13* (9), 2673–2681. https://doi.org/10.1021/acschembio.8b00610.

McBrayer, D. N.; Gantman, B. K.; Cameron, C. D.; Tal-Gan, Y. An Entirely Solid Phase Peptide Synthesis-Based Strategy for Synthesis of Gelatinase Biosynthesis-Activating Pheromone (GBAP) Analogue Libraries: Investigating the Structure–Activity Relationships of the *Enterococcus Faecalis* Quorum Sensing Signal. *Org. Lett.* **2017**, *19* (12), 3295–3298. https://doi.org/10.1021/acs.orglett.7b01444.

McBrayer, D. N.; Gantman, B. K.; Tal-Gan, Y. N-Methylation of Amino Acids in Gelatinase Biosynthesis-Activating Pheromone Identifies Key Site for Stability Enhancement with Retention of the Enterococcus Faecalis Fsr Quorum Sensing Circuit Response. *ACS Infect. Dis.* **2019**, *5* (6), 1035–1041. https://doi.org/10.1021/acsinfecdis.9b00097.

Nakayama, J.; Cao, Y.; Horii, T.; Sakuda, S.; Akkermans, A.; de Vos, W.; Nagasawa, H., Gelatinase biosynthesis-activating pheromone: a peptide lactone that mediates a quorum sensing in Enterococcus faecalis. *Mol Microbiol* **2001,** *41* (1), 145-154.

Sifri, C.; Mylonakis, E.; Singh, K.; Qin, X.; Garsin, D.; Murray, B.; Ausubel, F.; Calderwood, S., Virulence effect of Enterococcus faecalis protease genes and the quorum-sensing locus fsr in Caenorhabditis elegans and mice. *Infect Immun* **2002,** *70* (10), 5647-5650.

Ventola, C. L. The Antibiotic Resistance Crisis. *P T* **2015**, *40* (4), 277–283.