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

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Ⓢ *Supporting Information*

ABSTRACT: The growing prevalence of multiantibiotic-resistant bacteria necessitates looking at potential alternative approaches for attenuating infections by bacteria while reducing the rate of antibiotic resistance development. *Enterococcus faecalis* is responsible for a large percentage of clinical enterococci infections, and its pathogenicity has been demonstrated to be influenced by quorum sensing (QS). In this study, we report the systematic study of the relationship between backbone hydrogens and the ability to activate the FsrC receptor. We demonstrate that *N*-methylation was particularly well-tolerated at one site (Phe7) and granted stability against protease digestion, increasing the peptide half-life relative to the native signal by more than 6-fold. The inclusion of the *N*-Me-Phe7 modification may be useful for improving the pharmacological properties of *E. faecalis* QS inhibitors as part of the development of future therapeutic candidates.

KEYWORDS: *quorum sensing, Enterococcus faecalis, gelatinase biosynthesis-activating pheromone, metabolic stability, peptidomimetics*
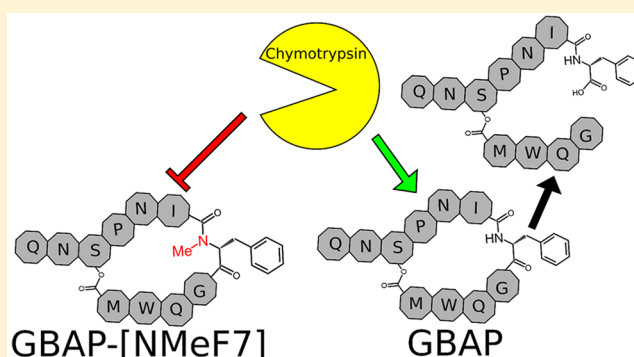
Enterococcus faecalis is an opportunistic pathogen present in the gut biome of humans and accounts for more than half of all enterococci infections, including nosocomial endocarditis, neonatal infections, and urinary tract infections.[1,2] *E. faecalis* is intrinsically resistant to some antibiotics, including vancomycin, an antibiotic of last resort.[1,3] Moreover, *E. faecalis* can transfer its resistance genes to other bacteria through a pheromone-responsive gene transfer system.[3,4] Therefore, there is an urgent need to discover nonantibiotic therapeutics for *E. faecalis* infections. The evolutionary pressure to evolve against therapeutics can be lessened by targeting pathways that are not required for cell survival. Such a strategy will decrease the rate by which antibiotic resistance is acquired while still attenuating infections by reducing the organism's pathogenicity.

In order to effectively attack their host and establish an infection, *E. faecalis* bacteria communicate with one another via quorum sensing (QS).[5,6] Gram-positive bacteria, like *E. faecalis*, release peptide signals into the environment at a constant rate.[6] Once an individual cell detects that the environmental concentration of that peptide has met a species-dependent threshold concentration, the transcription of genes involved in group-beneficial traits, such as virulence factor production, antimicrobial agent production, digestive enzyme secretion, bioluminescence, and biofilm formation, is activated.[5−7]

Because of the role QS plays in bacterial pathogenicity, this area has gained attention as a method to attenuate bacterial infections.[8−11]

The fsr QS circuit in *E. faecalis* relies on the production and response to an 11-amino-acid macrocyclic signaling peptide known as gelatinase biosynthesis-activating pheromone (GBAP).[5,12] GBAP contains an ester-linked macrocycle between the side chain of Ser3 and the *C*-terminus of Met11.[12] GBAP activates a membrane-bound histidine kinase receptor, FsrC. Receptor activation leads to phosphorylation of the response regulator, FsrA. Phosphorylated FsrA acts as a transcription factor that induces the transcription of the *fsrABDC*, *gelE*, and *sprE* genes, directly resulting in auto-induction of the QS circuitry, gelatinase production, and serine protease production, respectively.[1,5,13−15] Gelatinase directly affects *E. faecalis* adherence and biofilm formation, thus affecting processes such as translocation across intestinal epithelial cells, while SprE, a serine protease, has been implicated in similar processes.[1,14,16−18]

We have previously investigated the relative importance of each of the GBAP's residues on QS activation by modifying their functional groups (alanine scan) and chirality (D-amino

Table 1. EC$_{50}$ Values for *N*-Methylated GBAP Analogs^a

peptide #	peptide name	sequence	EC$_{50}$^{b} [95% CI]^c (nM)
1	GBAP^d	QN(SPNIFGQWM)	1.15 [0.825−1.59]
2	Ac-GBAP-Des(Q1N2)^d	**Ac**-(SPNIFGQWM)	1.01 [0.496−2.06]
3	Ac-GBAP-Des(Q1N2)[*N*-MeS3]	**Ac**-([***N*MeS3**]PNIFGQWM)	--
4	Ac-GBAP-Des(Q1N2)[*N*-MeN5]	**Ac**-(SP[***N*MeN5**]IFGQWM)	>1000
5	Ac-GBAP-Des(Q1N2)[*N*-MeI6]	**Ac**-(SPN[***N*MeI6**]FGQWM)	>1000
6	Ac-GBAP-Des(Q1N2)[*N*-MeF7]	**Ac**-(SPNI[***N*MeF7**]GQWM)	1.92 [1.46−2.54]
7	Ac-GBAP-Des(Q1N2)[*N*-MeG8]	**Ac**-(SPNIF[***N*MeG8**]QWM)	--
8	Ac-GBAP-Des(Q1N2)[*N*-MeQ9]	**Ac**-(SPNIFG[***N*MeQ9**]WM)	--
9	Ac-GBAP-Des(Q1N2)[*N*-MeW10]	**Ac**-(SPNIFGQ[***N*MeW10**]M)	322 [186−557]
10	Ac-GBAP-Des(Q1N2)[*N*-MeM11]	**Ac**-(SPNIFGQW[***N*MeM11**])	--
11	GBAP-[*N*-MeF7]	QN(SPNI[***N*MeF7**]GQWM)	6.30 [2.33−17.1]
12	linear GBAP	QNSPNIFGQWM	--

^{a}See the Supporting Information for details on the reporter strain used and methods. All assays performed in triplicate. ^{b}EC$_{50}$ values determined by testing peptides over a range of concentrations. ^{c}95% confidence interval. ^{d}Previously published analog shown for comparison; see ref 19 for details.

acid scan).[19] Information gained from this earlier work, combined with work done by others,[20] enabled us to rationally design potent activators and inhibitors of the fsr QS circuit.[21] Although these investigations have resulted in a greatly increased understanding of the structure−activity relationships (SARs) involved in QS activation, additional questions remained. For instance, the role of backbone interactions in GBAP activity had yet to be elucidated. In this study, we utilized an *N*-methylation scan approach to systematically evaluate the importance of amide protons in GBAP activity.[22−24] Through this analysis, we identified two *N*-methyl modifications of interest in the design of potential therapeutics. The first produces an analog that is a weak antagonist, while the second produces an analog with significantly improved stability compared to the native signal molecule while retaining its potency. The activator modification improved the stability to protease degradation when incorporated into the parent scaffold, suggesting that its inclusion in future peptide designs could potentially lead to peptides with enhanced pharmacological properties.

RESULTS AND DISCUSSION

Synthetic Strategies. Although we have demonstrated that tail modifications, i.e., modifications made to the two exocyclic residues, can be used to enhance the potency of GBAP analogs, we have also shown that the tail residues are not required to retain the activity of native GBAP.[19] As such, all peptides investigated here have the tail residues removed and replaced with an acetyl capping group. Most peptides were produced using our previously published entirely on-resin synthetic approach[19,21] with the incorporation of Fmoc-protected *N*-methylated amino acids at the appropriate point in the synthesis. However, two analogs required modification to their protocols in order to be synthesized successfully.

The first of these two was the *N*-MeS3 analog (**3**). Our synthetic strategy relies on the selective deprotection of the Ser3 side chain in order to form the lactone linkage in the completed macrocycle. However, the Boc protecting group commonly utilized in commercial *N*-methylated amino acids cannot be removed selectively. Thus, in order to prepare this analog, we used Fmoc-Ser(Trt)-OH and methylated the serine on the solid support after its addition to the peptide chain. This approach was successful with some modification of an existing protocol (see the Supporting Information for details).[25]

The second peptide that required an altered protocol was *N*-MeM11 (**10**). Because M11 participates in the lactone linkage in the final macrocycle and because a cis conformation is favored following an *N*-methylated amino acid,[26] the labile ester forming the macrocycle bridge between Ser3 and Met11 is well positioned for attack by the primary amine of the subsequent amino acid (tryptophan 10). This attack is stabilized by a six-membered ring intermediate and leads to loss of the methionine−-tryptophan dipeptide in the form of a diketopiperazine (DKP), causing a significant drop in yield as soon as the tryptophan's Fmoc protecting group is removed. In order to address this issue, we prepared a dipeptide composed of tryptophan and the amino acid that follows it in the synthesis (glutamine 9). By coupling this dipeptide to methionine following ester formation, we avoid the undesirable DKP formation. The details of the Fmoc-Gln(Trt)-Trp(Boc)-OH preparation on chlorotrityl resin and subsequent purification can be found in the Supporting Information. Table 1 shows the peptides prepared in this study and their EC$_{50}$ values.

N-Methylation Structure−Activity Relationship (SAR) Results. The SAR data for the *N*-methylated analogs were collected using our *β*-galactosidase cell-based bacterial reporter assay. Briefly, quantification of QS activation is measured in an *E. faecalis* strain bearing a plasmid containing pGelE-*lacZ*. Upon activation of the fsr QS circuitry, FsrA will bind pGelE and transcribe *lacZ*. Thus, *β*-galactosidase activity can be used to monitor QS response (full experimental details can be found in the Supporting Information).[19,21] Overall, the majority of *N*-methyl analogs exhibited significant reduction in activity, emphasizing the important role backbone protons have in GBAP activity, either through hydrogen-bond interactions with the receptor or by allowing conformational flexibility compared to the bulky methyl group (Table 1). Starting with the *N*-terminus, *N*-MeS3 (**3**) was found to be inactive as an activator and to have weak inhibitory activity (the signal of 50 nM competitor GBAP was reduced to around 60% at 10 *μ*M of **3**, the highest concentration tested). These results suggest that *N*-methylation at the Ser3 position could potentially enhance the inhibitory propensity of existing GBAP-based fsr inhibitors. Analogs **4** and **5** both had large drops in potency compared to native GBAP, with EC$_{50}$ values greater than 1000 nM. Similarly to **3**, analogs **7**, **8**, and **10** lost all activity. However, neither of these three analogs exhibit any inhibitory activity. Analog **9**
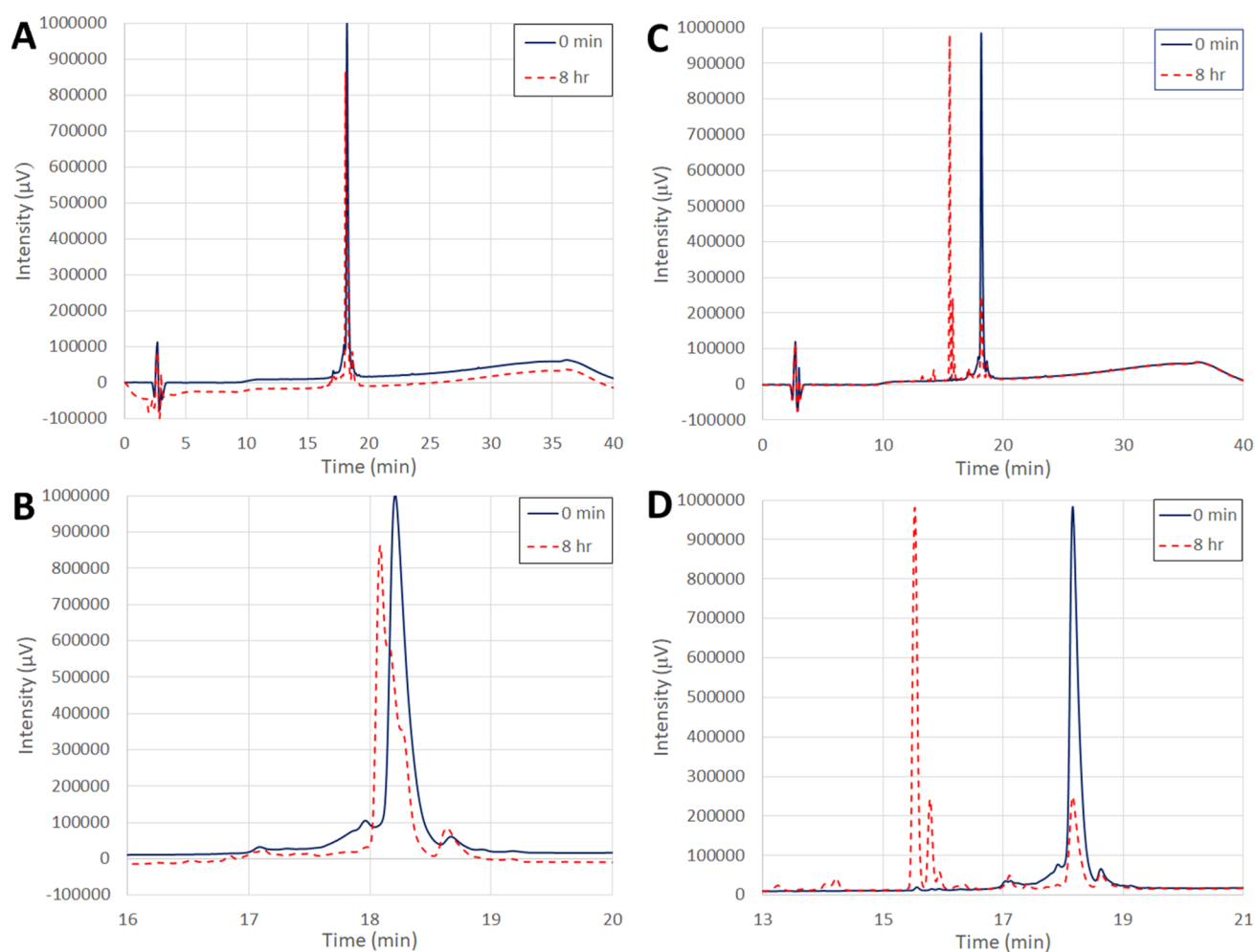
Figure 1. Comparison of GBAP in PBS at 37 °C without (A and B) and with (C and D) treatment with 50 nM chymotrypsin. Full analytical HPLC spectra (A and C) are compared with zoomed views (B and D) of the same respective spectra. Solid blue lines indicate the GBAP traces prior to incubation. Dashed red lines indicate the GBAP traces after 8 h of incubation. In both cases, the half-life for GBAP is approximately 4 h; however, the difference in products indicates that chymotrypsin can successfully digest GBAP.

also exhibited a substantial decrease in activity but had a measurable EC_{50} value of 322 nM (Table 1).

Interestingly, the N-MeF7 analog (**6**) was quite potent. With an EC_{50} value of 1.92 nM, it was comparably potent to native GBAP. This result reinforces the unique role the F7 position has in GBAP activity as this residue was found to be critical for activity (replacement with alanine resulted in a relatively inactive analog, GBAP-F7A, $EC_{50} > 1000$ nM)[19] yet was also the site previously found to retain potency when its chirality was inverted or when a larger amino acid was substituted.[19,21] This is especially interesting since Phe7 is a potential cleavage site for degradation by chymotrypsin.[27] Methylation at this site could therefore be applied to protect against degradation while simultaneously maintaining binding characteristics, allowing for the design of better drug leads. Due to the low solubility of the acetylated form of this analog (**6**) and to test that this modification can be successfully added to the native peptide (which would indicate potential effectiveness in other analogs as well), we prepared GBAP-N-MeF7 (**11**). This analog retains the native tail residues and lacks an acetyl capping group. As expected, it was found to have similar potency to the tail-removed analog (3-fold reduction), while having significantly improved solubility.

Stability Assays. In order to evaluate whether N-methylation at F7 makes the analog more resistant to degradation by proteases, we conducted stability assays to assess the length of time that the peptide remains intact when exposed to chymotrypsin or human plasma. We tested native GBAP (**1**) and linearized GBAP (**12**) as active and inactive controls whose stability can be compared with **11**.

In order to get a baseline for the peptide stability, we tested native GBAP in phosphate-buffered saline (PBS) at pH 7.2 and ammonium carbonate at pH 8 without the addition of proteases at 25 °C. Since we have previously observed that GBAP's terminal tail glutamine spontaneously degrades to pyroglutamate,[19] we wanted to be able to differentiate between this product and any others that might be observed. These experiments also addressed the feasibility of conducting structural studies with GBAP with confidence that degradation would not occur during data collection. We found that native GBAP undergoes rapid chemical degradation in the pH 8 buffer at 25 °C, involving not only conversion of the N-terminal glutamine to pyroglutamate but also slower opening of the macrocyclic ring due to hydrolysis of the ester linkage. Complete conversion to a ring-opened product containing the pyroglutamic residue was seen within 24 h at 25 °C. At pH 7.2, GBAP degradation was much slower at 25 °C. Approximately

6% of the native GBAP had degraded after 24 h. No ring opening was observed during those first 24 h in the pH 7.2 conditions at 25 °C.

Due to the chemical instability of GBAP in the pH 8 buffer, we conducted the protease experiments in the pH 7.2 PBS buffer system at 37 °C. This pH (7.2) also mimics better physiologically relevant conditions such as serum and plasma. We tested and compared GBAP, **11**, and **12** in the absence and presence of chymotrypsin (approximately 50 nM), an important protease of the digestive system where *E. faecalis* naturally resides, to assess their resilience against enzymatic degradation. Native GBAP chemically degraded much faster in the pH 7.2 buffer at the elevated temperature (37 vs 25 °C) with a half-life of approximately 4 h (at 24 h, GBAP had completely degraded). When GBAP was treated with chymotrypsin, significantly different degradation product peaks were produced compared to the chemical degradation, indicating that the peptide was indeed being cleaved by the protease (Figures 1 and 2). Analog **12** was hypothesized to
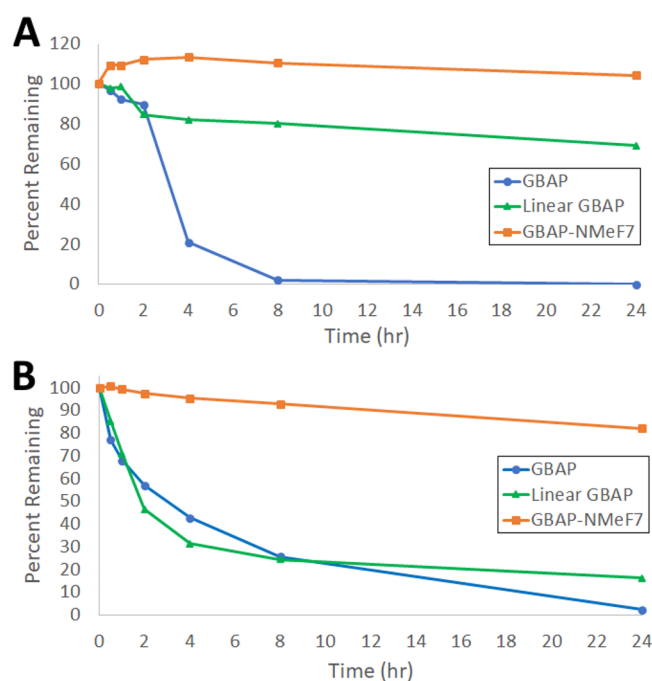

Figure 2. Degradation trends in PBS at 37 °C without (A) and with (B) treatment with 50 nM chymotrypsin. GBAP is shown in blue with circles; linear GBAP is shown in green with triangles, and GBAP-*N*-MeF7 is shown in orange with squares.

have similar or better chemical stability to GBAP (due to the lack of the ester bond found in GBAP's lactone ring) but was expected to have increased susceptibility to chymotrypsin compared to the other two macrocyclic peptides.

Indeed, **12** was chemically much more stable in the pH 7.2 buffer at 37 °C, with a half-life of more than 24 h (approximately 70% remained after 24 h). These results suggest that the conformation adopted by the macrocycle may help orient the tail residues to facilitate formation of the observed pyroglutamate degradation product. However, as predicted, **12** was much more susceptible to degradation by chymotrypsin with a half-life of only 2 h (Figure 2). **11** was very stable both in PBS buffer alone or in the presence of chymotrypsin with a similar amount remaining after 24 h in both cases. The results indicate that *N*-methylation at F7

induces remarkable stability against chymotrypsin in comparison to native GBAP. **11** had a half-life greater than 24 h (with approximately 80−90% peptide remaining undigested at the 24 h time point) when treated with chymotrypsin (Figure 2). These results further suggest that *N*-methylation at F7 results in conformational changes that make the formation of the pyroglutamate product less favored, while still allowing the peptide to retain its activity.

Lastly, we tested GBAP and **11** for their stability in human plasma. Overall, degradation in plasma was significantly faster for both analogs. However, a similar stability trend was observed, that is *N*-methylation at Phe7 resulted in an analog with higher resistance to degradation. GBAP exhibited very low stability in plasma with a half-life significantly lower than 30 min (first time point, <4% peptide remaining) and complete degradation in 1 h. GBAP-*N*-MeF7 (**11**) was more stable in plasma with a half-life just below 30 min (~30% peptide remaining) and complete degradation only after 2 h. The plasma stability results again highlight the utility of incorporating *N*-methylation as a means to improve the pharmacological properties of lead peptide scaffolds.

■ CONCLUSIONS

The *N*-methyl scan revealed several amide protons (S3, G8, Q9, and M11) that are vital for GBAP activity, either by allowing the peptide to adopt its active conformation or through direct interactions with the receptor. In addition, *N*-methylation at several other sites (N5, I6, and W10) significantly reduced potency, although agonist activity remained. Only F7 tolerated *N*-methylation, with only a minimal loss in potency.

The tolerance to modification at F7 proved advantageous as F7 is a likely cleavage location if the peptide is recognized by chymotrypsin. *N*-Methylation at F7 appeared to nearly eliminate cleavage by chymotrypsin, increasing the half-life of native GBAP from about 4 h in the presence of the same concentration of chymotrypsin to well over 24 h, as well as improve the peptide stability in human plasma. Combining the increased stability with the low impact that the modification has on the peptide activity has revealed a promising strategy for improving the enzymatic stability of future *E. faecalis* fsr QS modulators.

■ EXPERIMENTAL SECTION

General. All peptides were purified to ≥95% as confirmed by analytical reverse phase high-performance liquid chromatography (RP-HPLC). See Table S-1 for individual peptide purities.

Peptide Synthesis. Entirely on-resin fluorenylmethyloxycarbonyl (Fmoc)-based peptide synthesis[28−30] was conducted as we previously described (see the Supporting Information for full details). Due to the reduced nucleophilicity of *N*-methylated *N*-termini, longer coupling times and occasionally elevated temperature were necessary to attach the next amino acid. All amino acids were coupled for 4 h at room temperature. Synthesis continued if the chloranil test (see the Supporting Information) was found to be negative; otherwise, the attachment was reattempted but ran overnight at 50 °C.[19]

Serine was *N*-methylated on the growing peptide chain using an adapted procedure.[25] The peptide was synthesized using our standard procedures up until after the removal of Fmoc

from the Ser(Trt) residue. Four equivalents of ortho-nitrobenzenesulfonyl chloride (o-NBS chloride) was dissolved in 1 to 2 mL of DMF. Ten equivalents of 2,4,6-Collidine was added, and the mixture was shaken for 1 min. The resulting solution was then added to the resin and shaken for 15 min. This process was repeated once more before the resin was washed 5 times with DMF. This was sufficient to monoprotect the resin as indicated by a positive chloranil test result and a mostly negative Kaiser test result (roughly 10% of the assayed resin beads still stained blue). Six equivalents of DBU was then dissolved in 1–2 mL of DMF, added to the resin, and shaken for 10 min. Approximately 20–25 equiv of dimethylsulfate (DMS) was added, and the resulting mixture shaken for 1 h. The resin was briefly washed, and this process was repeated once more followed by washing with DMF 5 times and shaking for 2 min each. After this second treatment, both the Kaiser and chloranil tests were negative, indicating methylation of all available secondary and primary amines. To remove o-NBS, 10 equiv of β-mercaptoethanol was dissolved in 1–2 mL of DMF along with 5 equiv of DBU and added to the resin and shaken for 30 min. This process was repeated once, and the resin washed 5 times with DMF and shaken for 2 min each. After these steps, the Kaiser test was negative, but the chloranil test was positive, indicating successful monomethylation and o-NBS removal.

The synthesis of the peptide containing the N-MeM11 modification required the synthesis of the dipeptide Fmoc-Gln(Trt)-Trp(Boc)-OH to avoid reaction of the N-terminal amine of tryptophan with the macrocycle ester to form the corresponding diketopiperazine and peptide truncation at the serine branching point. In order to retain the protecting groups, the dipeptide was prepared using chlorotrityl resin as the solid support. First, the chlorotrityl resin was dried under vacuum for 48 h. 1.1 equivalents (relative to the manufacturer's reported loading for the resin) of Fmoc-Trp(Boc)-OH was dissolved in dry DCM, and 2.5 equiv of DIPEA was added. This solution was added to the resin in a 50 mL falcon tube (the screw-top cap prevents the cap popping off from the increased pressure when shaking DCM solutions) and shaken for 1 h. One milliliter of methanol was added, and the resin shaken for an additional 10 min. The resin slurry was decanted into a 20 mL solid phase reaction vessel and washed thoroughly three times each with DMF, DCM, and methanol. The resin was then dried under reduced pressure, and the loading of the resin determined (see the Supporting Information). The resin was allowed to stand in DCM for 30 min to swell. Three equivalents of Fmoc-Gln(Trt)-OH and 2.85 equiv of HATU were dissolved in DMF, and 4 equiv of DIPEA was added. The resulting solution was added to the resin and shaken overnight (approximately 18 h) followed by washes with DMF and DCM. The dipeptide was cleaved from the resin by treating with 1% TFA and 5% TIPS in DCM 4–5 times for 2 min. The resin was then washed 3 times with the same cleavage solution and 5 times with DCM. The combined cleavage and wash fractions were then washed 1 time with an equivalent volume of basic brine (approximately 250 mg/mL sodium bicarbonate saturated with sodium chloride); the DCM was dried over magnesium sulfate for approximately 2 h. The dried solution was filtered, and the DCM removed under reduced pressure. The residue was then dissolved in acetonitrile and water and lyophilized for at least 18 h. The resulting residue was then dissolved in the minimum amount of DCM and passed through a silica plug packed using 30% MeOH in DCM and eluted with the same solvent mixture. Fractions containing the product were verified with thin layer chromatography (TLC) and combined, and the solvent removed under reduced pressure. The residue was dissolved in acetonitrile and water and lyophilized a second time. This process yielded the desired, still protected dipeptide in ≥80% yield. The dipeptide was then treated the same as any other amino acid for the coupling step following N-MeM11 attachment (see above).

Peptide Purification and Verification. Peptide purification by RP-HPLC and verification by mass spectrometry (MS) were conducted as previously described (see the Supporting Information).[19]

β-Galactosidase Reporter Assay. The ability of the analogs to activate the QS circuit was evaluated as we previously described (see the Supporting Information for full experimental details).[19,21]

Chemical Stability Assays. Peptide solutions were prepared at 0.28 mM concentration in 1 mL of either PBS buffer (pH 7.2) or ammonium carbonate buffer (pH 8) and incubated at either 25 or 37 °C for 24 h. Time points were taken at 0 and 30 min and 1, 2, 4, 8, and 24 h. At each time point, a 100 μL aliquot was taken and mixed with 20 μL of ACN. Ninety microliters was then injected via an autosampler, and an analytical trace taken (see Peptide Purification and Verification section and SI for details on the analytical method). Traces were overlaid for comparison, and the percent of remaining peptide was calculated by comparing the integrated area under the undigested peak in each trace with that of the undigested peak in the 0 min time point trace (which was normalized to 100%). Note that, due to the very low chemical stability of GBAP in the ammonium carbonate buffer, all other peptides were only tested in the PBS buffer conditions.

Chymotrypsin Stability Assays. Due to the low chemical stability of GBAP in ammonium carbonate buffer (pH 8), the chymotrypsin degradation assays were conducted in PBS buffer (pH 7.2). A 1 mL, 0.28 mM peptide solution was prepared in the buffer. Five microliters of a 250 μg/mL chymotrypsin solution was added, giving a final chymotrypsin concentration of approximately 50 nM. The solution was incubated at 37 °C for 24 h. Time points were taken at 0 and 30 min and 1, 2, 4, 8, and 24 h. At each time point, a 100 μL aliquot was taken and mixed with 20 μL of ACN. Ninety microliters was then injected via an autosampler, and an analytical trace taken (see Peptide Purification and Verification section and SI for details on the analytical method). Trace analysis was conducted similarly to the chemical stability assays.

Human Plasma Stability Assays. Stability assays were done with human plasma by adapting a reported protocol.[31] A 500 μL, 0.56 mM peptide solution was prepared in PBS buffer (pH 7.2). This solution was combined with 500 μL of reconstituted human plasma to give a 0.28 mM final concentration of peptide. The resulting suspension was incubated at 37 °C for 24 h. Time points were taken at 0 and 30 min and 1, 2, 4, 8, and 24 h. At each time point, a 130 μL aliquot was taken and added to a 10 kDa microcentrifuge-compatible filter and tube assembly. The assembly was centrifuged at 18 000g for 15 min, giving approximately 100 μL of filtrate. Twenty microliters of ACN was added to the filtrate, and 90 μL of the resulting solution was injected via an autosampler; an analytical trace was then taken (see Peptide Purification and Verification section and SI for details on the

analytical method). Trace analysis was conducted similarly to the chemical and chymotrypsin stability assays.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.9b00097.

> Full details of experimental procedures, peptide characterization, and dose−response curves for all GBAP analogues (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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