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Review: Leonidas Zervas Award Lecture 2002‡

Turning Virulence on and off in *Staphylococci*

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Abstract: The progress made in a multidisciplinary research programme designed to elucidate the molecular basis of the interaction of *Staphylococcus aureus* secreted autoinducing peptides (AIPs) with their respective cell surface receptors is reviewed. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antibacterial agents; *S. aureus*; autoinducing peptides; AIPs

Staphylococcus aureus is one of the major nosocomial pathogens that are now presenting an increasing risk of developing resistance to all currently available therapeutics. Consequently, there is a pressing need to identify new types of antibacterial agents and it has been suggested that interference with the expression of virulence may represent a promising antibacterial modality [1,2]. It appears that *S. aureus* is a very good candidate for such an approach because it uses a global regulator, *agr*, activated by secreted autoinducing peptides (AIPs), to control the expression of most virulence genes [3,4]. These genes include the various secreted cytotoxic proteins and tissue degrading enzymes (e.g. toxic shock syndrome toxin-1 and α-, β- and δ-hemolysins) associated with the onset of disease [5]. In collaboration with Professor Richard P. Novick (New York University Medical Center), a multidisciplinary research programme was designed to elucidate the molecular basis of the interaction of these AIPs with their respective cell surface receptors. In this article, which is based on the Leonidas Zervas lecture given at the 2002 EPS in Sorrento, the progress made in this area over the past several years is reviewed.

The expression of most virulence genes in *Staphylococci* is under the control of the *agr* (accessory gene regulator) global regulon. The *agr* locus contains two divergent promoters, P2 and P3. There are four genes, *agr*A–D, in the P2 operon that code for the cytosolic, transmembrane and extracellular components of a density-sensing/autoinduction circuit [4] (see Figure 1). The *agr*D gene product is a ~50 aa pro-peptide that is processed and secreted through AgrB, an integral membrane protein. The resultant mature autoinducing peptide (AIP) is thought to bind to a polytopic receptor histidine kinase (RHK) coded by *agr*C, triggering a classical two-component signalling pathway [6] (Figure 1). Thus, binding the AIP leads to autophosphorylation of AgrC on a histidine residue [7], followed by trans-phosphorylation of the *agr*A gene product. Phosphorylated AgrA is assumed to activate transcription from the *agr* P2/P3 promoters [4,7]. The RNA transcript from the P3 promoter is responsible for the up-regulation of secreted virulence factors as well as the down-regulation of surface proteins [3,8]. The *agr* response is an example of quorum sensing since cell density-dependent accumulation of the AIP triggers AgrC activation.

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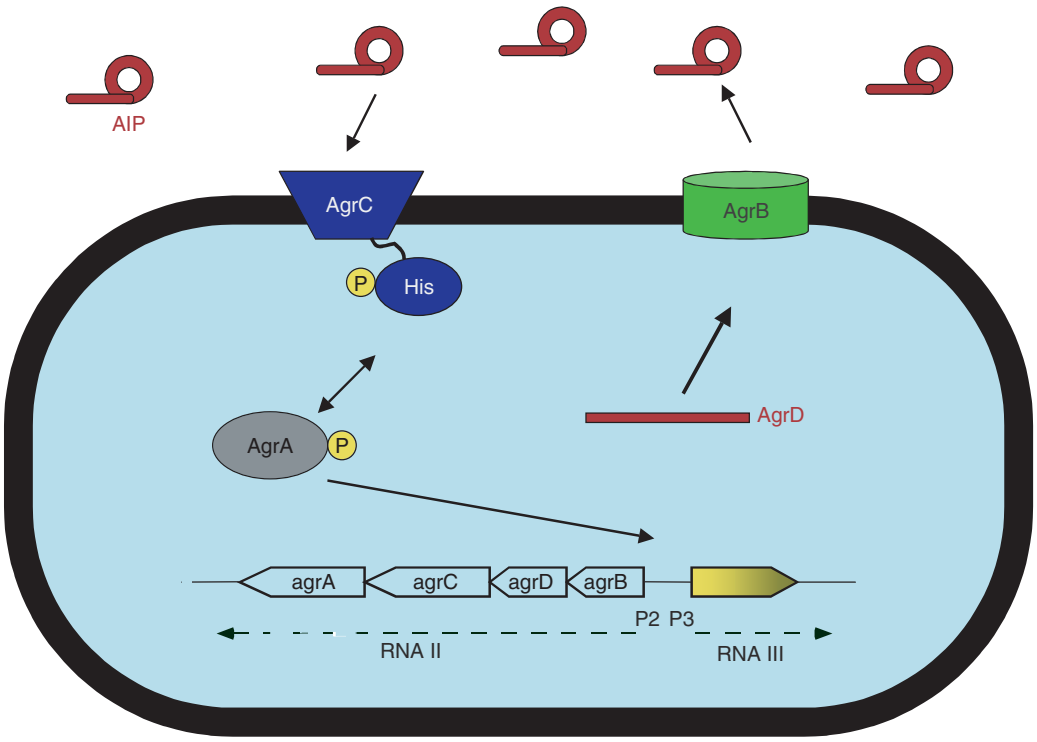

Figure 1 The *agr* autoinduction circuit in *S. aureus*.

BIOGRAPHY

Tom Muir was born in Stranraer in Scotland in 1967, and graduated BSc from the University of Edinburgh with First Class Honours in 1989. He was awarded the PhD Degree from the same University, where he was associated with Professor Robert Ramage FRS, in 1993. After postdoctoral work at the Scripps Research Institute, he was appointed to the faculty of the Rockefeller University New York, where he has been a Full Professor since 2002. He already has over 60 papers, half a dozen patents, and a dozen book contributions to his credit, and in the three years to 2002 gave around 50 invited lectures in the USA, Australia and Europe.

The sequence of the AIPs is highly variable (Table 1), resulting in at least four specificity groups of strains within *S. aureus* and many more (>25) in other staphylococci [6–11]. A group is defined by the fact that all strains within the group produce the same AIP. The *agrB*, *D* and *C* coding regions vary in concert to maintain the specificity of AIP processing and function [6]. This specificity results in four different receptors for the AIPs in *S. aureus*, designated AgrC-I, -II, -III and -IV, reflecting the group that expresses them. Remarkably, there is extensive crosstalk at the level of ligand-mediated signalling, as most AIPs activate their native receptor while competitively inhibiting activation of non-native receptors [6]. This inhibition is a form of bacterial interference that does not result in growth inhibition, but rather in the block of accessory gene functions, presumably resulting in an advantage for the strain producing the most abundant and/or the most potent AIP.

A combination of genetics, molecular pharmacology and chemical synthesis was used to study the mechanism of both activation and inhibition of the *agr* response by the AIPs. This led to a number of important insights. In one set of studies, a 'receptor-swapping' strategy (i.e. moving group-specific AgrCs into different genetic backgrounds) was used to demonstrate that AgrC and the AIPs are the only group-specific components of the system required for *agr* activation and inhibition [12]. In related studies, chimeric receptor constructs were then used to show that agonist and antagonist AIPs both bind to the sensor domain of AgrC that corresponds to the *N*-terminal polytopic integral membrane component

Table 1 Lineup of AgrD Propeptide Sequences from Various Staphylococci. The predicted sequences of the mature AIPs are shown in bold. Conserved residues are in red

SaureusI	MNTLFNLFFDFITGILKNIGNIAA	**YSTCDFIM** DEVEVPKELTQLHE-------
SaurII	MNTLVNMFFDFIIKLAKAIGIVG	**GVNACSSLF** DEPKVPAELTNLYDK------
SaurIII	MKKLLNKVIELLVDFFNSIGYRAAY	**INCDFLL** DEAEVPKELTQLHE-------
SaurIV	MNTLLNIFFDFITGVLKNIGNVAS	**YSTCYFIM** DEVEVPKELTQLHE-------
SlugdI	MNLLSGLFTKGISAIFEFIGNFSAQ	**DICNAYF** DEPEVPQELIDLQRK------
SlugdII	MNLLSGLFTKGISVIFEFIGNFSVQ	**DMCNGYF** DEPEVPQELIDLHRN------
SarlettaeI	MNLLNSFFSFFAKKFFELIGTVAG	**VNPCGGWF** DEPEVPEELTKYSE-------
SauricI	MMKLVNLLLSSTTSILQMVGNRQK	**AKTCTVLY** DEPEV-KELTQELEK------
SauricII	MMK-DNLLLSSTTSILQMVGNRSK	**TKTCTVLY** DEPEV-KELIQELEK------
ScapitisI	MIMNSLFNLIFKFFTVIFEFIGFVAG	**ANPCQLYY** DEPEVPEELSKLYE-------
ScapII	MIMDALFNLIFKFFTVIFEFIGFVAG	**ANPCALYY** DEPEVPDELSKLYE-------
ScapraeI	MMQIINLLFKVITAVFEKIGFIAG	**YSTCSYYF** DEPEVPKELLEIYKK------
ScaprII	MKMMQIFDLLFKVISAVFEKIGFLAG	**YRTCNTYF** DEPEVPKELFETYQK------
Scarnosus	MNFNMDILNGIFKFFAFIFEQIGNIAK	**YNPCVGYF** DEPEVPSELLDEQK-------
Sconc	MHIFESIINLFVKFFSVLGAISG	**GKVCSAYF** DEPEVPKEIKDLYK-------
Sconu	MNIFESIINLFAKFFAFIGTISS	**VKPCTGFA** DEPEIPKELTDLYK-------
SepiI	MNLLGGLLLKLFSNFMAVIGSAAK	**YNPCASYL** DEPQVPEELTKLDE-------
SepiII	MEIIFNLFIKFFTTILEFIGTVAG	**DSVCASYF** DEPEVPEELTKLYE-------
SepiIII	MNKLLGGLLLKIFSNFMAVIGNASK	**YNPCSNYL** DEPQVLPEELTKLDE------
SepiIV	MNKLLGGLLLKIFSNFMAVIGNAAK	**YNPCANYL** DEPQVLPEELTKLDE------

of the protein [13] (Figure 2). Based on a variety of pharmacological studies, including Schild-type analyses, it is believed that the binding of both agonist and antagonist AIPs to AgrC is reversible and competitive. However, the agonist–antagonist interaction is probably not a simple competitive one and instead it may involve negative cooperativity between binding sites in the presumed AgrC receptor dimer [13]. Moreover, agonist and antagonist AIPs share an overlapping binding site within the sensor domain of AgrC, but they clearly bind in subtly different orientations within the binding pocket [14].

Using the chimeric AgrC receptors fused within the sensor domain, the AIP binding site within the receptor is being defined. Specifically, the macrocyclic component of the AIP has been shown to interact with a region of the sensor domain proximal to the histidine kinase domain [15]. Analysis of the pharmacology of these chimeras indicates that a hydrophobic language underlies cross-communication between different receptors and the agonist or antagonist AIPs. This has led to a two-stage model for receptor activation in which there is an initial binding step, involving predominantly non-polar interactions between AgrC and the AIP, followed by an isomerization step leading to signal transduction. In this model, the first step would be common to both the agonist and antagonist AIPs, whereas the second step would be

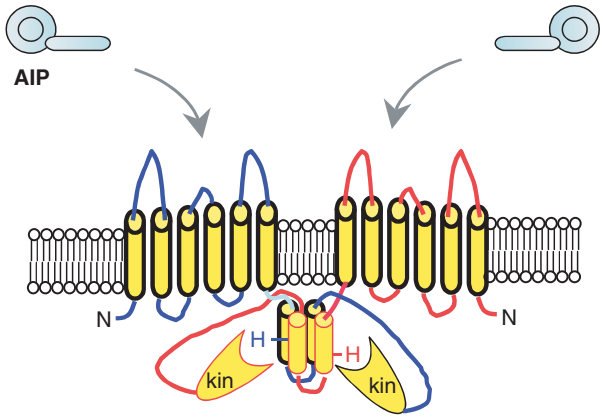

Figure 2 Schematic of the domain architecture of the receptor histidine kinase (RHK) AgrC. Topological mapping and biochemical analysis suggest that the sensor domain consists of five or six transmembrane helices, depending on whether the *N*-terminus is placed on the inside or the outside of the cell [7]. Mutagenesis studies indicate that the AIP binding site is located within the sensor domain [13–15]. Homology modelling and biochemical analyses indicate that the cytoplasmic region of the protein is composed of a *C*-terminal kinase domain (kin) and a helical linker domain, which contains the histidine phospho-acceptor [16]. Analogous to other RHKs [17], AgrC may exist as a pre-formed homo-dimer held together, at least in part, by the formation of a four-helix bundle structure involving the linker regions from each monomer.

group-specific and would most likely involve polar interactions [16].

In parallel with the molecular biology/molecular pharmacology studies summarized above, extensive structure–activity relationship (SAR) studies have been performed on the AIPs. Initially the focus was to characterize fully the chemical structure of the mature AIP. Previous studies performed in the Novick laboratory had suggested that the AIPs might contain an intramolecular thioester linkage between the cysteine sulfhydryl and the *C*-terminus [2,6]. Specifically, mass spectrometry analysis of a partially purified *S. aureus* group I AIP revealed a molecular weight that was exactly 18 Da lower than an octapeptide fragment of the group I AgrD pro-peptide (Table 1, sequence in bold). Since the only conserved residue within this region of the AgrD family is a cysteine, it was postulated that the mature AIP contains the thiolactone linkage illustrated in Figure 3.

A combination of chemical synthesis and *in vitro* and *in vivo* biological assays was used to establish that the mature AIPs contain a thiolactone structure [18]. A solid-phase synthetic strategy was developed that allowed these peptides to be generated in highly homogenous form and in excellent yield (see Figure 4). Key to this approach was the use of an acid-stable mercaptopropionamide-PEGA resin linker [19]. Following chain assembly using a Boc-N^{α} protection strategy, treatment with anhydrous HF afforded the unprotected peptide still linked to the resin via a thioester bond. Simply swelling this resin in aqueous buffer at ~pH 7 triggered a chemoselective ligation reaction and concomitant cleavage of the peptide from the support [18]. A similar type of approach can be used to cyclize the peptide in solution, in this case the linear peptide α-thioester is cleaved from the support prior to the ligation reaction [14]. As illustrated in Figure 4B, this intramolecular ligation reaction is extremely facile, perhaps as a result of structural properties in the linear peptide.

The availability of large quantities of these molecules has allowed the AIP/AgrC interaction to be studied in *S. aureus* cells using a transcriptional reporter assay [2,12]. Briefly, *agr*-null *S. aureus* cells are transfected with a plasmid encoding the AgrC/AgrA two-component system driven by the endogenous *agr* P2 promoter, and β-lactamase driven by the agr P3 promoter. Thus, activation of the AgrC-AgrA system leads to production of β-lactamase, which can be assayed colorimetrically. The use of this assay has revealed that the ED_{50} and IC_{50} values for activation and inhibition of the *agr* response, respectively, by the various AIPs are all in the low nanomolar range [14,18]. Moreover, a synthetic AIP was found to attenuate dramatically the spread of a subcutaneous abscess in *S. aureus* infected mice, thus confirming that the synthetic AIP can inhibit the *agr* response in an *in vivo* infection system [18].

A combination of chemical synthesis and structural and biological analyses has been used to study the structure–activity relationships (SAR) within the AIPs. This integrated approach has revealed some of the structural features important for the activation and inhibition activities of the AIPs (summarized in Figures 3 and 5), and has paved the way to the

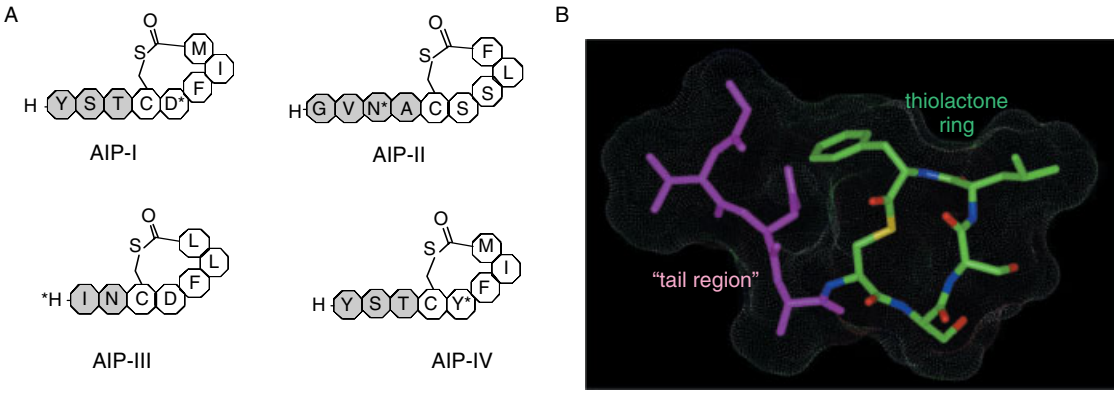

Figure 3 Stucture of the autoinducing peptides (AIPs). (A) Amino acid sequence of the *S. aureus* group I–IV AIPs [13,18]. Exocyclic (tail) and endocyclic (ring) residues are represented in shaded and open circles, respectively. Residues that are critical for receptor activation are marked with an asterisk (see text for details). (B) Energy minimized structural model of the *S. aureus* group II AIP with Connolly surface shown. The AIPs consist of a flexible 'tail region' and a constrained 16-membered thiolactone ring.

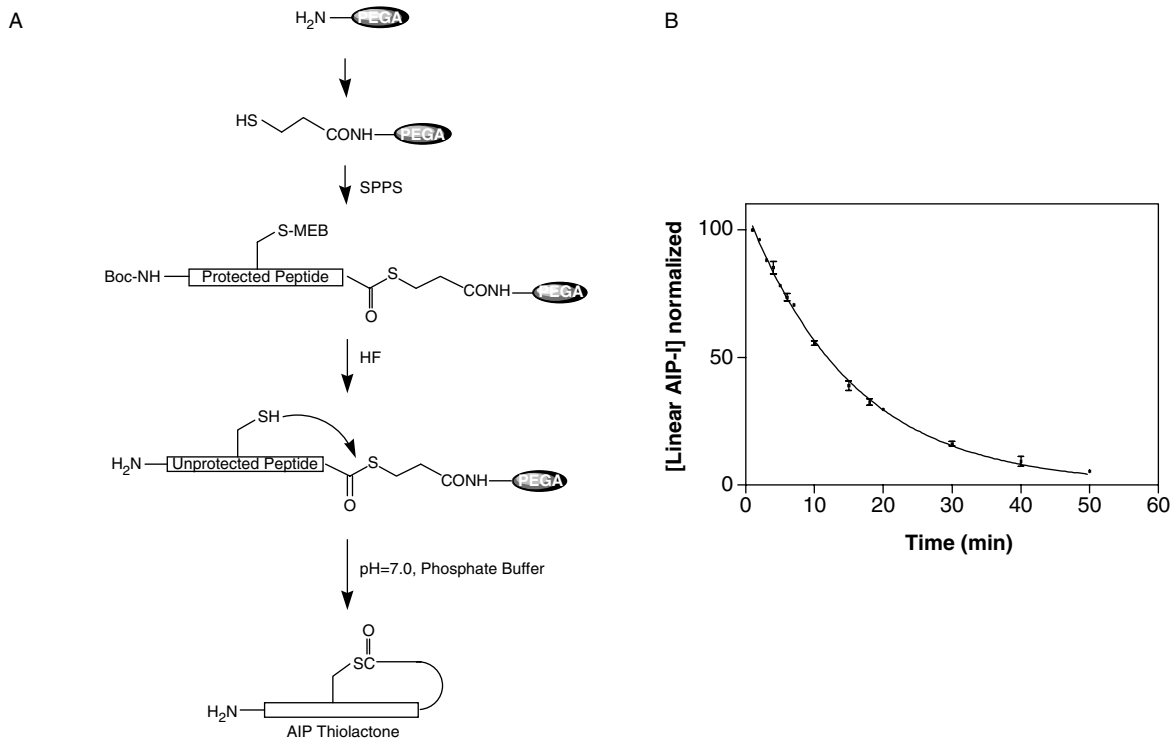

Figure 4 Synthesis of AIPs. (A) Solid-phase intramolecular chemical ligation strategy used to prepare AIPs. (B) Kinetics of thiolactone formation are extremely rapid. Shown is a plot of starting material consumption versus time for the cyclization of linear AIP-I in aqueous solution at pH 7. The half-life for this first order reaction is ~11 min.

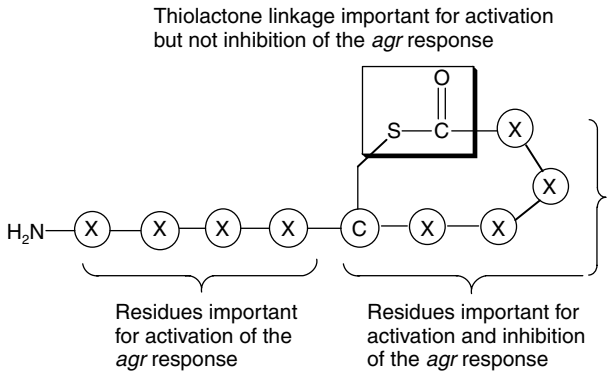

Figure 5 Summary of AIP-II SAR data.

rational design of global inhibitors of *S. aureus* virulence (Figure 7).

Our initial SAR studies involved scanning an alanine residue through the AIP-II sequence [18]. Perhaps surprisingly, this study revealed a rather simple partitioning of function within the molecule. Specifically, the residues in the tail region of AIP-II appeared to be important for the agonistic activity, but not antagonism, whereas residues in the macrocyclic part of the molecule appeared to play a role in both agonism and antagonism. This exocyclic–endocyclic delineation also extends to the structural properties of the molecule [14]. Two-dimensional NMR studies of AIP-II and the alanine-substituted analogues revealed that the two regions of the molecule are structurally independent; modifications in the ring do not affect the chemical shifts of residues in the tail and *vice versa* (Figure 6A). This is further underlined by studies of a truncated AIP-II analogue lacking the entire tail region [14]. In this case, removal of the tail had no effect on the chemical shifts of residues in the macrocycle (Figure 6B).

The macrocyclic structure in the AIP is critical for biological function. Linear versions of the AIPs neither activate nor inhibit the *agr* response, even at extremely high concentrations [9,18]. Moreover, the nature of the linkage forming the 16-membered macrocycle, comprising a thioester bond, is critical for certain aspects of biological activity, as the lactam and lactone analogues of AIP-I and AIP-II are potent cross-group inhibitors but activate receptors within their group only at very high concentrations [13,18,20]. NMR analysis of the AIP-II lactam analogue revealed dramatic differences in

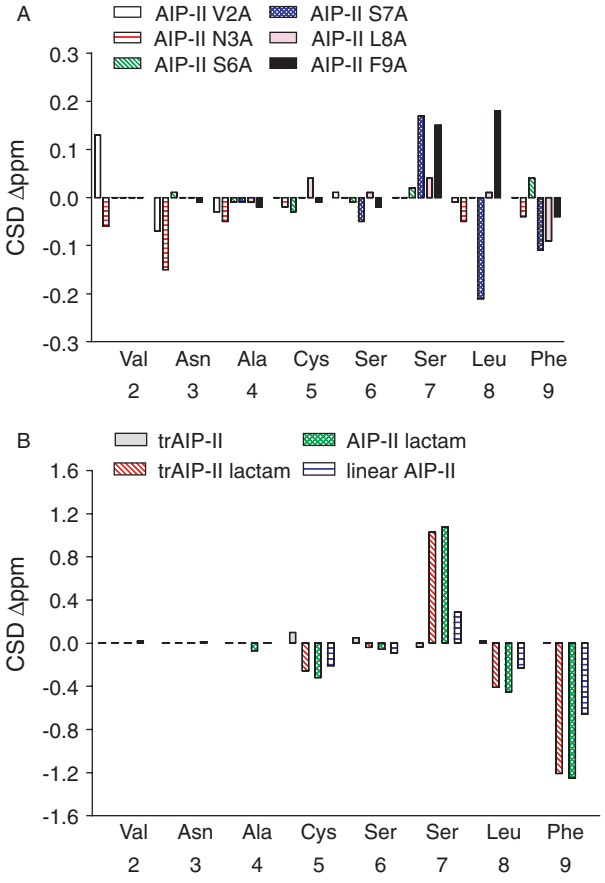

Figure 6 Chemical shift comparisons of some AIP analogues [14]. (A) The backbone amide ^{1}H chemical shifts of AIP-II alanine analogues are depicted as chemical shift differences (CSDs) relative to native AIP-II normalized to zero. (B) The backbone amide ^{1}H chemical shifts of trAIP-II thiolactone, AIP-II lactam, trAIP-II lactam and the linear free acid version of AIP-II are depicted as chemical shift differences (CSDs) relative to native AIP-II normalized to zero.

the backbone chemical shifts of residues within the ring (to roughly the same extent as linearizing the peptide), whereas the chemical shifts of the tail residues were essentially unaffected [14] (Figure 6B). This again points to the structural independence of the exocyclic and endocyclic regions of the molecule. Perhaps more importantly, these studies strongly suggest that the molecular recognition mechanisms underlying the receptor–agonist and receptor–antagonist interactions are different; modification of the thiolactone moiety dramatically affects the structure of the macrocycle, yet this perturbation results only in a loss of agonist activity.

The structure–activity studies performed by our group [12–16,18], and by others [9,20], have demonstrated that certain residues within the AIPs are critical for receptor activation. What complicates matters somewhat is that these key residues are not always located in the same positions in the peptides (summarized in Figure 3). For example, the key residue in AIP-II is asparagine-3, located in the tail. Replacement of this residue with an alanine converts an agonist of AgrC-II activation into a full antagonist [13]. Conversely, the key residue in AIP-1 is aspartic acid-5, located in the ring, and replacement of this residue with an alanine also converts the AIP into a full antagonist of its cognate receptor, AgrC-1 [14]. Moreover, deletion of the AIP-1 tail affords a partial agonist of the AgrC-1 receptor [14]. Interestingly, a chimeric peptide comprising the tail of AIP-II fused to the ring of AIP-1 was found to be an antagonist of both AgrC-I and AgrC-II activation [14]. On first analysis this result is surprising since this chimeric peptide contains the residues critical for activation of both receptors, as noted above. Thus, one is forced to conclude that these critical residues are not presented in the proper orientation within the ligand-binding pocket of the receptors; that is the tail and macrocycle must match in order for the AIP to activate the cognate receptor. This underscores the idea that cross-inhibition by the AIPs involves a binding mechanism that is subtly different from that of agonist AIP binding.

One of the long-term goals of our research programme is to develop small-molecule therapeutics based on pan-inhibition of *agr*-induced virulence in *Staphylococci*. Structure–activity studies, some of which are summarized above, have provided important insights that have allowed the rational design of AIP analogues that are global inhibitors of *S. aureus* virulence (some of these are shown in Figure 7). This is best illustrated through our work on AIP-II, where it was found that the residues in the tail of the molecule are critical for activation of the cognate AgrC-II receptor. Based on this finding it was reasoned that removal of the tail would afford a peptide that could still bind the receptor, but no longer activate the signalling response. Accordingly, it was demonstrated that the truncated version of AIP-II (**3**) was an inhibitor of all four *S. aureus* groups as well as some other *Staphylococci* species tested [12]. A global inhibitor was designed based on a truncated AIP-1 analogue (**5**) in which the key aspartic acid residue was replaced by an alanine. This compound inhibits *S. aureus* groups I–IV with IC_{50} values of 5 nM, 5 nM, 0.1 nM and 5 nM, respectively [14]. Importantly, molecules (**3**) and (**5**), are cyclic pentapeptides and are thus excellent starting

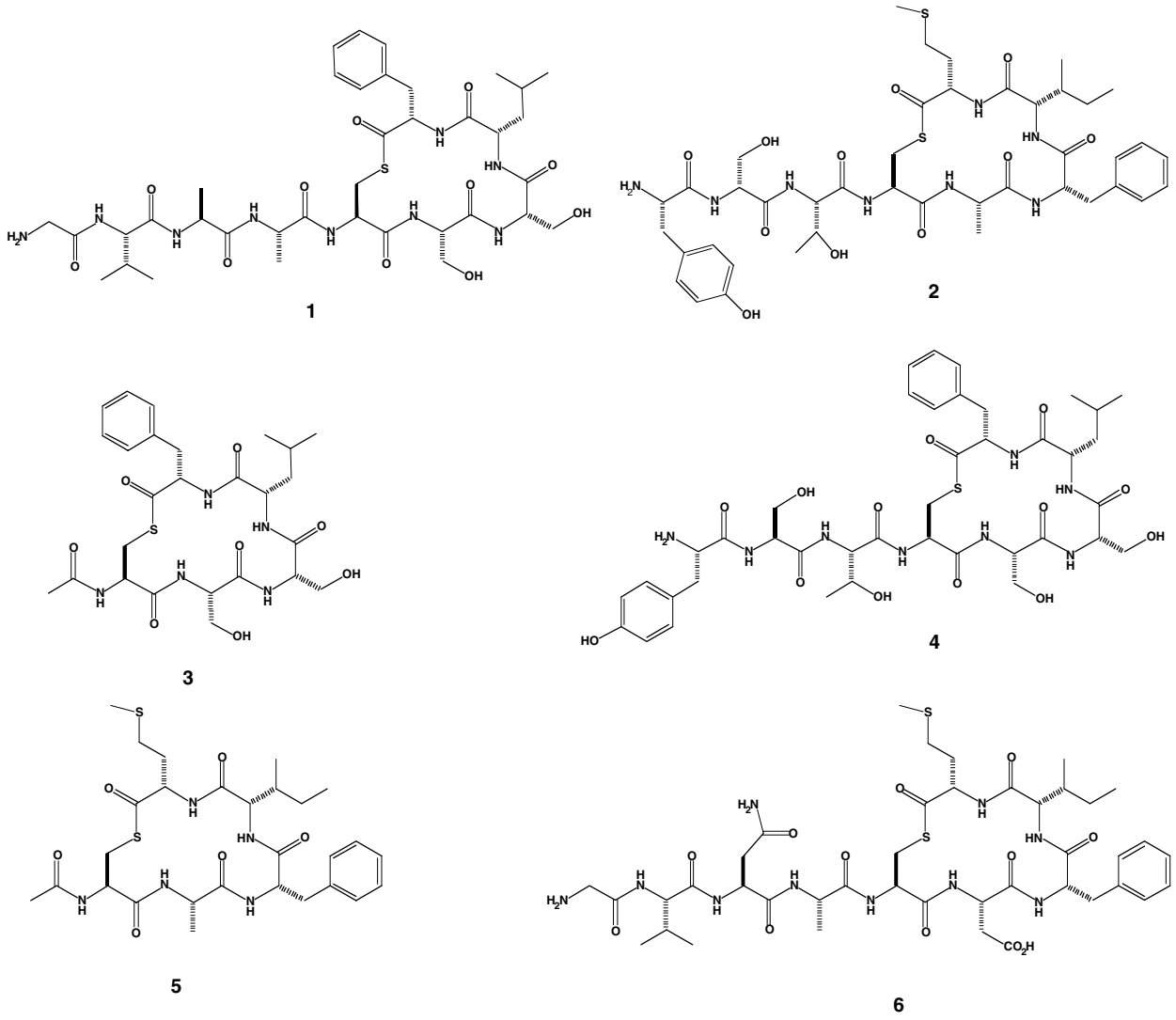

Figure 7 Chemical structures of six different global inhibitor peptides discovered or designed during the course of our studies.

points for peptidomimetic-type strategies designed to improve the bioavailability or potency of the initial compounds. For example, the effect of incorporating *N*-methyl amino acids, β-amino acids and D-amino acids into the cyclic peptide framework is currently being explored.

CONCLUSIONS AND FUTURE PERSPECTIVE

Based on the above studies there is now a basic understanding of the mechanisms underlying agonism and antagonism of the receptor histidine kinase, AgrC, by native AIPs. However, our understanding of how AIP binding then leads to AgrC autophosphorylation is still in its infancy. Indeed, it is expected that this difficult structural problem will be the focus of research efforts for many years to come. The biosynthetic mechanism by which the AgrD pro-peptide is converted into the mature AIP is equally poorly understood. There is good evidence that the integral membrane protein, AgrB, is responsible for both the post-translational modification of AgrD and the secretion of mature AIP [21]. However, the mechanistic details of this fascinating biotransformation remain to be elucidated by the groups working in this field.

In the course of our studies, a series of rules have been unearthed that allow the rational design of global inhibitors of virulence in Staphylococci.

Several peptides have been prepared with these desirable properties. Some of these molecules are of potential clinical use. Future work will involve improving the potency of these compounds as well as testing their efficacy in animal models of infection.

Acknowledgement

I would like to thank my collaborator Professor Richard P. Novick for introducing me into the fascinating world of bacterial signal transduction; I am extremely fortunate to have him as a colleague. The work described above was carried out by many dedicated, and talented, young scientists. In particular I would like to acknowledge the important contributions of Gholson Lyon, Jesse Wright and Patricia Mayville to the work. Finally I would also like to thank the National Institutes of Health and the Burroughs-Wellcome Fund for supporting this research.

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