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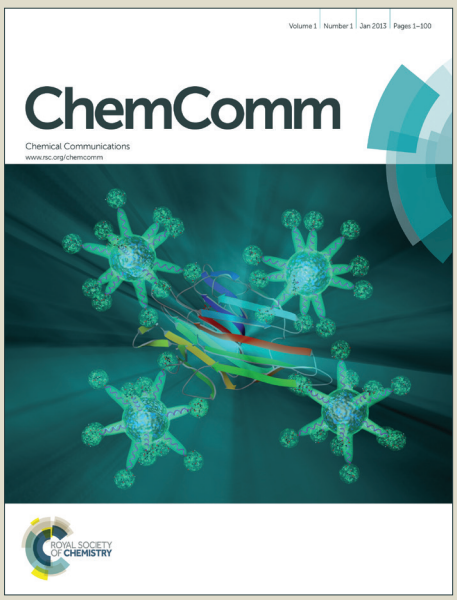

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ARTICLE TYPE

N-Methyl and Peptoid Scans of an Autoinducing Peptide Reveal New Structural Features Required for Inhibition and Activation of AgrC Quorum Sensing Receptors in *Staphylococcus aureus*

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We report the first *N*-methyl and peptoid residue scans of a full-length autoinducing peptide (AIP), AIP-III, used by *Staphylococcus aureus* for quorum sensing (QS). Biological evaluation of these AIP-III analogues uncovered new features of the AIP-III scaffold that can be tuned to develop chemical probes of QS in all four groups of *S. aureus* (I–IV).

Staphylococcus aureus is a notorious human pathogen that uses quorum sensing (QS) to assess its local population density.[1] Once a threshold cell density has amassed, the bacteria can activate group behaviours, including numerous virulence phenotypes, necessary for host infections.[2-5] *S. aureus* uses the accessory gene regulator (agr) system for QS, which is regulated by an excreted autoinducing peptide (AIP) signal and a transmembrane receptor histidine kinase AgrC. At a sufficiently high cell density – and thus extracellular AIP concentration – productive binding of the AIP to the extracellular sensor domain of AgrC activates QS signal transduction and alters the expression of group beneficial genes. Four unique AIP:AgrC pairs have been identified, leading to the classification of four *S. aureus* agr groups (I–IV).[6, 7]

As AIP:AgrC binding is essential for *S. aureus* QS, inhibition of AIP:AgrC interactions has attracted considerable interest as an approach to attenuate QS, and thereby virulence, in *S. aureus*. Both non-native AIP analogues that displace AIPs and antibodies that sequester AIPs away from their cognate AgrC receptor have been pursued.[8-10] Most past work has focused on AIP-I and AIP-II. We recently began examining the AIP-III signal (Fig. 1) and determined key residues on its heptapeptide scaffold essential for AgrC-III receptor interactions.[11] In addition, we identified a set of AIP-III analogues that were extremely potent inhibitors of all four AgrC receptors (I–IV) and were capable of completely blocking QS phenotypes in wild-type strains. These initial structure-activity relationship (SAR) studies suggested that AIP-III presents an excellent peptide scaffold for the development of QS modulators in *S. aureus*. We now seek to further explore the AIP-III structure and delineate features that can be further tuned to engender either enhanced agonistic activity in AgrC-III or increased inhibitory activities against AgrCs-I–IV. We are also interested in developing non-native AIP analogues capable of selectively inhibiting one (or two) AgrC receptors, to examine the role of QS in mixed populations of *S. aureus* groups. As a single *S. aureus* group is often prevalent in certain infection types,[4, 12] the facility of individual *S. aureus* groups to use QS as an interference mechanism has been postulated. The ability of native AIPs to cross-inhibit non-cognate AgrC receptors supports this hypothesis.[4, 13] Toward addressing these broad questions, we report herein our synthesis and biological evaluation of a set of new AIP-III analogues generated through *N*-methyl amino acid and *N*-substituted glycine (peptoid) scans of full-length AIP-III.

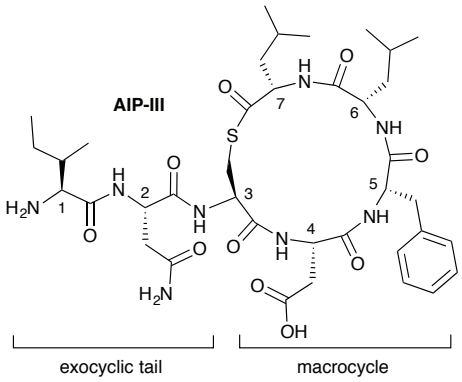

Figure 1. Structure of AIP-III. Residues numbered for clarity.

Our prior SAR[11] and structural studies[14] of AIP-III and analogues allowed us to develop a model by which AIP-III binds and activates the AgrC-III receptor. First, the three hydrophobic residues within the macrocycle form a triangular knob that is required for initial AgrC receptor recognition (Phe5, Leu6, and Leu7; Fig. 1). Second, the Asp4 side chain, as well as additional hydrophilic contacts, serve to properly orient the exocyclic tail relative to the macrocycle. Third, the side chain of Ile1 on the tail, when properly oriented, serves as a fourth hydrophobic anchor that is critical for AgrC-III activation. Modification of any one of these three facets yielded analogues that were incapable of AgrC-III activation, and many exhibited competitive antagonistic activity instead (against AgrCs-I–IV). Most notably, AIP-III with an Asp4→Ala4 modification (AIP-III D4A) is the most potent pan-group AgrC inhibitor reported to date.[11] Truncated analogues (lacking the tail) that retained the hydrophobic knob were all AgrC inhibitors, ranging from weak to strong.

This past work demonstrated that the majority of the side chain functionalities in AIP-III are essential for AgrC recognition. We sought to explore if other structural elements could be altered in

AIP-III to modulate its activity (i.e., backbone amide NHs and side chain positioning), and performed *N*-methyl (*N*-Me) and peptoid (n) scans of its primary sequence. In each peptoid analogue, the side chain of a specific residue is shifted from the C-α to the amide. We previously showed that a peptoid-peptide hybrid of truncated AIP-I was capable of modest QS inhibition in *S. aureus*;[15] peptoid AIP mimics are yet to be investigated further. Likewise, *N*-Me amide modifications remain largely unexamined in the context of AIPs. In the one reported study to date, George *et al.* performed an *N*-Me scan of the macrocycle of truncated AIP-II (tAIP-II) and discovered H-bonds essential for cognate and non-cognate AgrC inhibition,[16] underscoring the potential utility of such studies for investigating AIP SAR.

Table 1. Structures of the AIP-III analogues in this study and their IC_{50} values against AgrCs I–IV and EC_{50} values in AgrC-III.[a]

Peptide/ AIP-III Modification	Sequence	AgrC-I IC_{50} (nM)[b]	AgrC-II IC_{50} (nM)[b]	AgrC-III IC_{50} (nM)[b]	AgrC-IV IC_{50} (nM)[b]	AgrC-III EC_{50} (nM)[b]
AIP-III	I-N-(C-D-F-L-L)	5.05	5.63		8.53	406
N-Me-I1	***N*MeI**-N-(C-D-F-L-L)	60.8	12.8	>1000[c]	49.2	-[d]
N-Me-N2	I-***N*MeN**-(C-D-F-L-L)	8.04	3.19	--[e]	9.54	75.8
N-Me-C3	I-N-(***N*MeC**-D-F-L-L)	137	134	--	106	>4000
N-Me-D4	I-N-(C-***N*MeD**-F-L-L)	172	7.98	90.2	36.9	-
N-Me-F5	I-N-(C-D-***N*MeF**-L-L)	4.49	1.95	--	6.59	198
N-Me-L6	I-N-(C-D-F-***N*MeL**-L)	>1000[c]	--	162	81.6	-
N-Me-L7	I-N-(C-D-F-L-***N*MeL**)	--	--	>1000[c]	--	-
nI1	**nI**-N-(C-D-F-L-L)	44.0	4.25	>1000[c]	22.2	-
nN2 DKP	(I-**nN**)-(C-D-F-L-L)	126	4.28	25.1	84.8	-
nD4	I-N-(C-**nD**-F-L-L)	162	28.3	206	53.2	-
nF5	I-N-(C-D-**nF**-L-L)	13.8	75.6	>1000[c]	0.839	947
nL6	I-N-(C-D-F-**nL**-L)	--	--	>1000[c]	>1000[c]	-
nL7	I-N-(C-D-F-L-**nL**)	--	--	--	--	-

[a] See Supp. Info. for details of reporter strains and assay procedures. [b] See Supp. Info. for 95% confidence ranges. [c] 100% inhibition was not observed over the conc. tested. [d] No activation observed over the conc. tested. [e] No inhibition observed over the conc. tested.

We used a straightforward Boc/Fmoc hybrid solid-phase peptide synthesis protocol to construct the seven *N*-Me AIP-III analogues and six peptoid AIP-III analogues (each with one residue modifications, listed in Table 1; AIP-III nC3 not examined due to synthesis constraints). Peptide macrocyclization was performed post-cleavage using our reported method,[11] after which the peptides were purified to homogeneity by RP-HPLC (see Supp. Info. for full details). Interestingly, replacement of Asn2 with its peptoid counterpart resulted in almost quantitative formation of an amide bond between the amino terminus and the nAsn2 side chain to yield a diketopiperazine (DKP). This cyclic segment did not hinder the macrocyclization reaction, resulting in clean formation of a bicyclic AIP-III analogue, AIP-III nN2 DKP (Fig. 2A), which was also forwarded to biological analysis.

We examined the ability of the AIP-III analogues to modulate the AgrC receptors (I–IV) in cell-based assays using two sets of *S. aureus* reporter strains. To measure AgrC antagonism, we used *S. aureus* strains (groups-I–IV) harbouring P3-*gfp* reporter plasmids.[17] In these strains, the AIP:AgrC complex activates AgrA (the intracellular response regulator), which then binds the P3 promoter and induces *gfp* transcription. Thus, competitive AgrC inhibition by an exogenous peptide was quantified by measuring GFP fluorescence. For the AgrC agonism assay, we used a set of *S. aureus agr*-null strains each carrying a P3-*blaZ* reporter plasmid and *agrCA* from groups-I, -II, -III, or -IV.[13, 18] AgrC agonism was quantified by measuring β-lactamase activity.

The activities of the *N*-Me and peptoid AIP-III analogues in AgrCs-I–IV are summarized in Table 1. We first scrutinized their antagonism activity trends against *non-cognate* AgrC receptors relative to native AIP-III. Modifications to Ile1 yielded analogues that were ~2–10-fold less active than AIP-III (with only one exception), revealing the importance of both the Ile side chain position and an *N*-terminal primary amine in most non-cognate receptor interactions. However, the *N*-Me Asn2 analogue displayed analogous inhibitory activity as AIP-III, suggesting this amide NH was not important. We were unable to directly evaluate AIP-III nN2 (see above), but note that while AIP-III nN2 DKP significantly lost the ability to inhibit AgrC-I and -IV (>20-fold and 10-fold changes, respectively, relative to AIP-III), it displayed analogous activity as AIP-III against AgrC-II.

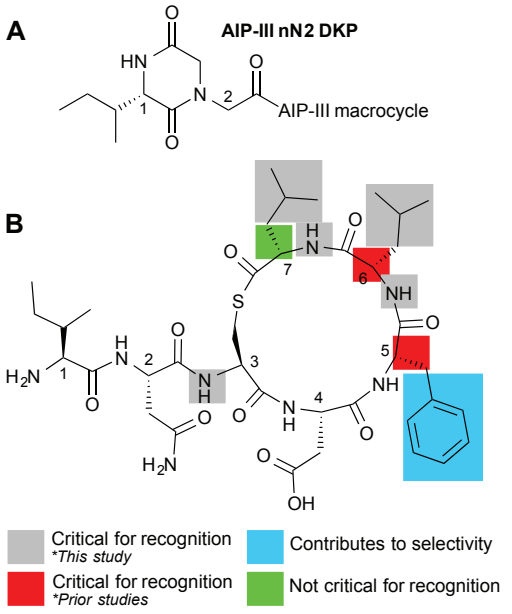

Figure 2. A. Structure of AIP-III nN2 DKP. B. Key SAR of AIP-III.

Turning to the AIP-III macrocycle, we found that *N*-Me modifications to both Cys3 and Asp4, and peptoid modification to Asp4, typically caused modest to large reductions in inhibitory activity relative to AIP-III (~4- to 30-fold changes, Table 1), indicating the significance of these two amide NHs and the Asp4 side chain position for certain non-cognate receptor interactions. These effects were the most apparent for AgrC-I, and the least apparent for AgrC-II (with AIP-III *N*-Me-D4 displaying almost comparable activity as AIP-III against this receptor). The results for Phe5 were perhaps more interesting. We previously observed that converting Phe5 to either D-Phe or Ala in AIP-III yielded a weakly active, non-cognate AgrC inhibitor;[11] however, in the current study we found that Phe5 could be replaced with either its *N*-Me or peptoid counterpart with virtually no change in antagonistic activity. In fact, AIP-III nF5 was the most potent inhibitor identified in this study (IC_{50} = 839 pM), with a 10-fold increased potency against AgrC-IV relative to AIP-III. These results are notable, as they suggest that the nPhe side chain can mimic that of L-Phe in the native AIP-III.

In contrast to Phe5, the amide NHs and side chain positioning of Leu6 and Leu7 in AIP-III were critical for non-cognate AgrC inhibition, as *N*-Me or peptoid modification of these *C*-terminal residues virtually abolished activity. George *et al.* observed

analogous results for tAIP-II when either of its two *C*-terminal residues (Leu4 and Phe5) were *N*-methylated.[16] These data also mesh with our earlier study, which showed that conversion of either Leu6 or Leu7 to Ala obliterated the resulting AIP-III analogue's inhibitory activity.[11] Interestingly, while the AIP-III D-Leu6 analogue was also inactive as a cross-receptor antagonist, the D-Leu7 analogue displayed analogous antagonistic activity as the native AIP-III. Together, these results indicate that the side chain of Phe5 (in the proper orientation), but not its amide NH, is a crucial component for non-cognate AgrC inhibition, whereas both the amide and the side chain are the important contributions at Leu6 and Leu7 (with the orientation of the Leu7 side chain less critical relative to Leu6). We propose, based on these results, that the AIP-III segment spanning from the Phe5 α-carbon to the Leu7 α-carbon (orientation controlled by the macrocycle) serves as a minimal requirement for non-cognate AgrC binding (Fig. 2B).

We next analysed the AgrC-III antagonism assay data to deduce any new structural features critical for AIP-III to bind (but not activate) its *cognate* receptor (Table 1). Modifications of Ile1 resulted in relatively inactive analogues, suggesting that a primary amine at the AIP-III N-terminus is important for not only non-cognate, but also cognate AgrC interactions. However, the ability of AIP-III nN2 DKP to effectively inhibit AgrC-III, despite the incorporation of the amino terminus within the DKP scaffold, weakens this hypothesis.[19] Indeed, AIP-III nN2 DKP was the strongest AgrC-III inhibitor identified in this study (IC_{50} = 25.1 nM). It is possible that a more basic, secondary amine at the N-terminal Ile1 has a detrimental effect on receptor binding. Within the macrocycle, modification of Asp4 yielded moderate AgrC-III inhibitors, as expected in view of our prior studies.[11] Modifications to either Leu6 or Leu7 resulted in largely inactive analogues, further emphasizing the importance of these two hydrophobic residues for AgrC interactions in general (Fig. 2B).

To streamline the AgrC agonism assays, we only evaluated AIP-III analogues that displayed <100% antagonism in the GFP reporter strains. None of these analogues were capable of activating *non-cognate* AgrC receptors (see Supp. Info.); however, four analogues were capable of AgrC-III activation (Table 1), and we analyse these data here. Most notably, the *N*-Me Asn2 analogue was ~4-fold more active than AIP-III (EC_{50} = 75.8 nM). This result, combined with the *antagonistic* activity observed for AIP-III nN2 DKP against AgrC-III, suggests that *N*-alkylation of Asn2 is highly beneficial for AgrC-III binding. In turn, *N*-methylation of Cys3 resulted in a >10-fold reduction in agonistic activity relative to AIP-III, highlighting again the significance of this amide NH for general AgrC receptor recognition. The *N*-Me and peptoid Phe5 analogues again yielded interesting results – both were able to activate AgrC-III as well as native AIP-III. This outcome strengthens our hypothesis about the minimal structural requirement for AIP-III:AgrC receptor binding, and extends it to now include AgrC-III (Fig. 2B).

Lastly, we examined the selectivities of the AgrC inhibitors identified herein. Few receptor selective AgrC inhibitors are known; prior work has uncovered four AgrC-III selective inhibitors and one AgrC-II selective inhibitor (selectivity defined as >15-fold stronger activity in one group relative to others).[11, 13] We discovered the first AgrC-IV selective inhibitor in this study, AIP-III nF5 (Table 1). As the sensor domains of AgrC-I and AgrC-IV have nearly 90% sequence homology,[5] the selectivity observed for AIP-III nF5 is significant, and reinforces the view that very subtle changes in the AIP-III structure can strongly tune its interactions with AgrC receptors.

In summary, we report the first systematic *N*-Me and peptoid scans of a full-length AIP from *S. aureus*, AIP-III. Evaluation of these new analogues in AgrC antagonism and agonism assays revealed key H-bond and side chain interactions that are critical for AgrC-I–IV receptor inhibition and AgrC-III activation. These SAR data allowed us to define a minimal structural requirement for AIP-III:AgrC interactions. In addition, we identified the first group-IV selective AgrC inhibitor, by replacing the AIP-III Phe5 residue with its peptoid counterpart. The analogues reported herein provide further insights into the mechanisms of AgrC activation and inhibition by AIPs, and constitute new – and potentially more biostable – tools to study QS in *S. aureus*.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Full details of peptide synthesis and characterization, biological assay protocols, dose response curves for AIP-III analogues. See DOI: 10.1039/b000000x/

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