Quorum Sensing

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Highly Stable, Amide-Bridged Autoinducing Peptide Analogues that Strongly Inhibit the AgrC Quorum Sensing Receptor in *Staphylococcus aureus*

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Abstract: *Blocking quorum sensing (QS) pathways has attracted considerable interest as an approach to suppress virulence in bacterial pathogens. Toward this goal, we recently developed analogues of a native autoinducing peptide (AIP-III) signal that can inhibit AgrC-type QS receptors and attenuate virulence phenotypes in Staphylococcus aureus. Application of these compounds is limited, however, as they contain hydrolytically unstable thioester linkages and have only low aqueous solubilities. Herein, we report amide-linked AIP analogues with greatly enhanced hydrolytic stabilities and solubilities relative to our prior analogues, whilst maintaining strong potencies as AgrC receptor inhibitors in S. aureus. These compounds represent powerful tools for the study of QS.*

Quorum sensing (QS) is a cell–cell signaling mechanism that is intimately connected to virulence in many common pathogens,[1] including *Staphylococcus aureus*.[2] QS enables bacteria to assess their population density and synchronize group behaviors at high cell number, allowing pathogens to overwhelm their host.[1] Bacteria with non-functional QS systems have been shown to be less virulent in numerous infection models.[3] Accordingly, inhibition of QS has garnered attention as a potential target to combat infection,[1,4] and substantial recent effort has been focused on QS in *S. aureus*.[2b,3a,b,4b]

QS in bacteria, at the most basic level, is regulated by small molecule or peptide signals and their cognate receptors.[1,2] Signal concentration increases with cell density, and once a threshold concentration is reached, productive signal:receptor binding alters gene expression levels to permit the initiation of group behaviors, such as toxin production, motility, or biofilm formation.[1] *S. aureus* uses an accessory gene regulator (agr) system for QS that is centered on the interaction between the autoinducing peptide (AIP) signal and its target transmembrane receptor, AgrC.[2] Four distinct AIP:AgrC pairs have been characterized in *S. aureus* strains, forming four QS specificity groups (I–IV). The *S. aureus* AIP signals share two main structural features: i) a macrocyclic thioester linked by a conserved cysteine and the C-terminus, and ii) an N-terminal exocyclic tail (Figure 1).[2b]

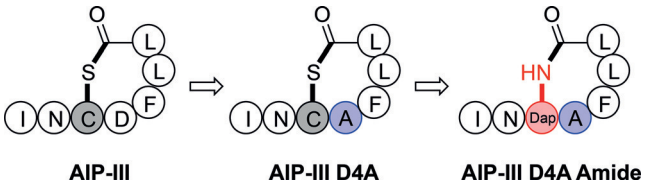

Figure 1. Structures of a native AIP (AIP-III), an inhibitor derived therefrom (AIP-III D4A), and a scaffold studied herein (AIP-III D4A Amide). Single letter codes are used for amino acids; Dap = L-diamino propionic acid.

To date, most efforts to block QS in *S. aureus* have focused on the AIP ligand, either with the intent to competitively inhibit AIP:AgrC binding[5] or, to a lesser extent, sequester the AIP ligand away from its target receptor.[4b] Blocking downstream transcriptional activation by AgrC has also been accomplished.[3b] These chemical strategies provide means to modulate the agr system with both temporal and spatial control, and gain insights into its role in *S. aureus* infections. Our laboratory has focused on blocking AIP:AgrC binding, and we recently reported analogues of AIP-III (Figure 1) that inhibit AgrC receptors and associated toxin production in *S. aureus*.[6] Despite their high potencies, these compounds (and many other reported AIP analogues)[5] have significant limitations, most notably the instability of the native thioester bridge and their relatively low solubilities in aqueous media.[7] Herein, we report an approach toward addressing these shortcomings through the development of new AIP-III analogues containing non-native amide bridges that are highly potent, stable, and soluble pan-group inhibitors of agr-type QS in *S. aureus*.

Our earlier studies identified AIP-III as a superior peptidic scaffold for the design of pan-group AgrC inhibitors.[6a] A seemingly simple modification to the native AIP-III structure, replacing Asp4 with Ala, converted the peptide

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into an extremely potent inhibitor, AIP-III D4A (Figure 1). Subsequent NMR studies revealed that this replacement drastically altered the structure of AIP-III.[6b] While AIP-III displayed a distinctive hydrophobic, triangular "knob" composed of three endocyclic side chains (Phe5, Leu6, and Leu7) and a separately positioned N-terminal tail domain presenting an "anchor point" necessary for activation, the D4A modification caused the peptide to fold up into a compact, amphipathic spheroid where the hydrophobic knob effectively sequestered the N-terminal tail.[6b] We propose that this compact, knob-like structure allows AIP-III D4A to compete with the native ligand for AgrC receptors and, in the absence of a correctly positioned tail, inhibit their function.

In the current study, we sought to leverage our past results and redesign AIP-III D4A to increase its utility as a probe to study QS in *S. aureus*. We reasoned that replacement of the thioester with an amide linkage (Figure 1) would increase its hydrolytic stability, and potentially its solubility, in aqueous media. Surprisingly, very few non-thioester AIP analogues have been studied to date.[5] We previously examined AIP-I peptoid-peptide hybrid analogues with lactam bridges,[8] but these compounds had limited activity. Muir,[3c,9] Williams,[10] and Otto[11] have studied lactam replacements in the native *S. aureus* AIP-I and AIP-II signals and the *S. epidermidis* AIP-I signal, and identified peptides capable of low to moderate non-cognate (that is, non-self) AgrC receptor inhibition and very weak cognate AgrC receptor activation. No other lactam-derived AIP analogues have been reported, to our knowledge.

To start, we developed a solid-phase synthetic route to amide-bridged AIP analogues in which the conserved Cys was replaced with L-diamino propionic acid (Dap; see the Supporting Information). We applied this route for the construction of three new AIP-III analogues (listed in Table 1): the amide versions of native AIP-III and our two most potent AgrC inhibitors, AIP-III D4A and its truncated analogue, tAIP-III D2A (lacking the N-terminal tail).[6a] Simple logP calculations on these peptides (Supporting Information) indicated they were less hydrophobic than their thioester precursors, suggesting that they could have enhanced aqueous solubilities.

We evaluated the amide-bridged peptides for their abilities to modulate AgrC receptor activity in *S. aureus* groups-I-IV using reporter gene assays. For AgrC activation, we used a set of *S. aureus agr*-null strains each harboring a P3-*blaZ* reporter and *agrCA* from groups-I, -II, -III, or -IV.[12] For AgrC inhibition, we used *S. aureus* strains (groups I–IV) with functional agr circuits and P3-*gfp* reporter plasmids.[13] In these strains, AgrC activation or inhibition was quantified by measuring β-lactamase activity or GFP fluorescence, respectively.

None of the amide-bridged AIPs were capable of AgrC activation in any of the groups (at concentrations up to 40 μM). This result for AIP-III Amide is congruent with previous studies of the AIP-I and AIP-II lactams, both of which are only very weak cognate receptor agonists.[9,10] Turning to the inhibition data (Table 2), AIP-III Amide was found to be a very weak to moderate inhibitor of the four AgrC receptors. Interestingly, AIP-III was most potent against AgrC-III, which contrasts with the lack of self-inhibition observed previously for the AIP-I and AIP-II lactams.[3c,9,10] These results, when compared to the relatively potent non-cognate receptor antagonism displayed by the native AIP-III signal (Table 2), underscore the importance of the thioester linkage for strong non-cognate AgrC receptor inhibition by AIP-III.

Gratifyingly, and in contrast to AIP-III Amide, the amide-bridged analogues of our lead inhibitor, AIP-III D4A, had largely comparable inhibitory activities to their parent thioester counterparts (Table 2). With the exception of tAIP-III D2A Amide against AgrC-II (15-fold reduction in activity compared to tAIP-III D2A), the activity differences between the amide-bridged analogues and their thioester counterparts did not exceed a 5-fold reduction, and, in most cases, the activity differences were within error. These results suggest that the AIP-III D4A and tAIP-III D2A scaffolds are more tolerant to linkage modifications relative to the native AIP-III signal. Notably, these compounds represent the first lactam-bridged AIP analogues with strong, pan-group inhibitory activities (picomolar to low nanomolar IC_{50} values) against *S. aureus* AgrC-type receptors.

We next examined the 3D structures of the lactam AIP-III analogues to gain insights into the origins of their biological activities. Specifically, we were interested to determine if the consensus structural features that we previously identified as critical for AgrC-III inhibition and activation (see above) also applied to these amide-bridged analogues.[6b,d] We used NMR spectroscopy to elucidate the 3D solution structures of the

Table 1: Peptides evaluated in this study.[a] Dap = L-diamino propionic acid.

Peptide name	Sequence
AIP-III Amide	Ile-Asn-(Dap-Asp-Phe-Leu-Leu)
AIP-III D4A Amide	Ile-Asn-(Dap-Ala-Phe-Leu-Leu)
tAIP-III D2A Amide	Ac-(Dap-Ala-Phe-Leu-Leu)
AIP-III[b]	Ile-Asn-(Cys-Asp-Phe-Leu-Leu)
AIP-III D4A[b]	Ile-Asn-(Cys-Ala-Phe-Leu-Leu)
tAIP-III D2A[b]	Ac-(Cys-Ala-Phe-Leu-Leu)

[a] See Table S-1 for MS and HPLC characterization data. [b] Control peptides; reported in Ref. [6a].

Table 2: IC_{50} values of the amide-bridged AIP analogues against AgrCs I–IV determined using *S. aureus* fluorescence reporter strains.[a]

Peptide name	Inhibition [IC_{50} nM][b]			
	AgrC-I	AgrC-II	AgrC-III	AgrC-IV
AIP-III Amide	>1000	>1000	75.2	299
AIP-III D4A Amide	1.20	2.19	0.0551	0.192
tAIP-III D2A Amide	1.50	13.9	0.216	0.189
AIP-III[c]	5.05	5.63	–[d]	8.53
AIP-III D4A[c]	0.485	0.429	0.0506	0.0349
tAIP-III D2A[c]	0.257	0.900	0.329	0.0957

[a] See the Supporting Information for details of the reporter strains and methods, and antagonism dose response curves. [b] See the Supporting Information for 95% confidence ranges. [c] Control peptides, data from Ref. [6a]. [d] Receptor agonist.

peptides, utilizing conditions analogous to those in our past report (see the Supporting Information).[6b] Starting with AIP-III Amide, as this analogue was incapable of AgrC-III activation and instead moderately inhibited AgrC-III, we expected it to possess a hydrophobic, knob-like motif to enable interaction with AgrC-III, but to lack an appropriate fourth anchor point in the correct orientation. The NMR data revealed that this analogue did possess a knob motif analogous to AIP-III (Figure 2A). However, unlike AIP-III

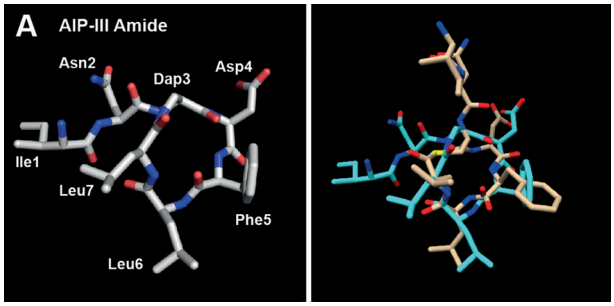

Figure 2. A) Heavy atom lowest energy structure of AIP-III Amide. B) Overlay of AIP-III Amide (cyan) and AIP-III (tan) structures. AIP-III structure from Ref. [6b].

where the exocyclic tail projects from the top of the triangular knob to presumably correctly orient the activating anchor (Figure 2B), the exocyclic tail in AIP-III Amide projects from the left side of the triangular knob (Figure S-3A) and the Ile1 side chain is located in close proximity to the Leu7 side chain (Figure S-4A). We propose that this alternate tail orientation for AIP-III Amide hinders AgrC-III activation; it may also present a steric blockade that prevents the knob from productively binding to AgrC receptors in general, thus providing an explanation for the relatively low to moderate activity of this peptide overall in any of the *S. aureus* groups.

As AIP-III D4A Amide exhibited activities comparable to those of its parent thioester (AIP-III D4A) in the reporter assays, we expected this analogue to possess a largely similar structure in solution. We observed just this. Although the conformation of the AIP-III D4A Amide macrocycle was somewhat different than that of AIP-III D4A (Figure 3), the overall structure of AIP-III D4A Amide revealed an obvious

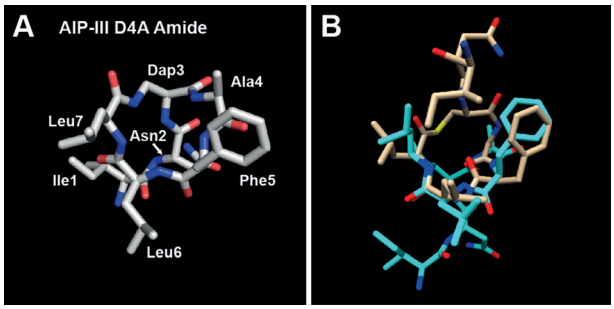

Figure 3. A) Heavy atom lowest energy structure of AIP-III D4A Amide. B) Overlay of AIP-III D4A Amide (cyan) and AIP-III D4A (tan) structures. AIP-III D4A structure from Ref. [6b].

endocyclic knob motif (Figures S-3B and S-5), with the tail residing behind the macrocycle and projecting to the left side, and the Ile1 side chain distanced from Leu7 (relative to AIP-III Amide; Figure S-4B). We reason that this orientation of the AIP-III D4A Amide tail disallows activation, but does not restrict competitive AgrC binding (and thereby the observed strong inhibition), in contrast to the AIP-III Amide tail (compare Figure S-4A vs. S-4B).

Turning to tAIP-III D2A Amide, this peptide had similar inhibitory activities as its thioester counterpart (tAIP-III D2A) against AgrC-III and -IV, and was only slightly less potent against AgrC-I and -II. We thus expected to observe a triangular knob motif for this analogue as well, similar to that we had previously observed for tAIP-III D2A.[6b] The NMR analyses of tAIP-III D2A Amide revealed a very structured conformation (Figures 4A and S-3C; backbone

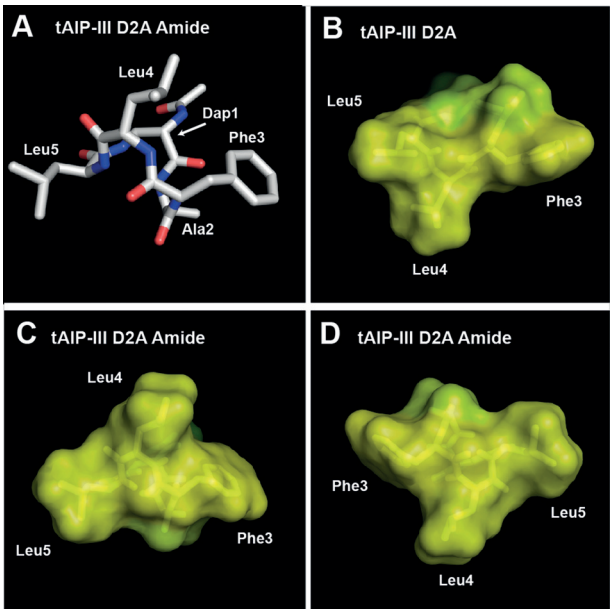

Figure 4. A) Heavy atom lowest energy structure of tAIP-III D2A Amide. Space-filling models of B) tAIP-III D2A (from Ref. [6b]), C) tAIP-III D2A Amide, and D) tAIP-III D2A Amide (showing the alternate triangular knob) displaying hydrophobic (yellow) and hydrophilic (green) surfaces.

atoms RMSD of 0.01 Å for the 10-structure ensemble). Interestingly, this amide analogue displayed a "reversed" triangular knob motif when compared to tAIP-III D2A (Figure 4B,C). When viewed with the Phe3 and Leu5 residues to the right and left, respectively, the Leu4 side chain in tAIP-III D2A Amide is positioned higher than those for Phe3 and Leu5 (Figure 4C), as opposed to being lower as in tAIP-III D2A (Figure 4B). It is tempting to speculate that tAIP-III D2A Amide binds AgrC receptors in an opposite way relative to other AIP-III derivatives: the Phe3 residue interacts with the AgrC binding pocket usually occupied by Leu5, and vice versa (compare Figure 4C vs. 4D). In our prior study, we hypothesized that the roles of the hydrophobic side chains of AIP-III (as part of the knob or the anchor) may be interchangeable.[6b] Additional studies are needed to fully

explore these possibilities. Nevertheless, the 3D structures of the three amide-bridged AIP-III analogues described herein, when coupled with their biological activity profiles in the AgrC reporter assays, support the general structural trends that we have previously identified for AgrC inhibition and activation.

Lastly, we sought to determine whether the replacement of the thioester with an amide confers increased overall stability to the peptides. We had observed empirically that all three amide-bridged AIPs were more soluble in aqueous media relative to the parent thioesters, congruent with the logP calculations. To probe stability, we initially chose to compare the stability of AIP-III D4A to its amide analogue, AIP-III D4A Amide. However, AIP-III D4A could not be solubilized at the concentrations necessary for the stability studies, further highlighting the solubility limitations of this compound. We thus compared the stability of the native AIP-III to AIP-III D4A Amide instead.

To monitor hydrolytic stability, solutions of the two peptides (0.33 mM) were prepared in different buffers (PBS, pH 7.4 and ammonium carbonate, pH 8.0) and incubated at 37° C. To monitor proteolytic stability, a mixture of trypsin and chymotrypsin was added to the peptides in ammonium carbonate prior to incubation. HPLC was used to quantitate the amount of intact peptide over time. In PBS, 30% of the AIP-III thioester was degraded after 72 h, while AIP-III D4A Amide remained intact (Figure 5A). In the more alkaline ammonium carbonate solution, the stability of the thioester-linked peptide dramatically decreased, and AIP-III was fully degraded in ~8 h. In contrast, AIP-III D4A Amide was found to be significantly more stable at pH 8.0, with >90% intact peptide remaining after 24 h (and >60% remaining after 72 h).[14] Treatment of the peptides with trypsin/chymotrypsin in ammonium carbonate yielded similar results as in the buffer alone (Figure 5B). This result was not unexpected, as cyclization is a common method to confer proteolytic stability to linear peptides. Nonetheless, the replacement of the thioester with an amide linkage markedly increased the overall hydrolytic stability of AIP-III D4A Amide. Further, the amide replacement increased the aqueous solubilities of all of the amide-bridged AIP analogues and significantly facilitated their purification and handling.[15]

In summary, the AIP-III analogues reported here constitute robust new tools to study the role of agr-type QS in *S. aureus* virulence, especially in biologically relevant environments. Their activity profiles also further underscore the utility of the native AIP-III scaffold for the generation of potent AgrC inhibitors.

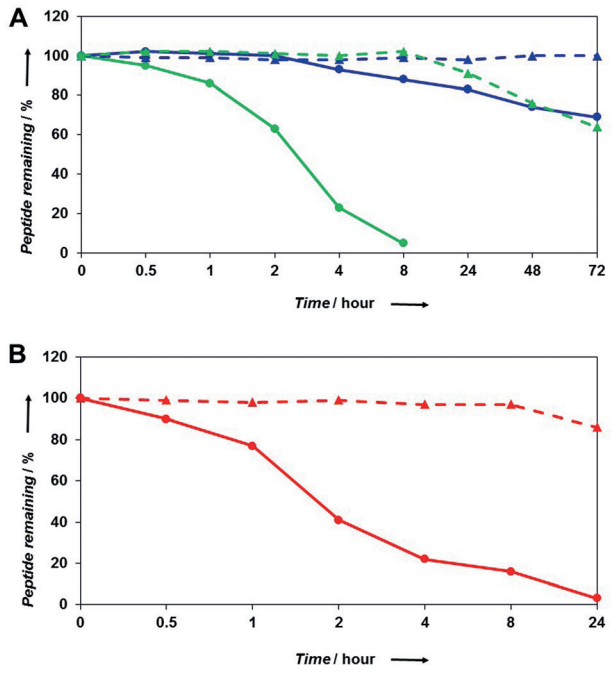

Figure 5. Peptide stability studies as monitored by HPLC. AIP-III and AIP-III D4A Amide data are shown as circles with solid lines or triangles with dashed lines, respectively. A) Stability in aqueous solutions (blue is PBS, pH 7.4; green is ammonium carbonate, pH 8.0). B) Stability towards enzymatic degradation by trypsin/chymotrypsin (red). See the Supporting Information for methods.

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