N-Methylation of Amino Acids in Gelatinase Biosynthesis-Activating Pheromone (GBAP) Identifies Key Site for Stability Enhancement with Retention of the *Enterococcus faecalis* Fsr Quorum Sensing Circuit Response

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Supporting Information.

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Full Experimental Methods

General: Unless otherwise specified, all coupling reactions were manually conducted in 6 mL polypropylene reaction vessels containing porous frits at their base for ease of filtering and washing the resin between reaction steps. All reference to equivalents used are relative to the initial resin loading. All bioassays were conducted with *Enterococcus faecalis* reporter strain TX5274, expressing β-galactosidase as the reporter. *N*,*N*-dimethylformamide (DMF) and *N*-methyl-2-pyrrolidone (NMP) were used interchangeably as solvents.

Chemical Reagents and Instrumentation: All chemical reagents and solvents were purchased from Sigma-Aldrich or Chem-Impex and used without further purification. Water (18 MΩ) was purified using a Millipore Analyzer Feed System. Solid-phase resin was purchased from Advanced ChemTech.

Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed using a Shimadzu system equipped with a CBM-20A communications bus module, two LC-20AT pumps, an SIL-20A auto sampler, an SPD-20A UV/VIS detector, a CTO-20A column oven, and an FRC-10A fraction collector. All RP-HPLC solvents (18 MΩ water and HPLC-grade acetonitrile (ACN)) contained 0.1% trifluoroacetic acid (TFA). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) data were obtained on a Bruker Microflex spectrometer equipped with a 60 Hz nitrogen laser and a reflectron. In positive ion mode, the acceleration voltage on Ion Source 1 was 19.01 kV. Exact mass (EM) data were obtained on an Agilent Technologies 6230 TOF LC/MS spectrometer. The samples were sprayed with a capillary voltage of 3500 V and the electrospray ionization (ESI) source parameters were as follows: gas temperature of 325 °C at a drying gas flow rate of 8 L/min at a pressure of 35 psi.

Initial Resin Deprotection and Loading Determination: Unless otherwise noted, peptide synthesis was conducted using Rink Amide MBHA (Knorr) resin with a loading capacity of 0.89 mmol/g. Peptide synthesis was conducted on either 50 mg (0.045 mmol) or 100 mg (0.09 mmol) resin scales. Resin loading was verified by measuring Fmoc removal during the initial deprotection of the resin. 2 mL of 20% piperidine in DMF was added to the resin, which was subsequently shaken at 200 rpm for 8 min. The 2 mL solution was collected, and an aliquot (10-20 μL) was taken and diluted to 3 mL in DMF before the absorbance was read at 290 nm. This step was repeated after the resin was subjected to a second, identical deprotection step (for a total of 16 min of deprotection). The combined moles of removed Fmoc were determined using an extinction coefficient of 5253 $M^{-1}cm^{-1}$ and corrected for the dilution factor used.[1] Division by the resin mass verified the resin loading to be as stated by the manufacturer.

Fmoc Removal: Fmoc removal was accomplished with treatment of the resin by 2 mL of 20% piperidine or 2% piperidine and 2% 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF and shaking at 200 rpm for 7 min. This process was repeated once more (for a total of 14 min deprotection time). The resin was then washed with shaking three times with DMF for 1 min. The solution from the first 7 min reaction was collected, an aliquot taken, diluted to 1 mL in DMF, and the absorbance read at 290 nm after blanking with a solution of 20% piperidine that had been similarly diluted. This absorbance was compared with that of the first deprotection solution value from the original deprotection of the resin as a means of following the resin loading throughout the synthesis.

HATU and DIPEA-Facilitated Coupling: Most amino acid coupling steps were accomplished by using (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (HATU) as the coupling reagent and *N*,*N*-diisopropylethylamine (DIPEA) as the base. 3 equiv of the desired amino acid was combined with 2.85 equiv of HATU before being dissolved in approximately 1.3 mL of DMF. 2.85 equiv of DIPEA was then added, and the mixture briefly shaken, before addition to the resin and reaction for 1.5, 2, 4, or 16 h. Immediately following coupling, the resin was washed with shaking three times with DMF for 1 min.

Selective Deprotection of the Serine OTrt Protecting Group: Before the lactone linkage reaction could be conducted, the OTrt protecting group on Ser was selectively removed. The resin was washed three times with dichloromethane (DCM) for 1 min, followed by treatment with a 2 mL solution of 1% TFA and 5% triisopropylsilane (TIPS) in DCM for 2 min. The treatment with the TFA solution was repeated an additional three times (for a total deprotection time of 8 min). Following deprotection, the resin was washed four times with DCM for 1 min, then four times with shaking with DMF for 1 min prior to beginning the next reaction step.

Peptide Acetyl Capping: Peptide analogues incorporated an *N*-terminal acetyl cap. 10 equiv of acetic anhydride and 7 equiv of DIPEA in 2 mL DMF were added to the resin, which was then shaken at 200 rpm for 15 min. The resin was washed with shaking three times with DMF for 1 min prior to conducting additional synthetic steps. For the acetyl capping of *N*-methyl serine, the time was increased to 1 hr and the coupling repeated once more to ensure complete reaction.

DIC with Catalytic DMAP-Facilitated Coupling: Formation of the lactone "branch" in the peptide was accomplished *via* reaction with a symmetrical anhydride formed from the preliminary reaction of the desired amino acid with *N*,*N*'-diisopropylcarbodiimide (DIC). 10 equiv of the desired amino acid was dissolved in DCM that had been dried over 3 Å molecular sieves. To this vigorously stirred solution was added 5 equiv of DIC dropwise at 0 °C. The flask was then sealed with continued stirring for 25 min. After the initial reaction was complete, additional dry DCM was added as necessary to dissolve any precipitate, and the solution was allowed to warm back to room temperature with additional stirring for 10 min. The DCM was then removed *via* rotary evaporation under reduced pressure and the resulting solid dissolved in 2 mL DMF that had been dried over 3 Å molecular sieves. The resulting solution was then added to the resin and the mixture shaken for 25-30 min at 200 rpm. At this point, 0.04 equiv of 4-Dimethylaminopyridine (DMAP) was added as an 18.6 - 26.3 mg/mL solution in DMF to the resin mixture. The resin mixture was shaken for an additional 3.5 h. The resin was then washed with shaking three times with DMF for 1 min before coupling the next amino acid.

Palladium-Catalyzed Removal of OAlloc Protecting Group: The resin was washed three times in DCM for 1 min before being dried down and placed under argon. Approximately 5 mL of DCM dried over 3 Å molecular sieves in a 15 mL polypropylene centrifuge tube was sparged with argon for 3 min. 10 equiv of phenyl silane was then added and sparging continued for an additional 2-3 min. 0.5 equiv of tetrakis(triphenylphosphine)palladium(0) was added and sparging continued for 4 min. The resulting solution was added to the resin and the reaction vessel was sealed with parafilm. The reaction vessel was then placed in a 50 mL polypropylene centrifuge tube wrapped in aluminum foil. The air in the centrifuge tube was displaced with argon before the centrifuge tube was sealed with parafilm. The 50 mL tube was then shaken

at 200 rpm for 2 h. The resin was washed with shaking four times with 0.5% sodium diethyldithiocarbamate trihydrate in DMF for 2 min and four times with shaking with DMF for 1 min before proceeding onto the next reaction step.

Pyoxim and DIPEA-Facilitated On-Resin Peptide Cyclization: The final cyclization of the peptide was accomplished through reaction with pyoxim and DIPEA. 1.25 equiv of pyoxim was dissolved in 1.3 – 2 mL of DMF. To this solution was added 2.5 equiv of DIPEA. The mixture was briefly shaken and then added to the resin. The resulting mixture was shaken at 200 rpm for 4 h. The solution was drained, and a second, identical coupling reagent solution was added to the resin. The mixture was shaken at 200 rpm for 16 h. Completion of the cyclization was verified using the Kaiser test (see below). After the reaction was complete, the resin was washed with shaking three times with DMF for 1 min and three times with DCM for 1 min. The resin was then dried prior to final cleavage.

Kaiser Test: The Kaiser (ninhydrin) test for primary amines was conducted as previously described.[1] Briefly, a small amount of dried resin was placed in a small glass culture tube. To the dried resin was added 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 (KCN) in pyridine. The tube was then heated for 6 min at 110-120 °C. Blue or purple coloration in the beads and/or solution is indicative of an incomplete reaction, due to the presence of unreacted primary amines.

Chloranil Test: The chloranil test for primary and secondary amines was conducted as previously described.[1] Briefly, a small amount of dried resin was placed in a small glass culture tube. To the dried resin was added 2 drops of 2% (v/v) acetaldehyde in DMF and two drops of 2% (w/v) p-chloranil in DMF. The mixture was allowed to stand for 4-6 min at room temperature. Dark blue to green-stained resin beads indicate the presence of unreacted primary and/or secondary amines.

Final Cleavage and Precipitation: The resin was transferred from the original reaction vessel to a 15 mL polypropylene centrifuge tube. 3 mL of 2.5% water and 2.5% TIPS in TFA was added and the tube was shaken at 200 rpm for 3 h. The resin was filtered through a cotton plug in a polypropylene syringe and washed with a small amount of TFA. The filtrate was collected in a 50 mL polypropylene centrifuge tube. 45 mL of -20 °C 1:1 ether:hexane solution was then added as a precipitant and the sealed tube mixed by upending several times before being placed in a flammables-compatible freezer at -20 °C for 10 min. The 50 mL tube was centrifuged in a Beckman Coulter Allegra 6 centrifuge equipped with a GH3.8 rotor at 2990 rpm (approx. 2550 x g) for 5 min in order to pellet the crude peptide. The supernatant was decanted off and the pellet dissolved in approximately 5-10 mL of 1:1 ACN:water. The solution was frozen by placing the tube in an acetone/dry ice bath, and then lyophilized for at least 18 h.

Purification by HPLC: Crude peptide was purified using RP-HPLC. The crude pellet was dissolved in either 4 mL (0.045 mmol scale) or 8 mL (0.09 mmol scale) of 3-50% ACN in water (depending on solubility) and purified in 4 mL portions on a Phenomenex Kinetex 5 µm C18 semi-preparative column (10 mm × 250 mm, 110 Å). The crude purification was accomplished with a gradient from 20% ACN to 60% ACN or from 30% ACN to 70% ACN (for more hydrophobic peptides) over 40 min. These conditions were typically sufficient to purify the peptide to ≥ 80-90%. Fraction purity was determined through analysis on a Phenomenex Kinetex 5 µm analytical C18 column (4.6 mm × 250 mm, 110 Å). Typically, 90 µL of either a 2-fold-diluted fraction sample or a 50:50 mix of aliquots from two different

HPLC fractions (to verify peak identity between fractions) was injected. The gradient used for analytical analysis was from 5% ACN to 95% ACN over 24 min. Fractions with similar purity were combined, frozen using an acetone/dry ice bath, and lyophilized for at least 18 h. Secondary purification to get the peptide to ≥ 95% purity was conducted on the same semi-preparative column mentioned above and generally used a 10% "wide" gradient centered on the solvent composition at which the desired peak eluted over 40 minutes (for example, a peak eluting at approximately 30% ACN would be run from 25% ACN to 35% ACN). Before the final masses and yields of purified peptides were determined, they were dissolved in 30% acetic acid in up to 50% ACN in water to displace and permit removal of any residual TFA. The solution was then frozen in an acetone/dry ice bath, and lyophilized for at least 18 h before the yield was determined.

Peptide Verification with Mass Spectrometry: During purification, peaks were verified to contain the desired peptide by MALDI-TOF MS. Samples were prepared using α-Cyano-4-hydroxycinammic acid as matrix and aliquots were taken directly from the preparative HPLC fractions. Final verification of the peptides was conducted by obtaining their exact masses with a high resolution ESI-TOF MS (**Table S-1**). 8 - 30 μM stock solutions were prepared in either water or 50% ACN in water. The instrument was calibrated before each run and an internal reference mass standard was used.

β-Galactosidase Reporter Assay: To quantify the activation of the QS circuit, as seen by activation of the *gelE* promoter, a β-galactosidase reporter assay was used. This assay was conducted as previously described for the determination of EC_{50} values.[2] Briefly, *E. faecalis* TX5274 bacteria were grown on Mueller-Hinton Broth 2 (MHB-2) plates without antibiotics overnight at 37 °C. An isolated colony was then transferred to 5 mL of brain heart infusion (BHI) broth containing 500 μg/mL of kanamycin and shaken overnight at 37 °C in ambient atmosphere. These overnight cultures were then diluted 50-fold into BHI broth with a final concentration of 500 μg/mL kanamycin. The cells were then grown with shaking at 37 °C for 1 h before 198 μL aliquots were placed in the wells of a 96-well plate containing either 2 μL dimethyl sulfoxide (DMSO) or 2 μL experimental peptide dissolved in DMSO and statically incubated at 37 °C for 2 h. The experimental peptide concentrations were varied along a 9-point, 5-fold serial dilution starting with 10 μM and ending with 0.052 nM. In cases where the initial sigmoidal curve gave few baseline points, the starting point for the 9-point dilution series was adjusted and the dilution varied between 2-fold, 3-fold, or 5-fold as necessary. Each well was treated with 2 μL DMSO solution of experimental peptide, GBAP positive control, or DMSO negative control. After the incubation time had elapsed, the absorbance at 600 nm (A600) was read. The wells were then treated with 20 μL of 1% Triton X-100 in water for 30 min at 37 °C to lyse the cells. After lysis, 100 μL of the lysate was transferred to a new well containing 100 μL substrate buffer containing 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). The enzyme reaction was run for 30 min at 37 °C before being quenched with 20 μL of 1 M sodium carbonate in water. The absorbance of quenched reaction wells was read at 420 nm (A420) and 550 nm (A550), allowing for the calculation of the activity in Miller units (Equation 1).

$$\text{Equation 1: } Miller\ Unit = 1000 * \frac{A420 - c * A550}{t * v * A600}$$

Where c is a species-specific correction factor (1.6 for *E. faecalis*), t is the substrate reaction time in minutes, and v is the volume of lysed cell culture used in the enzyme reaction in

milliliters.[3] The equation allows for correction of background absorbance due to initial cell density, cell lysate turbidity, and enzyme reaction time.

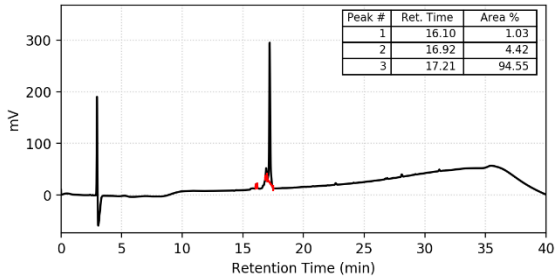
After Miller unit determination, the average value for the DMSO negative control was subtracted and the results normalized to the average value of the GBAP positive control. The EC_{50} value was determined through fitting using nonlinear regression with GraphPad Prism using Equation 2.

$$\text{Equation 2: } y = min + \frac{\max - min}{1+10^{\log(EC50-x)}}$$

Where min is the average minimum signal and max is the average maximum signal. If the maximum concentration (10 μM) activity of a peptide was less than or equal to 50% relative to the GBAP positive control, that peptide was tested as a potential competitive inhibitor. The IC_{50} of antagonistic peptides was determined similarly. However, each sample also included 50 nM of GBAP as a competitor standard. The 50 nM concentration of GBAP was determined to reproducibly give 90 - 95% of the GBAP maximum signal. After Miller unit determination, the DMSO negative control was subtracted and the results were normalized to the signal produced by the concentration of competing GBAP used in the absence of any potential inhibitor. All experiments were conducted in triplicate and repeated on three separate days.

HPLC Traces for GBAP Analogues

Ac-GBAP-Des(Q1N2)[NMeS3]



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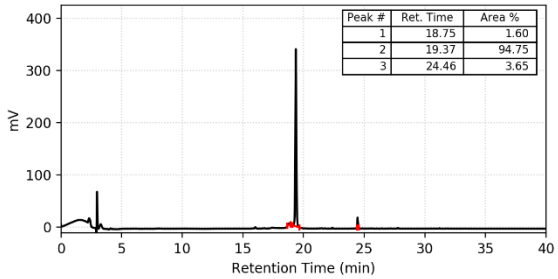

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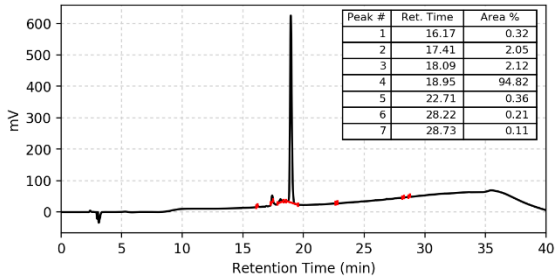

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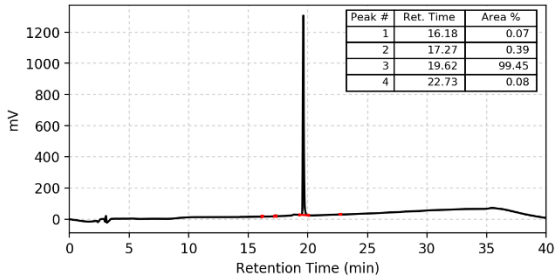

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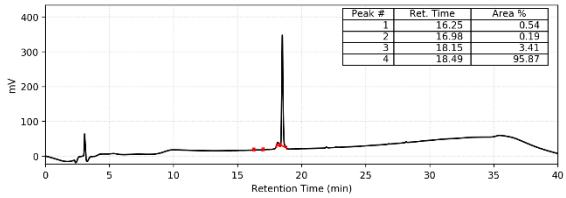
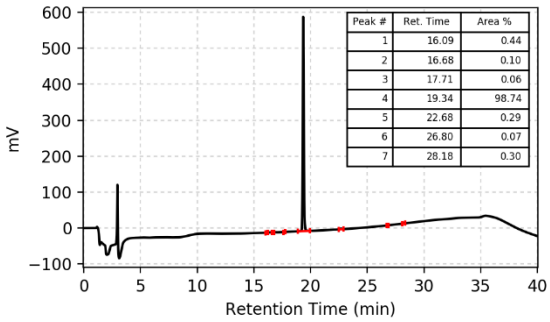


Ac-GBAP-Des(Q1N2)[NMeQ9]



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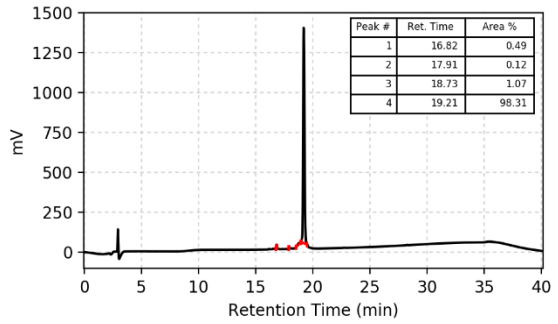
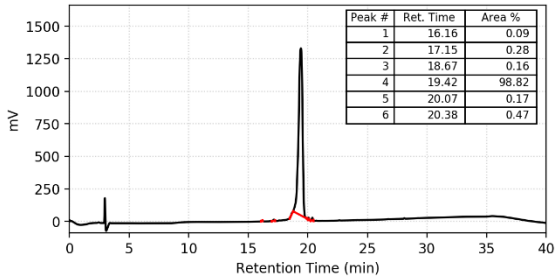
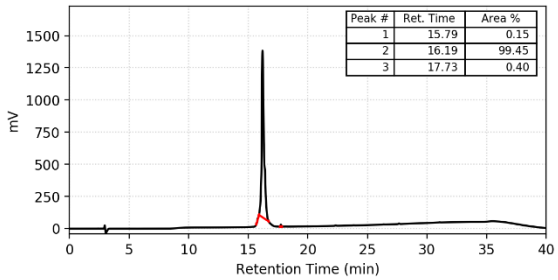


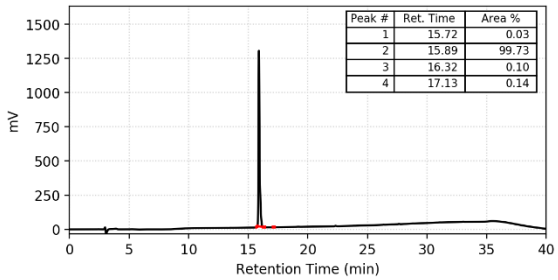
Ac-GBAP-Des(Q1N2)[NMeM11]



GBAP-[NMeF7]



Linear GBAP



MS and HPLC Data for GBAP Analogues

Table S1. HRMS and HPLC data for all analogues

Peptide #	Peptide Name	Calc. EM Mass ([M+H]$^+$)	Obs. EM Mass ([M+H]$^+$)	Purity (%)
3	Ac-GBAP-Des(Q1N2)[NMeS3]	1117.5135	1117.5161	≥ 95
4	Ac-GBAP-Des(Q1N2)[NMeN5]	1117.5135	1117.5150	≥ 95
5	Ac-GBAP-Des(Q1N2)[NMeI6]	1117.5135	1117.5125	≥ 95
6	Ac-GBAP-Des(Q1N2)[NMeF7]	1117.5135	1117.5145	≥ 99
7	Ac-GBAP-Des(Q1N2)[NMeG8]	1117.5135	1117.5150	≥ 96
8	Ac-GBAP-Des(Q1N2)[NMeQ9]	1117.5135	1117.5143	≥ 99
9	Ac-GBAP-Des(Q1N2)[NMeW10]	1117.5135	1117.5088	≥ 98
10	Ac-GBAP-Des(Q1N2)[NMeM11]	1117.5135	1117.5161	≥ 99
11	GBAP-[NMeF7]	1317.6045	1317.5983	≥ 99
12	Linear GBAP	1321.5994	1321.5930	≥ 99

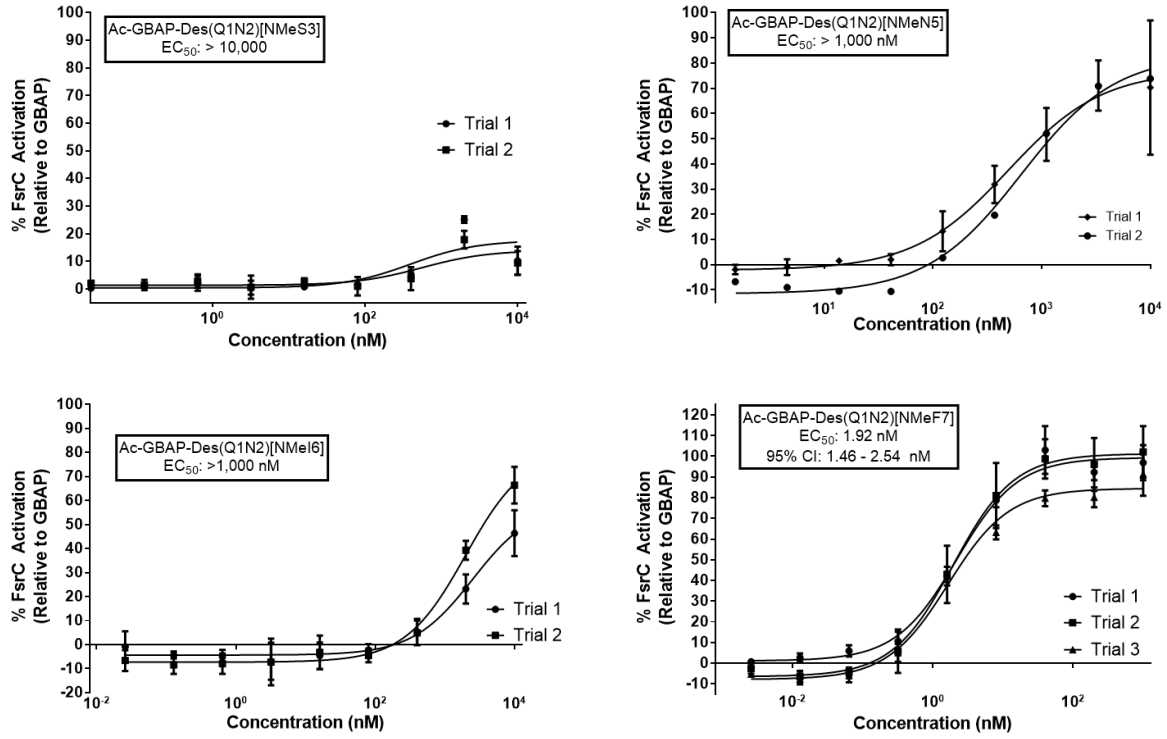
EM = Exact Mass. See above for methods

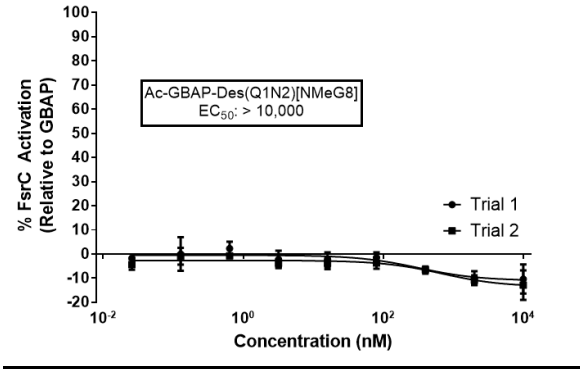
Dose Response Curves

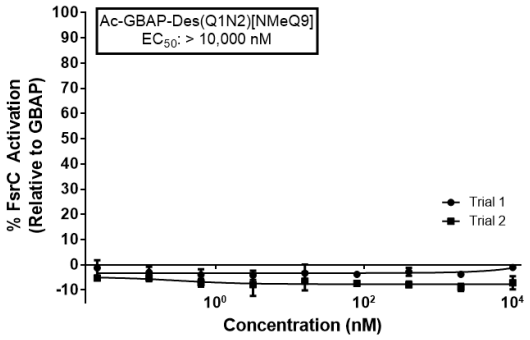
GBAP analogues were tested to determine their EC_{50} or IC_{50} values over varying concentrations. Each dose response experiment was performed in triplicate on three separate occasions* (i.e., experiments (Trial) #1-3; shown for each peptide below). Error bars indicate standard error of the mean of triplicate values. In each plot, the peptide, as well as its EC_{50} or IC_{50} value (in nM) and 95% confidence interval (95% CI) values (in nM), when calculable, are indicated in the boxed area. IC_{50} values were determined using 50 nM of exogenous GBAP as a competitor and were only conducted for analogs whose max signal was less than 50% of GBAP's maximum.

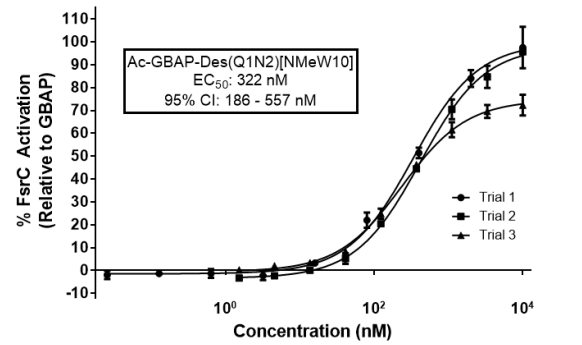
*Only two trials were conducted for peptides that were found to be inactive (EC_{50} or IC_{50} > 1,000 nM). EC_{50} data for linear GBAP (inactive) is not shown.

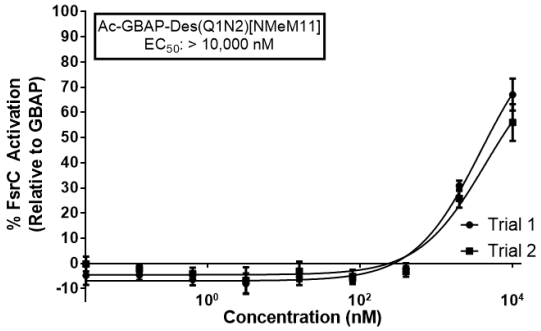
Activation Dose Response Curves

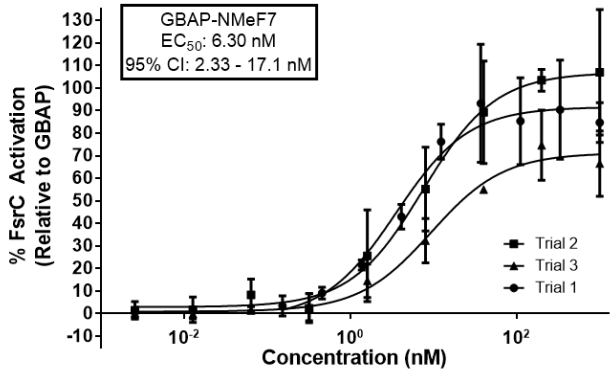




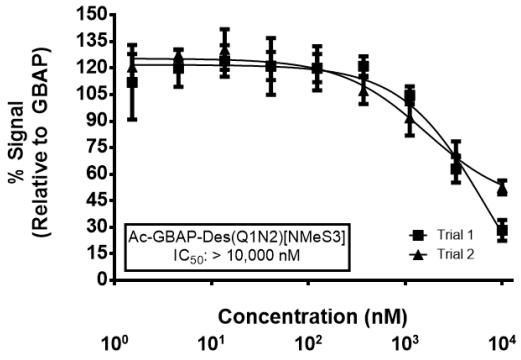


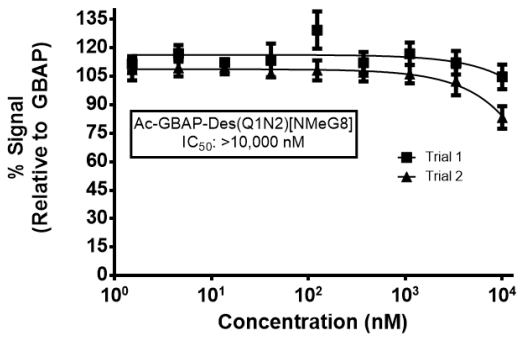


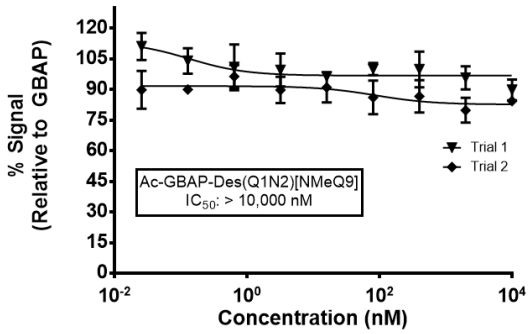




Inhibition Dose Response Curves







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[1] Chan, W. C., and White, P. D. (2000) *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, Vol. 222, Oxford University Press, Oxford.

[2] McBrayer, D. N., Gantman, B. K., Cameron, C. D., and Tal-Gan, Y. (2017) 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 19*, 3295-3298 DOI 10.1021/acs.orglett.7b01444.

[3] Qin, X., Singh, K. V., Weinstock, G. M., and Murray, B. E. (2001) Characterization of fsr, a Regulator Controlling Expression of Gelatinase and Serine Protease in Enterococcus faecalis OG1RF, *J Bacteriol 183*, 3372-3382 DOI 10.1128/JB.183.11.3372-3382.2001.