Interception of quorum sensing in *Staphylococcus aureus*: a new niche for peptidomimetics

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Pathogenesis in *Staphylococcus aureus* is dependent on local cell density and is regulated in part by small macrocyclic peptides. Natural and artificial peptide inhibitors of this quorum sensing response have been synthesized and evaluated in structure–activity relationship studies. These investigations have illuminated the quorum sensing mechanism and set the stage for the design of biostable, peptidomimetic inhibitors that could be developed ultimately as therapeutics.

Staphylococcus aureus is a prevalent and highly adaptable Gram-positive bacterium responsible for numerous clinical infections.[1] The emergence of antibiotic resistance in this species and others has therefore become a serious concern in the medical community.[2] Unfortunately, pathogens continue to adapt more quickly than new antimicrobial agents can be developed to control them. One appealing approach to this problem is to target bacterial systems associated with virulence rather than essential cellular processes. It is hoped that this strategy will reduce selective survival pressures and slow the development of resistance.[3] Regardless of long-term efficacy, this tactic provides new targets for therapeutics that could preemptively deactivate the defenses of a developing colony. These defensive mechanisms, such as biofilm formation and secretion of virulence factors, pose the greatest threat to the host, rather then common commensal behaviors.

The discovery of a global regulatory system for virulence in *S. aureus* mediated by small autoinducing peptides (AIPs) has provided an avenue for interrupting these defenses.[4,5] AIPs function as extracellular signaling molecules that allow individual cells to sense the surrounding population density. Once a "quorum" of cells has been achieved, the bacteria modulate their gene expression to facilitate cooperative behaviors that confer survivability to the developing colony. AIP mimics that perturb this system would be useful chemical probes and could potentially be developed for therapeutic applications. Organic chemists, particularly those working in the field of peptidomimetics, are in a unique position to design and synthesize these compounds. Here, we provide a brief review of the progress that has been made towards understanding quorum sensing in *S. aureus* and discuss the implications for the design of new peptidomimetic AIP analogues.

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Helen Blackwell was born in Cleveland, Ohio and attended Oberlin College for her undergraduate studies, graduating with highest honors in chemistry in 1994. She pursued her graduate training in organic chemistry at the California Institute of Technology with Professor Robert Grubbs. Helen received her PhD in 1999 and then spent three years as a postdoctoral fellow in the lab of Professor Stuart Schreiber at Harvard University. In 2002, she returned to the Midwest and joined the faculty of the University of Wisconsin-Madison, where she is presently an Assistant Professor of Chemistry. Research in her group is focused broadly on the development of new combinatorial chemistry methods and their application to design molecular tools to ask important questions in bacteriology and ecology.

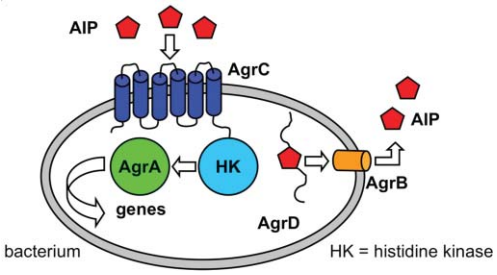


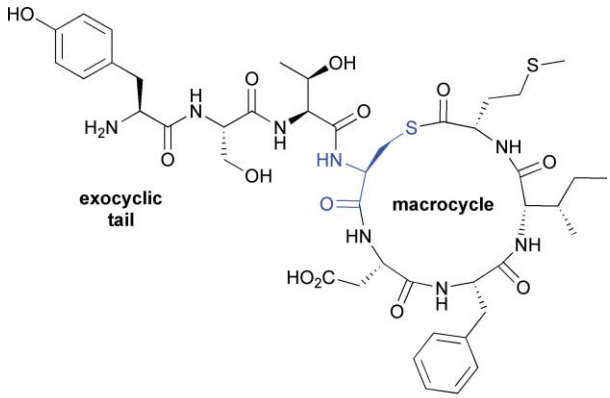
Fig. 1 Proposed mechanism of the two-component *agr* autoinduction system in *S. aureus.* AgrB processes the propeptide AgrD to generate an AIP and secretes it into the extracellular environment. The AIPs bind to the AgrC receptor, a histidine-kinase that phosphorylates the intracellular response regulator AgrA. This second signalling component then promotes gene transcription that induces virulence and produces the *agr* proteins, completing the autoinduction circuit.

The *Staphylococcal agr* system

The quorum sensing system in *S. aureus* is encoded by the accessory gene regulator (*agr*) locus and is shown schematically in Fig. 1. Each bacterium secretes AIPs that accumulate in the extracellular environment. Once these ligands reach a threshold concentration, they will bind to a cognate receptor protein located on the cell exterior named AgrC. AIP binding activates a two-component intracellular signaling system that ultimately triggers the virulence response and also up-regulates production of the four *agr* proteins: AgrA through D (Fig. 1). This up-regulation creates a positive feedback loop in which both the extracellular and intracellular signaling components are amplified. Although several of the Agr proteins are viable targets for therapeutic control, the surface receptor protein AgrC is especially attractive in this regard, as inhibitors need not permeate the cell membrane. This greatly relaxes the typical pharmacokinetic design constraints requiring low molecular weight and sufficient hydrophobicity for membrane permeability. However, the situation of AgrC within the membrane also is responsible presumably for the dearth of crystal structures that would permit rigorous, structure-based design of inhibitors. Fortunately, Nature has effectively provided peptidic leads for further antagonist development—the *agr* system has evolutionarily diverged into four distinct subgroups (I–IV), each with its own unique AIP that typically cross-inhibits the quorum sensing response in the other three competing strains of *S. aureus.* Researchers have seized the opportunity to delineate structure–activity relationships (SARs) in these systems in order to better understand quorum sensing and develop agents to intercept this signaling network. These relationships are likely to prove useful in the development of new peptidomimetic inhibitors.

Synthesis and SAR studies of AIPs

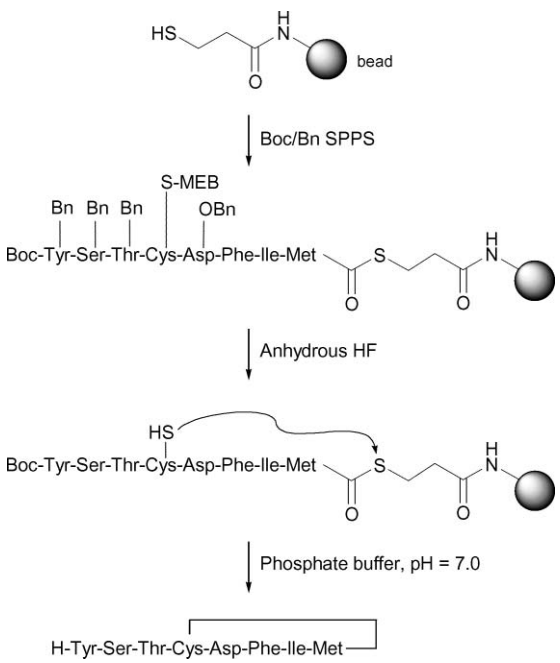
SAR studies require precise but flexible control of AIP structure in order to effectively probe ligand–receptor interactions. This has spurred the development of efficient chemical routes to both natural AIPs and rationally modified analogues. The wild-type AIPs in *S. aureus* are biosynthesized from the propeptide AgrD and are characterized by a thiolactone macrocycle and conjoined linear "tail" (Fig. 2). These unique molecules have been chemically synthesized using solid-phase peptide synthesis techniques and



S. aureus AIP-I		Y	S	T	C	D	F	I	M
AIP-II	G	V	N	A	C	S	S	L	F
AIP-III			I	N	C	D	F	L	L
AIP-IV		Y	S	T	C	Y	F	I	M

Fig. 2 Top: Chemical structure of AIP-I. Bottom: Peptide sequences of AIPs I through IV. The cysteine residue that forms the thiolactone is highlighted in blue.

a variety of cyclization strategies. The most commonly used methodology is shown in Scheme 1, in which the peptide is assembled using *N*-Boc–*O*-Bn chemistry, culminating with a cyclization–cleavage step.[6] Solution-phase cyclizations were later developed in order to better monitor the kinetics of the cyclization reaction.[7,8] A solid-phase synthetic route to AIPs utilizing the popular *N*-Fmoc strategy has yet to be reported. Such a route would not only make the natural peptides accessible to more researchers, but would facilitate the synthesis of novel hybrid peptide–peptidomimetic structures; for example, peptoid and β-peptide units could be incorporated easily using this strategy.



Scheme 1 Solid-phase synthesis and cyclization of AIPs (AIP-I shown.)

Numerous such hybrids of peptidomimetics and α-peptides have been reported already in the literature[9–11] and could be used to great advantage in this context (see below).

In initial studies, the biological activities of chemically synthesized AIPs I and II were confirmed to be identical to those of the naturally-derived ligands.[6] Lactone, lactam, and acyclic AIP analogues were also synthesized and assayed for activity in order to derive preliminary SARs.

These studies established that the macrocycle is crucial for biological activity, as the linear structures functioned neither as activators nor inhibitors of the quorum sensing response. The lactam and lactone derivatives were found to be effective cross-strain inhibitors, although the lactams remained weak agonists of their cognate receptors.[12,13] The labile thioester linkage in AIPs led to the initial speculation that a covalent bond could be made to the AgrC receptor, but subsequent experiments demonstrated that AIP-binding is a reversible and competitive process.[13] Alanine scans, in which single amino acids are replaced systematically with alanine, were performed on AIP-II and revealed that although residues in both the macrocycle and the tail are required for *agonism*, only the macrocyclic residues are essential for *antagonism.* This finding strongly implied that ligand binding to the receptor need not be coupled to activation and suggested a strategy for designing an inhibitor of the *agr* response that was soon exploited. Specifically, a truncated derivative of AIP-II consisting of only the macrocycle was synthesized and found to be an antagonist (IC_{50}: 10–272 nM) in all four *agr* systems.[14] Interestingly, the truncated AIP-I remained a modest group I agonist, intimating that this strategy is not general for all AIPs.[7] Overall, these studies showed a trend in which agonism seemingly required precise orientations of functionality in both the macrocycle and the tail, while antagonism was subject to relatively fewer constraints.

Subsequent alanine and D-amino acid scans of AIP-I underscored the importance of the endocyclic residues for antagonism as well.[12] Notably, replacement of the endocyclic aspartate with alanine converted AIP-I into a potent group I inhibitor, while replacement of the more hydrophobic phenylalanine or isoleucine residues seriously compromised the activity of the ligand. In contrast, replacement of phenylalanine with the non-natural D-enantiomer had little effect. These observations were soon rationalized based upon the results of studies carried out by Muir, Novick, and co-workers using chimeric AgrC receptors, in which the two halves of the receptor domain had been systematically exchanged with those of the other four *agr* groups.[15,16] The responses of the chimeric receptors to both natural and synthetic AIPs were then assessed. The authors hypothesized that the ostensibly independent binding and activating modes might be localized in different areas of the receptor, and that these experiments would elucidate the mechanisms of each. The selectivity of the receptor for its cognate AIP ligand was indeed found to reside mostly in the distal subdomain (relative to the *N*-terminus), while the cross-inhibitory response was closely associated with the proximal subdomain (Fig. 3). This trend, in conjunction with the observation of an increasing hydrophobicity gradient from tail to macrocycle in nearly all of the AIPs,[17] lead to the commonly accepted model of AgrC activation and inactivation shown above. The macrocycles of both agonists and antagonists bind to a relatively promiscuous hydrophobic pocket in the putative dimeric receptor. Activation requires additional *specific* contacts between the distal subdomains and the AIPs, which presumably triggers a conformational change in the receptor that, in turn, initiates signal transduction.[15]

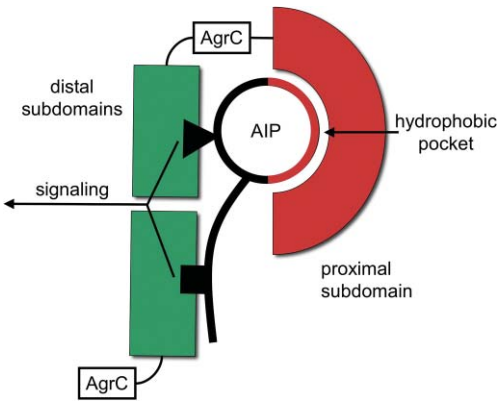


Fig. 3 Model of AgrC–AIP interactions. Antagonists need only contain residues that bind to a promiscuous hydrophobic pocket (circle) in the proximal subdomain (red), while agonists must also make specific contacts (square, triangle) to the distal subdomains (rectangles) to activate the signaling cascade.

Designing peptidomimetic effectors of the *agr* response

The SAR studies outlined in the previous section provide a framework for designing non-native inhibitors of AgrC function. Although effective peptide-based inhibitors have been identified, the generally low bioavailability and *in vivo* instability of peptides make these molecules poor candidates for therapeutic development.[18] This is particularly the case for AIPs, which contain thioester linkages that are likely to impart half-lives of less than three hours in biological media[19] Replacement of the thioester with a lactam or lactone linkage, however, significantly alters biological activity (see above), presumably by perturbing both the conformation of the macrocycle and the stereoelectronic profile of the carbonyl.[8] While the relative importance of such changes is not yet well understood, the undesirable conformational effects of thioester replacement conceivably could be minimized by incorporation of non-peptidic units. These strategies are being explored in our laboratory and others; few peptidomimetic inhibitors of AgrC have been reported to date, however, and the field remains largely wide-open.[20]

Several peptidic antagonists have been developed that may provide insight for designing peptidomimetic inhibitors. AIP-II is an attractive target for mimicry, as its truncated, or "tail-less", derivative **1** was observed to exhibit universal cross-inhibition of AgrC function in all four subgroups (shown in Fig. 4a, below).[14] Alanine scans of AIP-II, however, did not yield a cross-group inhibitor superior to the natural peptide, although some potent group I antagonists were discovered.[6] One strategy for improving activity would be to incorporate residues with more hydrophobic character near the *C*-terminus to increase affinity for the hydrophobic pocket. Although relatively non-discriminatory, binding in this pocket is apparently still subject to some steric constraints, as the 9-naphthylalanine derivative of AIP-II (**2**) exhibited significantly reduced inhibitory activity.[15] However,

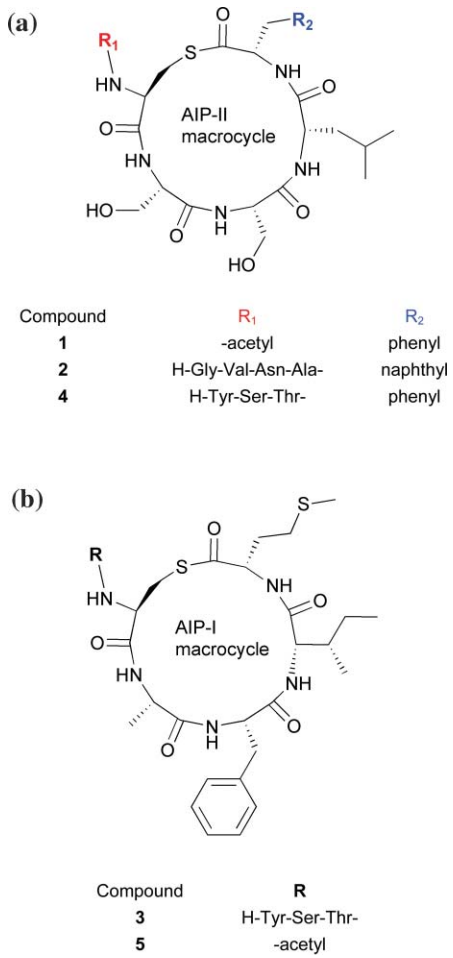


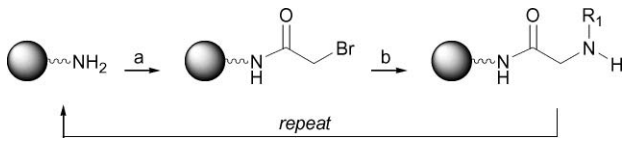
Fig. 4 Structures of designed universal inhibitors of the quorum sensing system in *S. aureus* (**1–5**).

other more compact means of increasing hydrophobicity, such as halogenated side chains, could be exploited in this context.

Williams and co-workers have recently focused their attention on developing analogues of a different AIP—AIP-I.[12] Solution-phase NMR data indicated that substitution of the endocyclic aspartate with alanine (**3**, Fig. 4b) does not significantly affect the conformation of the macrocycle, but instead perturbs the tail relative to the macrocycle such that it facilitates universal antagonism across the *agr* subgroups. Therefore, the researchers proposed that non-native tails could be effectively utilized as components of new inhibitors. A hybrid AIP **4**, in which the AIP-I tail and AIP-II macrocycle were fused, was shown to be significantly more potent than **1** and demonstrated the legitimacy of this concept.[8] Analogously to AIP-II, replacement of the tail in **3** with an acetyl group (**5**) also increased its cross-inhibitory activity, yielding a highly potent cross-inhibitor (IC_{50}: 0.1–5 nM).[7] The tolerance for residue substitution in this macrocycle, coupled with the conformational constraints it enforces, invites the incorporation of peptidomimetic units to generate isosteric, but biostable, adaptations of these inhibitors. *N*-Methylated α-amino acid, β-amino acid, D-amino acid, and peptoid units (see below) are all promising candidates that have been or are currently being investigated within this context.[20]

A role for peptoids

Recent experiments suggest that quorum-sensing inhibition during the first few hours of infection may be crucial for virulence suppression in *S. aureus*.[19] This finding underscores the therapeutic potential of biostable inhibitors that could be developed as "vaccines" to prevent pathogenesis. Moreover, the retention of weak agonistic activity by several lactone and lactam analogues suggests an alternative approach in which virulence is prematurely up regulated, triggering an immune response before the bacterial colony is well-established. The high proteolytic stability of peptoids makes this class of peptidomimetics an attractive candidate for further research in this area.[21] They are also among the more versatile foldamers in terms of their synthetic scope, which continues to expand as demonstrated by our group and others.[22,23] Peptoids are synthesized using the "submonomer" synthesis protocol (Scheme 2), in which a wide variety of amines can be incorporated to generate structural diversity.[24] We believe this flexibility can be utilized to tune uniquely the conformational and stereoelectronic properties of AIP mimics in both rational design and combinatorial formats. Little is currently known about the synthesis and conformational preferences of cyclic peptoid systems. Therefore, the development of peptoid cyclization chemistry would not only benefit quorum sensing research, but would also open the door to a new class of macrocycles that could be exploited across a broad spectrum of peptidomimetic applications.[25,26]



Scheme 2 Submonomer methodology for peptoid synthesis on amine-functionalized solid-support. *Reagents*: a. bromoacetic acid, *N*,*N*'-diisopropylcarbodiimide, DMF. b. primary amine building block NH_2R_1, DMF.

Summary and perspective

In summary, we contend that peptidomimetics are poised to play a leading role in the advancement of *S. aureus* virulence control and will likely pervade the next generation of non-native quorum sensing effectors. Several design strategies have been suggested, including replacement of labile thioester and/or peptide linkages and incorporation of non-natural amino acids.[27] Peptidomimetic systems will facilitate the implementation of these design strategies and permit the straightforward integration of a wide variety of non-natural functionality. This tremendous versatility also entreats the use of combinatorial techniques to further explore the mechanism of AgrC function. Indeed, it has been suggested that several of the more promiscuous AgrC chimeras discussed above could be used to screen for lead compounds in high-throughput assays.[15] Clearly, an understanding of the complex behavior of the AgrC receptor is still in its infancy and will benefit from further fundamental studies involving peptidomimetic–receptor interactions. Quorum sensing control promises to be an exciting therapeutic avenue, and chemists undoubtedly will continue to advance this field.

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