Journal of Medicinal Chemistry

Volume 47, Number 19 September 9, 2004

Miniperspective

Virulence Regulation and Quorum Sensing in Staphylococcal Infections: Competitive AgrC Antagonists as Quorum Sensing Inhibitors§

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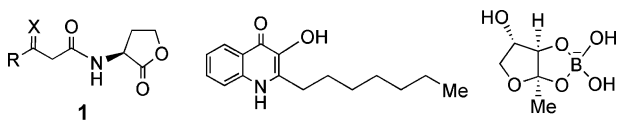
Received March 30, 2004

Introduction

Bacteria have evolved sophisticated mechanisms for cell-to-cell communication, which in response to environmental cues results in the coordinated modulation of gene expression.[1] This primitive multicellular behavioral process, known as quorum sensing, is mediated in a cell population density dependent manner. It utilizes diffusible chemical signal molecules as "words" within the languages of bacterial chemical communication. These low-to-medium molecular weight signal molecules, sometimes referred to as pheromones or autoinducers, regulate the transcription of target genes encoding diverse functions (e.g., pathogenicity and secondary metabolite production). This is achieved by the signal molecule binding to a cognate sensor kinase or response regulator protein.[1] The archetypal example of quorum sensing in Gram-negative bacteria is the regulation of luciferase-encoding genes in the bioluminescent marine bacterium *Vibrio fischeri*.[2,3] However, diverse quorum sensing systems have been extensively characterized in both Gram-negative and Gram-positive bacteria.[1]

In Gram-negative bacteria, e.g., *Aeromonas hydrophila*, *Erwinia carotovora*, *Pseudomonas aeruginosa*, *Yersina enterocolitia*, and *Yersina pseudotuberculosis*, the *N*-acyl-L-homoserine lactones (AHLs, **1**) are the most extensively employed quorum sensing signal molecules. Structurally, the acyl chain in AHLs **1** is of various lengths (C_4−C_{14}), saturation levels, and oxidation states at the C-3. In bacteria, AHLs **1** are usually but not always biosynthesized by a member of the LuxI synthase family and sensed by a member of the LuxR, response regulator protein family of transcription factors, in which the molecular recognition specificity is apparently defined by the chemical architecture of the AHL *N*-acyl chain.[1,4,5] For example, in the human pathogen *P. aeruginosa*, two different AHL−LuxR complexes[6] activate the transcription of genes encoding the LuxI homologues LasI and RhlI and numerous virulence factors,[1,4] including proteins that promote structured biofilm formation.[7] Recently, 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS, **2**)[8] and furanosyl borate diester (AI-2, **3**)[1c,9] have been identified as new Gram-negative quorum sensing signal molecules. Apparently, PQS **2** is a component of the *P. aeruginosa* AHL-dependent quorum sensing hierarchy and plays an important role in co-regulating the expression of virulence factors. Quorum sensing systems are now recognized as novel antimicrobial targets with significant potential for controlling infections including those associated with biofilm formation.[10]



Whereas Gram-negative bacteria employ low molecular weight signal molecules, side chain to tail cyclic

§ This article is dedicated to Professor Barrie W. Bycroft as celebration of his 65th birthday.

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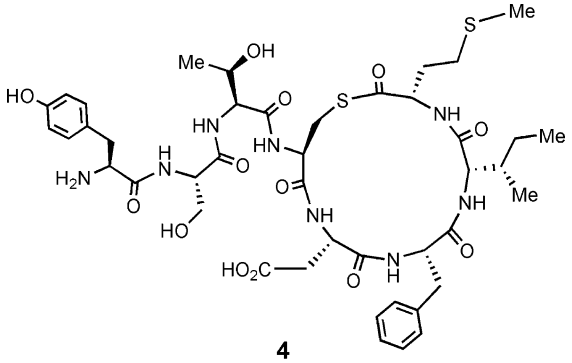
10.1021/jm0400754 CCC: $27.50
Published on Web 07/16/2004



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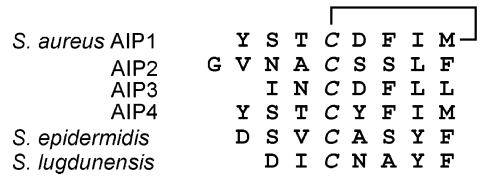


Figure 1. Chemical structure (top) of AIP1 **4** and the schematic structures (bottom) of characterized staphylococcal AIPs.

peptides (ca. 900 molecular weight) are engaged as quorum sensing signal molecules by Gram-positive bacteria such as *Staphylococcus aureus*.[1,11] These autoinducer peptides (AIPs) are typified structurally by a 16-membered side chain to tail macrocyclic peptide to which is attached N-terminally a short linear peptide; an example is the modified octapeptide AIP1 **4** employed by *Staphylococcus aureus*.[11,12] The thiolactone macrocyclic structure is derived from the condensation of a Cys sulfhydryl group to the C-terminal carboxylic acid. To date, several variants of this quorum sensing signal molecule have been reported, and while the primary amino acid sequences for the staphylococcal AIPs are different, they share a common central Cys that is located four residues from the C-terminal amino acid of the processed peptide (Figure 1).[11−13] Functionally, the AIPs are sensed by the two-component signal transduction system (TCSTS) comprising of AgrC, a transmembrane sensor kinase, and AgrA, a response regulatory protein. Interaction of the AIP with its cognate AgrC results in activation of the TCSTS, thus resulting in up-regulation of the agr (accessory gene regulator) mediated quorum sensing system that controls the expression of virulence factors including several exotoxins. In fact, *S. aureus* strains can be divided into four AIP groups (Figure 1) on the basis of their ability to cross-activate or inhibit agr expression.[11,12] The observed inhibitory activities display some degree of selectivity, since a *Staphylococcus epidermidis* AIP can inhibit the agr responses in *S. aureus* groups I−III but not group IV. Conversely, AIP4 (from *S. aureus* group IV) is the only natural staphylococcal peptide capable of inhibiting, albeit weakly, the *S. epidermidis* agr response.[14]

The recent report of clinical isolates of *S. aureus* with high-level resistance to vancomycin (MIC = 1024 μg mL^{-1}!), which was the result of an in vivo interspecies transfer of the van cluster to a multiresistance conjugative plasmid,[15] reinforces the concerns posed to public health by the emergence of *S. aureus* strains resistant to multiple antibacterial agents. These important concerns have stimulated interest in novel chemotherapeutic strategies that attenuate virulence, i.e., the capacity of the bacteria to cause disease, thus facilitating the clearance of the infectious bacterium by the host innate defense mechanisms. In this context, the staphylococcal quorum sensing system employed to control virulence is an attractive target for the design of novel anti-infective agents. We will herein discuss the staphylococcal agr quorum sensing system and analyze the quorum sensing signal molecules and the discovery of AgrC competitive antagonists as quorum sensing inhibitors.

Staphylococcus aureus and *Staphylococcus epidermidis*

S. aureus, one of the most successful opportunistic human Gram-positive pathogens, is responsible for postoperative wound infections, bacteraemia, pneumonia, osteomyelitis, mastitis, acute endocarditis, and deep abscesses in various organs.[16] The organism produces an arsenal of cell surface colonization factors, extracellular material, and exoproteins, some of which are known to contribute to virulence. Cell-wall proteins, e.g., coagulase, protein A, and the fibronectin-binding proteins, that promote adherence to infected tissues are produced early in the exponential phase of growth but are subsequently down-regulated. In contrast, production of most secreted proteins including the exotoxins and tissue-degrading enzymes occurs at the end of exponential growth. Among the *S. aureus* exotoxins are α-toxin, δ-toxin, and toxic shock syndrome toxin (TSST-1), and the staphylococci also secrete proteases, lipases, and hyaluronate lyase. Several elements are involved in the temporal regulation of these virulence factors, and in the context of quorum sensing the most extensively characterized is the agr locus.[11] The agr regulon effectively controls the balance of virulence factor expression during the colonization and invasion phases of the staphylococcal infection. Furthermore, intracellular replication is now considered to play an important role in the persistence of invasive staphylococcal infections by providing protection against host defenses and antibacterial agents. The hosts' endothelial and epithelial cells can act as nonprofessional phagocytes and promote staphylococcal internalization. By use of bovine mammary epithelial cells, it was recently established that induction of the internalized *S. aureus* agr regulon facilitated endosomal escape and replication within the cytoplasm.[17]

In contrast to *S. aureus*, infections caused by *S. epidermidis* are of a less acute and life-threatening nature. *S. epidermidis* has historically been considered as an innocuous commensal bacterium that colonizes the human skin and is therefore part of the normal human microbial flora. However, *S. epidermidis* is now recognized as an important human pathogen and is the predominant cause of infections associated with indwelling medical devices, as well as the primary cause of many nosocomial infections.[18,19] The main virulence mechanism of *S. epidermidis* is its capacity to form biofilms at the site of infections and the chronic nature of such infections.[19] Notably, several processes that contribute to the establishment of staphylococcal biofilms are under the control of the agr-mediated[13] quorum sensing system.[20] Factors that influence biofilm

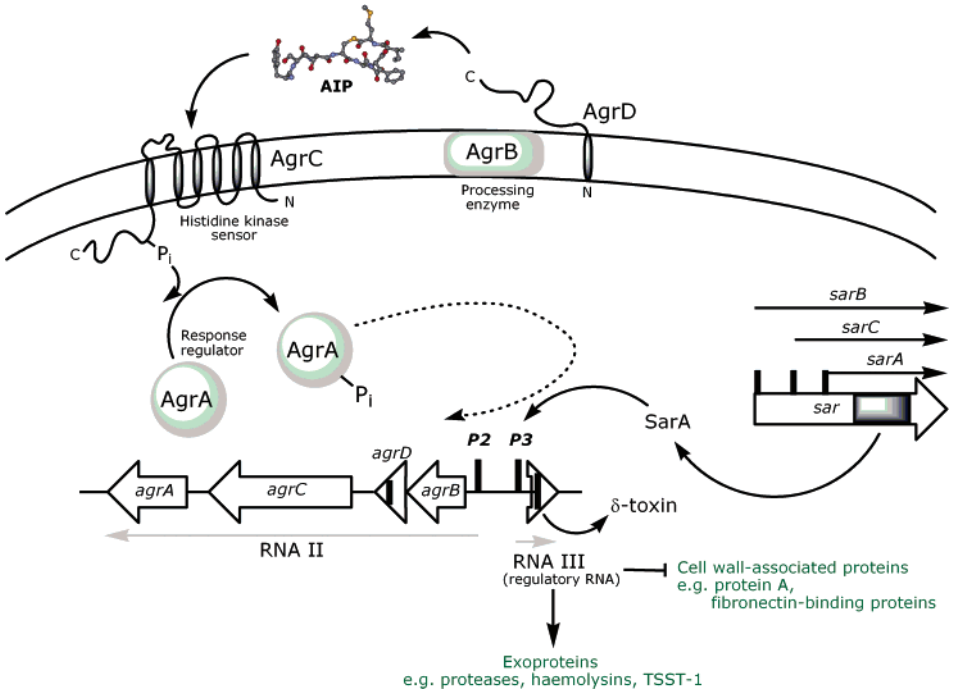


Figure 2. Schematic representation of the staphylococcal quorum sensing system showing the agr locus and its regulatory pathways. Also shown are the translated proteins of the RNAII transcript, as well as the AIP derived from the post-translational modification of AgrD by the membrane-bound AgrB. The AgrC−AgrA forms the crucial agr TCSTS.

formation by *S. epidermidis* will have obvious implications on the therapeutic use of agents that block the staphylococcal quorum sensing system.

The agr Loci in *S. aureus* and *S. epidermidis*

The agr locus or regulon in *S. aureus* consists of two divergent operons, which is transcribed under the control of the promoters P2 and P3 (Figure 2). The P2 transcript RNAII is polycistronic (agrBDCA) comprising the genes that encode proteins of a quorum sensing system[11] that, at threshold concentration of the AIP, activates the transcription of P2 (resulting in signal amplification) and P3-initiating production of the 517-nt transcript RNAIII.[21] Being the effector of the agr system, RNAIII initiates the transcription of genes that encode a variety of exoproteins, e.g. hla (encoding α-haemolysin), saeB (enterotoxin B), tst (TSST-1), ssp and spr (serine proteases), and represses the genes encoding cell surface proteins, e.g., spa (protein A) and fnb (fibronectin-binding protein).[11,12] In addition to its regulatory role, RNAIII is also an mRNA in which an internal segment hld is independently translated into the 26-residue amphipathic peptide *δ*-toxin (also known as *δ*-haemolysin).[11,21] The secondary structure of RNAIII is hypothesized to contain 14 hairpin structures, and the 3′ end centered around the conserved stemloop-13 is evidently important for repression of spa transcription.[21,22] Conversely, the 5′ end of RNAIII appeared to be involved in the transcriptional initiation of hla and ssp.[22]

A functionally analogous agr locus is found in *S. epidermidis*, which showed an overall homology of 68%, and the RNAII transcript is organized in a manner similar to that of the *S. aureus agr* locus.[13,23] It is worth noting that in *S. epidermidis*, the agr locus negatively regulates the transcription of atlE (encoding a cell surface associated autolysin protein that facilitates the primary attachment process in biofilm formation) and up-regulates the expression of hld (*δ*-toxin with detergent-like property, which attenuates the later stages of biofilm formation).[20] *S. epidermidis* agr mutants display high levels of atlE expression throughout growth, resulting in enhanced biofilm formation. In contrast, although *S. aureus* agr mutants showed enhanced biofilm formation, the expression of AltE is apparently unaffected,[24] which reflects subtle differences in the genes regulated by the agr systems of the two staphylococcal species.

The intergenic 120 base pair (bp) region between the agr promoters P2 and P3 is likely to contain the transcription factor binding site(s), which is required for bidirectional activation of both operons. However, the binding of either AgrA or AgrA-PO_4^{3-}, the "end products" of the TCSTS, to the agr promoters or any of the intergenic elements has not been demonstrable; AgrA also does not appear to contain any promoter-binding domain.[11] *S. aureus* was recently shown to possess another global regulatory locus sar in which the translated product SarA contains a putative DNA-binding domain. SarA has the capacity to bind a consensus motif within the agr promoter, resulting in initiation of RNAII and RNAIII transcripts (Figure 2).[25] Hence, it is speculated that AgrA and SarA function in a cooperative manner to mediate transcriptional activation; incidentally, the sar locus and specifically SarA is also capable of regulating directly, in an agr-independent manner, several virulence determinant genes. A recent transcriptional profiling study, which embraces over 86% of the *S. aureus* genome, established that 104 genes were up-regulated and 34 genes were down-regulated in a cell population density and agr-dependent manner.[26] The analysis also revealed that RNAII and RNAIII transcripts were decreased by ca. 2.6-fold in a sar mutant, thus suggesting that SarA is required for

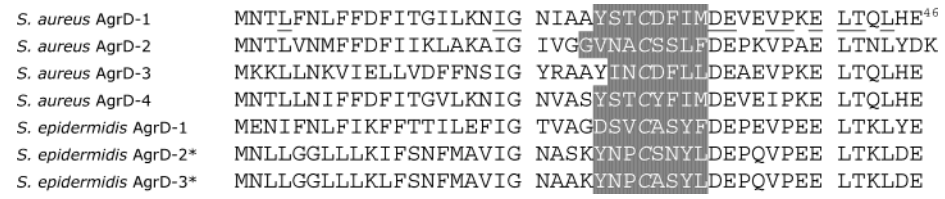


Figure 3. Alignment of the amino acid sequences of the *S. aureus* and *S. epidermidis* AgrDs from the different agr specificity groups. The structural domains are highlighted in gray boxes, which on post-translational modification will afford the AIPs; the central Cys residue that participates in the formation of the crucial thiolactone bond is in italics. Conserved residues are underlined. The asterisk (∗) indicates that the putative structural domain is highlighted and the mature AIP has not been experimentally demonstrated.

wild-type levels of agr transcription; in fact, 56% of the agr-up-regulated genes were similarly affected and are therefore indirectly regulated by SarA.[26]

At the functional level, the AgrB and AgrD proteins are essential for the production of the AIP quorum sensing signal molecule (Figure 2).[11,12] AgrB is responsible for the post-translational processing of AgrD, which involves formation of an intramolecular thioester bond via a putative "activated" peptide−enzyme acyl adduct, followed by an endoproteolytic cleavage.[27] Because of the polymorphic nature of the RNAII transcript,[28] *S. aureus* can be divided into four distinct groups that are characterized by the unique primary sequence of the AIPs derived from the propeptides AgrDs;[11,12,29] the *S. epidermidis* agr introduces a further level of divergence (Figure 3; also see Figure 1).[13,30] Apparently, AgrB is anchored in the cytoplasmic membrane and the hypervariability in two-thirds of its C-terminal domain imparts the discriminatory processing of cognate AgrD.[27,28] The processing of AgrD is facilitated by its N-terminal amphipathic domain-mediated anchoring to the cytoplasmic membrane; the membrane colocalization appeared to be a prerequisite for efficient maturation of the propeptide AgrD.[31]

The AgrA and AgrC proteins constitute a classical TCSTS where AgrC is a histidine kinase sensor, i.e., the receptor for AIP, and AgrA is a response regulator that is phosphorylated by AgrC (Figure 2). The AIP sensor, AgrC, contains several motifs typical of histidine kinases and is predicted to be a transmembrane protein. Functional analyses indicated that AgrC comprises two large domains: an N-terminal sensor domain (ca. 200 residues of a 46 kDa protein) comprising transmembrane helices and extracellular loops; a cytoplasmic C-terminal histidine kinase domain.[32,33] Using transmembrane protein topology prediction programs, others[28,33] and we (using the program located at http://www.cbs.dtu.dk/services/TMHMM-2.0/) propose that the N-terminal region of AgrC comprises of six transmembrane helices. This prediction agrees essentially with a recent PhoA fusion-based analysis,[32] except the analysis suggested that the N-terminus is extracellular; the precise location of the first helix undoubtedly requires further investigation. The binding of cognate AIP to the sensor protein results in N-phosphorylation of a histidine residue in the C-terminal cytoplasmic domain of AgrC, which in turn is predicted to mediate phosphorylation of the AgrA. Although it has previously been proposed that the interaction between AIP and its cognate receptor AgrC is mediated via an irreversible trans-thioesterification reaction,[34] it has now been comprehensively established that the AIP−AgrC interaction is reversible.[12,14,33,35]

To date, not much is known about the structure or the molecular target(s) of AgrA-PO_4^{3-}. In concert with covariation of AgrBD, sequence analysis of AgrCs revealed hypervariable regions in the N-terminal domain of AgrC,[28,29] especially in the hypothesized extracellular loop regions that are responsible for discriminatory recognition of species- and group-specific AIPs. In contrast, staphylococci AgrAs were found to be much more conserved.

Staphylococcal AIPs

The *S. aureus* AIP1 **4** was first reported in 1995, during which a dehydrated macrocyclic structure was proposed,[11] and was subsequently confirmed by total chemical synthesis and ^{1}H NMR analysis.[12,34] The thiolactone peptide **4** showed good chemical stability[11a] but low solubility in aqueous media. Our recent preliminary studies confirmed that AIP1 is relatively stable to many endoproteases, including chymotrypsin, thermolysin, proteinase K, and V8 serine protease. Consistent with the concept of cell population density sensing, a threshold concentration of AIP1 (EC_{50} = 10.2,[34] 19,[12] or 28 nM;[35] the slightly different values are the result of different staphylococci constructs used in functional reporter assays) is required to trigger activation of the cognate AgrC-1 and hence agr-dependent responses in *S. aureus* group I.[11,33] Conversely, the AIP1 **4** is a potent reversible[33] and competitive antagonist of *S. aureus* AgrC-2 (IC_{50} = 3.4[34] or 25 nM[35]) and AgrC-3 (IC_{50} = 3−4 nM[34,35]). Since AIP1 represses agr-mediated responses in other *S. aureus* groups, an interference mechanism was proposed in which the inhibitory effects correlate with the ability of a group-specific strain to compete successfully against other groups at the site of infection.[11] However, recent studies established that although strain replacement was associated with a change in the agr group, cocolonization of mutually inhibiting groups was also observed.[36,37] In fact, the *S. aureus* group I accounts for a major proportion (up to 90%) of the staphylococcal strains found in both hospitalized and healthy individuals.[36−39]

We recently investigated the solution structure of AIP1 in [$^2H_6$]-DMSO using ^{1}H NMR (600 MHz) spectrometry. The unambiguous and complete sequence-specific resonance assignment of the AIP1 **4** was initially achieved by using a series of two-dimensional NMR techniques. By determination of the temperature dependence of the backbone N*H* chemical shifts over the range 288−306 K, relatively low-temperature coefficient values were observed for the backbone amide N*H*s of

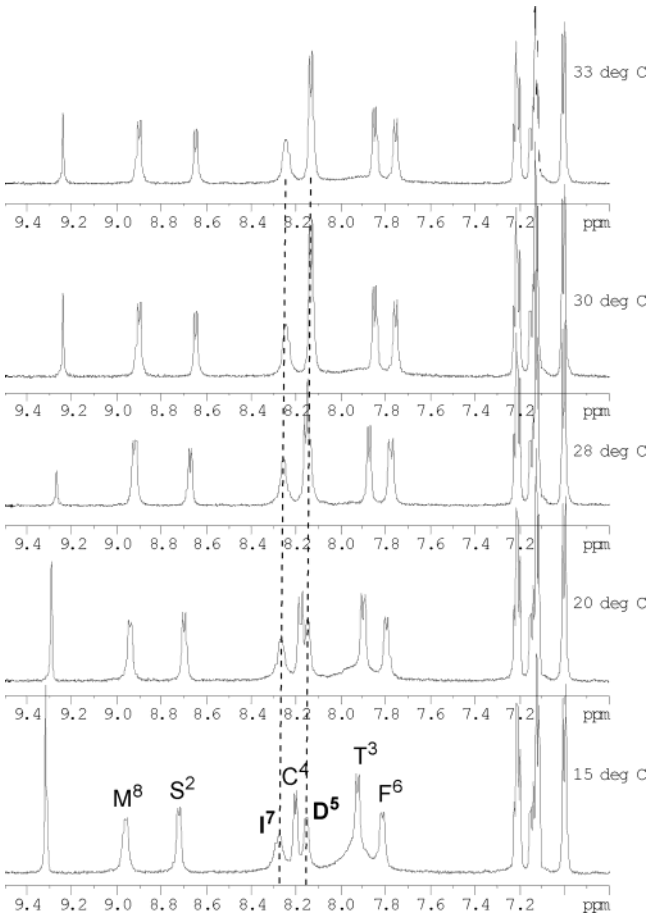


Figure 4. Part of the ^{1}H NMR spectra (600 MHz) of AIP1 in [$^2H_6$]-DMSO at 288−306 K showing the backbone amide regions. The chemical shifts of the highlighted resonance signals assigned to the N*H* of Asp^5 (D^5) and Ile^7 (I^7) were least affected by changes in temperature.

Asp^5 (-1.2×10^{-3} ppm/K) and Ile^7 (-1.5×10^{-3}, cf. others (-3.6 to -4.7) $\times 10^{-3}$ ppm/K) (Figure 4), indicating that these protons are solvent-shielded or involved in intramolecular H bonds. We propose that the 16-membered macrocyclic region of AIP1 adopts a somewhat constrained conformation, while the exocyclic tripeptide (i.e., the "tail") region is "unstructured" and solvent-exposed.

Structure−activity studies of AIP1 **4** established that an intact macrocyclic structure is essential for binding to AgrC and that the macrocyclic is the "address" region required for molecular recognition and the exocyclic tripeptide is the "message" region necessary for receptor activation.[12] In addition to a defined topology imposed by the thiolactone ring, structural elements within the endocyclic segment Phe^{6}Ile^{7}Met^8 make significant contributions to AgrC-1 binding, since removal of any of the side chain functional groups resulted in profound reduction in activity (i.e., (Ala^6)AIP1, (Ala^7)AIP1, and (Ala^8)AIP1 displayed $EC_{50} = 9-11\ \mu M$).[12] Unexpectedly, the methionyl sulfoxide-8 analogue showed no binding activity to AgrC-1, even at high micromolar concentrations. The D isomers are tolerated for both Phe^6 and Met^8, which suggests that the complementary hydrophobic pocket in AgrC-1 is presumably large enough to accommodate different stereo-orientations.[12] The identification of the competitive antagonist, *N*-acetyl-(*des*-Tyr^{1}Ser^{2}Thr^3)AIP1 provided further support for the hypothesis that the macrocyclic domain is the "address" region of the ligand.

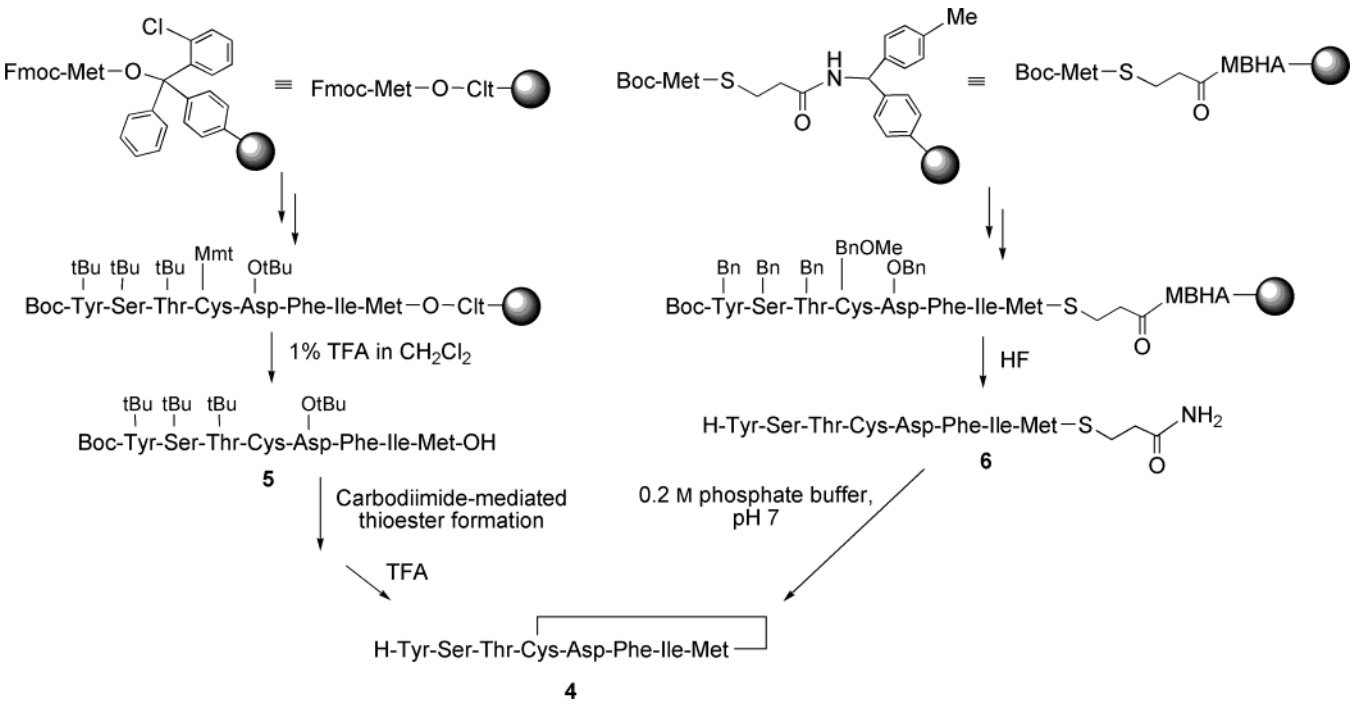
Structurally, AIP2 comprises the critical macrocyclic pharmacophore to which is appended an exocyclic tetrapeptide "tail" (see Figure 1). The autoinducer peptide is a potent agonist of self-AgrC-2 ($EC_{50} = 3.6$[34] or 30 nM[35]) and competitive antagonist of *S. aureus* AgrC-1 ($IC_{50} = 2.9$[34] or 40 nM[35]), AgrC-3 ($IC_{50} = 3.2$[34] or 1 nM[35]), and AgrC-4 ($IC_{50} = 86$ nM[35]); like AIP1, the AIP2 showed no demonstrable activity against *S. epidermidis* AgrC.[14b] Detailed structure−activity relationship studies established that the AIP2 interacts with its cognate receptor AgrC-2 in a manner similar to that observed for the AIP1−AgrC-1 interaction. For example, systematic replacement of the endocyclic Leu^8 and Phe^9 (equivalent to positions 7 and 8 in AIP1) with Ala resulted in inactive analogues, either as agonist of AgrC-2 or as antagonist of AgrC-1.[34] It is also worth noting that the lactam analogues, derived from the macrocyclization involving an *S*-2,3-diaminopropionyl residue instead of Cys, of both AIP1 and AIP2 displayed very weak agonism of self-AgrC-1 and AgrC-2 ($EC_{50} > 5\ \mu M$);[12,33] presumably, the low activity is the consequence of significant perturbation of the macrocyclic conformation.

The *S. aureus* groups III and IV are minor strains and are generally but not exclusively associated with menstrual toxic shock (mediated by TSST-1)[39] and exfoliative (a scalded-skin condition mediated by the exfoliatin toxin)[12,29,40] syndromes, respectively. As anticipated, the *S. aureus* AIP3[41] is an activator of self-AgrC-3 ($EC_{50} = 26$ nM) but competitively inhibits AgrC-1 ($IC_{50} = 70$ nM), AgrC-2 ($IC_{50} = 6$ nM), and AgrC-4 ($IC_{50} = 150$ nM);[35] in fact, the chemical structure of AIP3 was only recently established by multistage "matrix-assisted laser desorption ionization (MALDI) ion trap" mass spectrometry,[41] which undoubtedly is an invaluable tool for identifying new naturally occurring AIP variants. In line with the "message" role of the exocyclic "tail", the length of the exocyclic peptide (based on the propeptide AgrD-3) profoundly affects activation of cognate AgrC-3.[35] The signal molecule AIP4 (AgrC-4 $EC_{50} = 13$ nM)[35] differs from AIP1 by one amino acid residue, in which the Asp^5 (in AIP1) is replaced with Tyr^5 residue,[12,29] and is a potent blocker of AgrC-2 ($IC_{50} = 4$ nM) and AgrC-3 ($IC_{50} = 1$ nM).[35] However, the activity of AIP4 toward AgrC-1 in *S. aureus* group I is controversial; it has been reported as a weak inhibitor[12,37] ($IC_{50} = 7\ \mu M$[12]), as well as an activator.[29,34]

Among the AIPs from *S. aureus*, AIP4 is the only ligand that displays weak inhibition of the *S. epidermidis* agr system. Moreover, qualitative studies suggest that the *S. epidermidis* AIP is a weak inhibitor of *S. aureus* AgrC-1 and -2 ($IC_{50} \approx 300$ nM) but apparently a potent antagonist of AgrC-3 ($IC_{50} < 25$ nM).[14]

The observed inhibitory effects of native staphylococcal AIPs on specific agr systems are reflected at the cellular level. For example, near-complete attenuation of acute subcutaneous infections in mice were demonstrated when *S. aureus* group I was coadministered with AIP2 (5.6 nmol, i.e., total body concentration of ca. 0.2 μM for a 25 g mouse).[34] Furthermore, the *S. epidermidis* AIP (at final concentration of 0.1 μM), because of its capacity to inhibit the agr system, promotes biofilm

Scheme 1. Summary of the Two Main Strategies Used for the Chemical Synthesis of Side Chain to Tail Thiolactone Peptides[a]



[a] The overall synthetic strategies, based on either Fmoc/tBu (left) or Boc/Bn (right) chemistry, are illustrated using AIP1 as the example of a desired thiolactone peptide.

formation by *S. aureus* group I on polystyrene.[24] Similarly, biofilm formation by *S. epidermidis* is enhanced by AIP4 (0.1 μM) but unaffected by AIP1 (up to 1.0 μM).[20] These observations indicate that AgrC antagonists are potentially useful in the treatment of acute staphylococcal infections but are likely to be detrimental in most biofilm-associated/chronic infections (including but not limited to infections associated with in-dwelling medical devices).

Solid-Phase Strategies for the Chemical Synthesis of AIPs

Scheme 1 outlines the two main solid-phase approaches for the total chemical synthesis of side chain to tail thiolactone peptides. Both strategies, based on either Fmoc/tBu (Scheme 1, left) or Boc/Bn (Scheme 1, right) chemistry, have been used successfully for the synthesis of native AIPs and analogues thereof and should provide synthetic access to new AIP molecules.

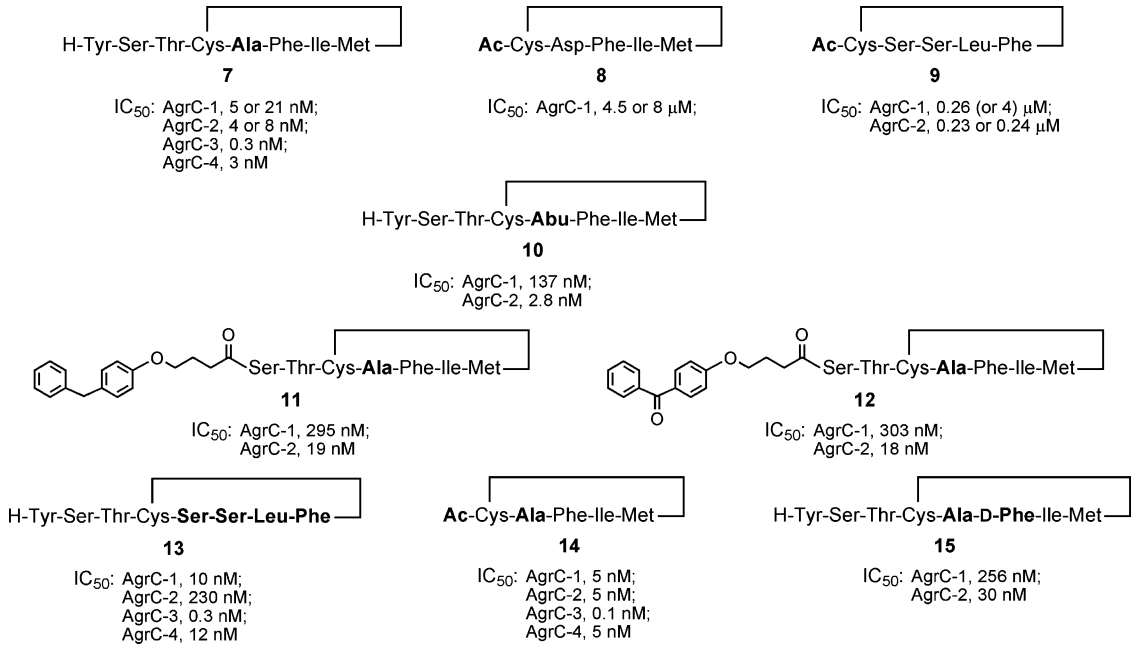
In the Fmoc/tBu strategy (Scheme 1, left), the hyperlability of *S*-4-methoxytrityl and 2-chlorotrityl(polystyrene) ester moieties to acidolysis (1% v/v TFA in CH_2Cl_2) was exploited for concomitant chemoselective unmasking of the Cys sulfhydryl group and release from the solid support. The partially protected peptide **5** was then macrocyclized using a dialkylcarbodiimide reagent, followed by TFA-mediated global deprotection to afford the desired thiolactone peptide.[12−14,42] We have recently reported a variant of this strategy, which is based on a branched approach.[42] For the Boc/Bn strategy (Scheme 1, right), the C-terminal Boc-protected methionine was used initially to acylate the tethered 3-mercaptopropionic acid. Peptide assembly was then carried out using the standard Merrifield Boc/Bn methodology, followed by HF-mediated global deprotection and release of the modified peptide thioester **6**. Macrocyclization was achieved, in aqueous buffer at pH 7, via an intramolecular trans-thioesterification to yield the required thiolactone peptide **4**.[35] The latter synthetic strategy is likely to be more robust than the Fmoc/tBu-based strategy, since the macrocyclization step utilizes linear precursors that do not contain steric-hindering side chain protecting groups. In this context, recent developments in the Fmoc/tBu solid-phase synthesis of globally deprotected peptide thioesters[43] could be exploited for the routine chemical synthesis of AIPs.

New AgrC Antagonists as Quorum Sensing Inhibitors

During the course of structure−activity studies on AIP1 and AIP2, a number of unexpected observations were found to be useful pointers for the design of new AgrC antagonists. These are the following. (i) The endocyclic C-terminal segments Phe[6]Ile[7] and Leu[8]Phe[9] in the octapeptide AIP1 and nonapeptide AIP2, respectively, make crucial interactions with the AgrC.[12,34] (ii) Replacement of the endocyclic amino acid residue adjacent to Cys (i.e., Asp[5] in AIP1 and Ser[6] in AIP2) with an Ala residue resulted in potent competitive AgrC antagonists;[12,34,35] in fact, (Ala[5])AIP1 **7** (Chart 1) was found to be a potent inhibitor of all *S. aureus* AgrCs at $IC_{50} = 0.3-21$ nM.[12,35,42] (iii) The truncated analogues, lacking the exocyclic "tail", *N*-acetyl-(*des*-TyrSerThr)-AIP1 **8** and *N*-acetyl-(*des*-GlyValAsnAla)AIP2 **9** are weak antagonists of AgrC-1 ($IC_{50} = 4-8$ μM[12]) and AgrC-2 ($IC_{50} = 244$ nM[33]); apparently, although the exocyclic "tail" is important for AgrC activation, elements within the "tail" increase the affinity of the macrocyclic "address" region for the receptor AgrC.

Our recent ^{1}H NMR analysis of the global antagonist (Ala[5])AIP1 **7** revealed the noticeable presence of two well-defined resonance signals at δ 4.95 (doublet) and

Chart 1



5.24 (triplet), which are assigned to the Thr[3] CβO*H* and Ser[2]CβO*H*, respectively, in the exocyclic region. This suggests that these hydroxyl functionalities are solvent-shielded and that the protons are exchanging at a very slow rate, if at all. In contrast, the corresponding CβO*H*s in native AIP1 were observed as broad singlets. Determination of the temperature coefficient for the backbone amide N*H*s confirmed that the macrocyclic regions in (Ala[5])AIP1 **7** (the Ala[5] and Ile[7] display comparatively lower $\Delta\,\delta T^{-1}$ values) and AIP1 **4** are conformationally similar. We therefore propose that replacement of the Asp[5] in AIP1 with hydrophobic residues such as Ala results in insignificant changes in the conformation of the macrocyclic region but facilitated decisive changes in the orientation of the "tail" with respect to the macrocyclic and that the exocyclic "tail" contributes to an increased affinity to *S. aureus* AgrC.

A limited number of analogues have been evaluated, which are based on (Ala[5])AIP1 **7** as the starting template. The hydrophobicity and steric constraints imposed by the Ala[5] residue appeared to be optimal for AgrC-1 interaction, since its replacement with either Gly or Abu had detrimental effects.[12,42] However, the Abu[5] analogue **10** seemed to show marginally improved affinity for AgrC-2 (IC_{50} = 2.8 nM).[42] Similarly, when the Tyr[1] residue is replaced by *des*-aminotyrosine surrogates, the analogues **11** and **12** displayed a decrease in inhibitory activity against AgrC-1 but retained excellent antagonistic properties against the *S. aureus* AgrC-2 (IC_{50} = 18−19 nM).[42] Since the analogue **12** contains a photophore tag (the alkoxybenzophenone moiety) and showed good-to-excellent binding affinity to both AgrC-1 and -2, the AIP analogue could be used to obtain a detailed map of the AgrC ligand-binding site(s). Another noteworthy analogue is the chimeric peptide AIP1^{N}/2^{C} **13**, in which the exocyclic "tail" from AIP1 is fused to the macrocyclic domain of AIP2. The chimera AIP1^{N}/2^{C} **13** is a potent antagonist of all four *S. aureus* AgrCs (its potencies exceed those displayed by the truncated AIP2 **9** (comprising essentially the thiolactone)[35]), thus providing further support that an exocyclic "tail" can be exploited to increase the binding affinity to AgrC.

Remarkably, the analogue *N*-acetyl-(Ala[5],*des*-(Tyr-SerThr)AIP1 **14** is an effective antagonist against all *S. aureus* AgrCs (IC_{50} = 0.1−5 nM) at levels comparable to that of its parent derivative (Ala[5])AIP1 **7**.[35] It is likely that the thiolactone peptide **14** (molecular weight 607) represents the minimum structure for effective binding to AgrC. In recent developments, we established that the D-Phe-containing analogue **15** retains, though somewhat lowered, binding potency toward AgrC-2.

Conclusions

With the increasing prevalence of multiantibiotic resistance in *S. aureus*, development of new therapeutic strategies is of paramount importance. In this context, the staphylococcal quorum sensing system offers a unique and exciting prospect for the development of novel anti-infective agents that have the capacity for attenuating virulence and that circumvent the resistance mechanisms that protect *S. aureus* from conventional antibiotics.[10−12] Although there are at least 17 putative TCSTS in *S. aureus*, the agr TCSTS (AgrC−AgrA) is the central component of a true quorum sensing system and plays a major role in the pathogenesis of staphylococcal infections. Furthermore, the agr TCSTS in *S. epidermidis* and to a lesser extent that in *S. aureus* are likely to influence the chronic nature of biofilm-associated infections. Recently, a number of potent competitive AgrC antagonists, i.e., agr TCSTS inhibitors, have been reported.[11,12,33−35,42] We anticipate that these initial structure−activity studies will provide an invaluable platform for the development of both selective and global inhibitors of *S. aureus* and *S. epidermidis* agr quorum sensing systems. The effectiveness of these antagonists will obviously require future studies using appropriate experimental animal infection models.

Biographies

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Barry J. Coyle received his B.Sc. in Chemistry and Biology (2000) and his Ph.D. in Chemistry (2004) from National University of Ireland Maynooth. He is currently conducting postdoctoral studies under the guidance of Weng Chan and Paul Williams. His research interests are synthetic, structural, and biological studies of new transition metal complexes and quorum sensing autoinducer molecules.

Paul Williams (B.Pharm., Ph.D.) is Professor of Molecular Microbiology and is the Director of the Institute of Infection, Immunity & Inflammation. He is internationally known for his research in bacterial quorum sensing and has (co)authored over 180 research and review articles, book chapters, and patents. His current research interests focus primarily on the regulation of gene expression through cell−cell communication (quorum sensing) in pathogenic bacteria with the aim of identifying new targets for novel anti-infective agents. He was awarded the Royal Pharmaceutical Society of Great Britain's Conference Science Medal in 1992 and the Pfizer prize in Pharmaceutical Sciences in 1994.

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JM0400754