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Letter

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Structure–Activity Relationship Study Based on Autoinducing Peptide (AIP) from Dog Pathogen *S. schleiferi*

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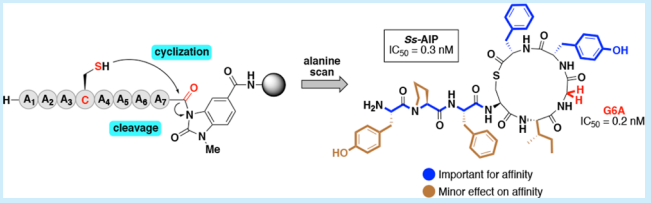
Supporting Information

ABSTRACT: Herein, an effective protocol for solid-phase synthesis of peptide thiolactones by concomitant ring closure and cleavage from the solid support is reported. The strategy was applied for mapping the importance of the structural features in *S. schleiferi* AIP (**5**) by performing an alanine scan and truncation of this natural compound. This furnished some of the most potent inhibitors of accessory gene regulator (*agr*)-I in the human pathogen *S. aureus* reported to date.



The increased emergence of bacterial pathogens that are resistant to so-called last-resort antibiotics, in combination with the lack of novel antibiotics brought to market, represents a major challenge for the public health sector and requires the development of alternative treatments.[1] An alternative to conventional antibiotic treatment could be to target the virulence of the pathogen rather than the viability, which may circumvent evolutionary pressure and, in turn, potentially also resistance development.[2] In the pathogenic bacterium *Staphylococcus aureus*, virulence gene expression is regulated through a quorum sensing (QS) system, an intercellular communication process that generates population-wide behavioral responses depending on the cellular density.[3] Inhibiting QS signaling in *S. aureus* was identified as a potential new drug target in the late 1990s[4] and has been shown to attenuate infections.[5]

The communication from cell to cell is based on secreted autoinducing peptides (AIPs), which are detected by the cognate QS system and activate the system when sufficiently high concentrations are present.[4,6] Thus, these peptide pheromones are extracellular signaling components, which are expressed as part of the accessory gene regulator (*agr*), a chromosomal locus consisting of two divergent operons transcribed from the P2 and P3 promoters, respectively (Figure 1A).[3b] The RNAII transcript encodes AgrB and AgrD, responsible for AIP synthesis, as well as a two component signal transduction system with membrane bound AgrC histidine kinase and the AgrA response regulator.[3a] The P3 promoter expresses RNAIII, which is the effector molecule of *agr* and regulator of the expression of virulence factors.[7] This population-controlled induction of the QS system allows the bacteria to grow to a certain density before engaging in a synchronized attack on the host, turning virulent and invasive.[3d]

S. aureus strains may be divided into four groups (I–IV) based on their secreted AIP (**1**–**4**; Figure 1B). The AIPs have a length of 7–9 amino acids and are cyclized by thiolactone formation between the C-terminal carboxylate and the cysteine residue in the fifth position from the C-terminal.[4] AIPs activate their cognate AgrC receptor and competitively inhibit the *agr*-dependent signaling circuit of other groups (Figure 1A).[8] The function of this cross-inhibitory mechanism remains unclear, but niche competition between groups has been hypothesized. The *agr*-dependent QS signaling is conserved for other staphylococci, and interspecies communication between strains through *agr* inhibition has been reported.[9] Structure–activity relationship (SAR) studies based on *S. aureus* AIP-I, -II, and -III (**1**–**3**),[3,4,10] as well as other staphylococcal AIPs, have been investigated in the search for potent QS inhibitors and to study the biology of *agr* signaling in staphylococci.[11] In a recent study, we demonstrated cross-talk between *S. aureus* and 14 other staphylococcal strains and identified the dog pathogen *S. schleiferi* as a strong suppresser of *S. aureus* virulence through *S. schleiferi* AIP (**5**, Figure 1B).[9d] The exceptionally strong interspecies communication between *S. aureus* and *S. schleiferi* is intriguing and could be indicative of evolutionary niche competition. In this Letter, we therefore investigate the structural requirements of the activity of the *S. schleiferi* AIP against *S. aureus* through chemical synthesis of AIPs and analogues.

The first synthetic AIP molecules from *S. epidermis* were prepared by thiolactonization using dicyclohexylcarbodiimide

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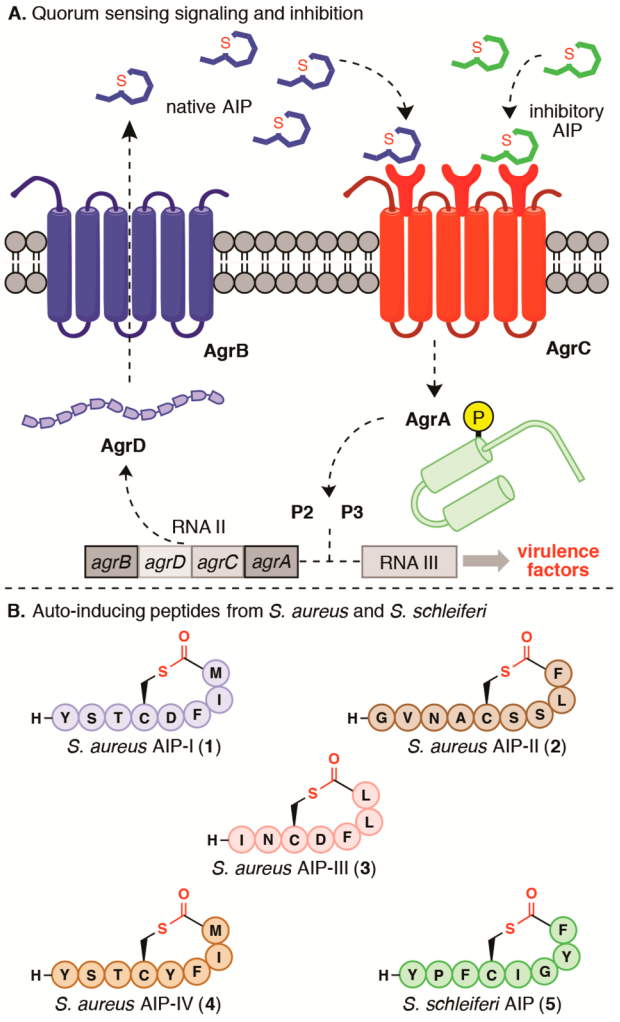

Figure 1. Quorum sensing signaling in *S. aureus* and structures of selected AIPs. (A) Membrane-bound peptidase AgrB converts the AIP precursor peptide AgrD to the mature AIP and releases it into the extracellular space. The AIP activates AgrC and thus initiates phosphorylation of AgrA. AgrA binds to promoters P2 and P3 leading to up-regulation of RNA-II–III transcription. (B) *S. aureus* AIPs I–IV (**1**–**4**) and *S. schleiferi* AIP (**5**).

and 4-dimethylaminopyridine.[12] The following year, Muir and co-workers conducted the first extensive SAR study on *S. aureus* AIPs using an elegant, chemoselective on-resin trans-thioesterification.[4] Stemming from these general approaches, sophistication of the synthesis of macrocyclic thiodepsipeptides has since resulted in methods involving Fmoc-based latent thioesters,[10d] metal-mediated thioesterification,[10e] and solid-supported carbodiimide reagents.[11a] Recently, Blackwell and co-workers applied an *N*-acyl-benzimidazolinone (Nbz) (**7**) approach, using the N^3-Fmoc-3,4-diaminobenzoic acid (Dbz)[13] linker (**6**) developed by Dawson and co-workers (Figure 2A).[14] We adopted this strategy for the synthesis of *Ss*-AIP (**5**);[9d] however, in our hands, this protocol provided insufficient purities when attempting to prepare AIP-I (**1**) as the control compound. Cyclization of the crude linear peptide resulted in mixtures that were difficult to separate due to acylation of the second aniline of the Dbz linker (**6**) (Figure 2A and Figure S1). To avoid these challenging HPLC purifications, we then applied the second generation linker, which is *N*-methylated on the 4-positioned aniline of the linker (MeDbz, **8**).[15] This optimization step resulted in significant reduction of by-products and gave good yields for *Ss*-AIP (**5**), but for AIP-I (**1**), small amounts of impurities were still present (Figure S1). We therefore envisioned that the cyclization could be performed on-resin to release only the cyclized product into solution while leaving the byproducts attached to the solid support (Figure 2C). Thus, the MeDbz linker was coupled directly to the ChemMatrix resin (**10**), enabling global deprotection of the resin-bound linear peptide after Nbz-formation (**11**). After extensive washing of the deprotected and MeNbz-activated resin-bound peptide (**11**), swelling the resin in DMF–phosphate buffer (0.1 M, pH = 6.8) (1:1, v/v) for 2 h at 50 °C furnished crude purities of 70–80% in LC–MS scale test reactions. However, more consistent results were achieved with an increased strength of the phosphate buffer (0.2 M, pH = 6.8) in MeCN (1:1, v/v), presumably due to the remaining TFA. Finally, we added 5% of an acid-labile MeDbz resin to the MeDbz-ChemMatrix resin (**11**) during compound synthesis to enable analysis of the efficiency of the SPPS by TFA cleavage. This still leaves 95% of the material on the resin for the cyclization–cleavage event. Independently, a preliminary account of similar cyclization–cleavage of MeNbz-activated resin-bound peptides was reported by Albericio and co-workers while this manuscript was in preparation.[16] However, this is, to the best of our knowledge, the first demonstration of this strategy for preparation of full AIPs.

Using our optimized protocol, we performed a SAR study on *Ss*-AIP (**5**) and compared its inhibitory abilities against *agr*-I to one of the most potent pan-group inhibitors of *agr* in *S. aureus* reported, AIP-III D4A (**3a**).[10e] The specificity group I of *S. aureus* represents one of the most common and was therefore chosen for preliminary evaluation of the compound series. We synthesized the control compounds AIPs I–IV (**1**–**4**) and AIP-III D4A (**3a**) and performed an alanine scan of the *S. schleiferi* AIP (**12**–**18**) to investigate the role of specific residues on the inhibition of *agr*-I. Furthermore, a truncated and *N*-acetylated analogue (**19**) was included to determine the importance of the exocyclic tail, inspired by the potent global inhibitor, truncated AIP-I D2A, reported by Muir and co-workers.[10b] The crude cyclization products were obtained in purities of 56–87% , and compounds were isolated by preparative HPLC in yields of 8–45% based on the resin loading (Table 1).

With the reference compounds and *S. schleiferi* AIP analogues in hand, we determined their ability to inhibit AgrC-I using a previously reported β-lactamase reporter assay (Table 1).[5a] As expected, the cognate AIP-I (**1**) and *agr*-I activator AIP-IV (**4**) did not affect the β-lactamase activity.[10b] The determined IC_{50} values for native *agr*-I inhibitors AIP-II (**2**) at 12 nM, AIP-III (**3**) at 8 nM, and AIP-III D4A (**3a**) at 0.16 nM were comparable to previously reported 1.6, 5.1, and 0.5 nM, respectively.[10e]

The *Ss*-AIP (**5**) has several interesting structural features, including a flexible glycine residue in the macrocycle and a proline residue in the exocyclic tail, which are not present in AIP-I–IV (**1**–**4**). Nevertheless, our alanine scan based on *Ss*-AIP (**5**) recapitulated several requirements for AgrC–AIP interaction established through previous SAR studies of AIP-I–IV (**1**–**4**)[3b,d] (Table 1).

(*i*) Compounds *Ss*-AIP Y7A (**17**) and *Ss*-AIP F8A (**18**) exhibited loss in affinity of 10-fold and 4000-fold, respectively, showing that hydrophobic residues at the C-terminal are important for tight binding. (*ii*) The *Ss*-AIP analogues **12**–**15** were only slightly less potent than the natural compound. Thus,

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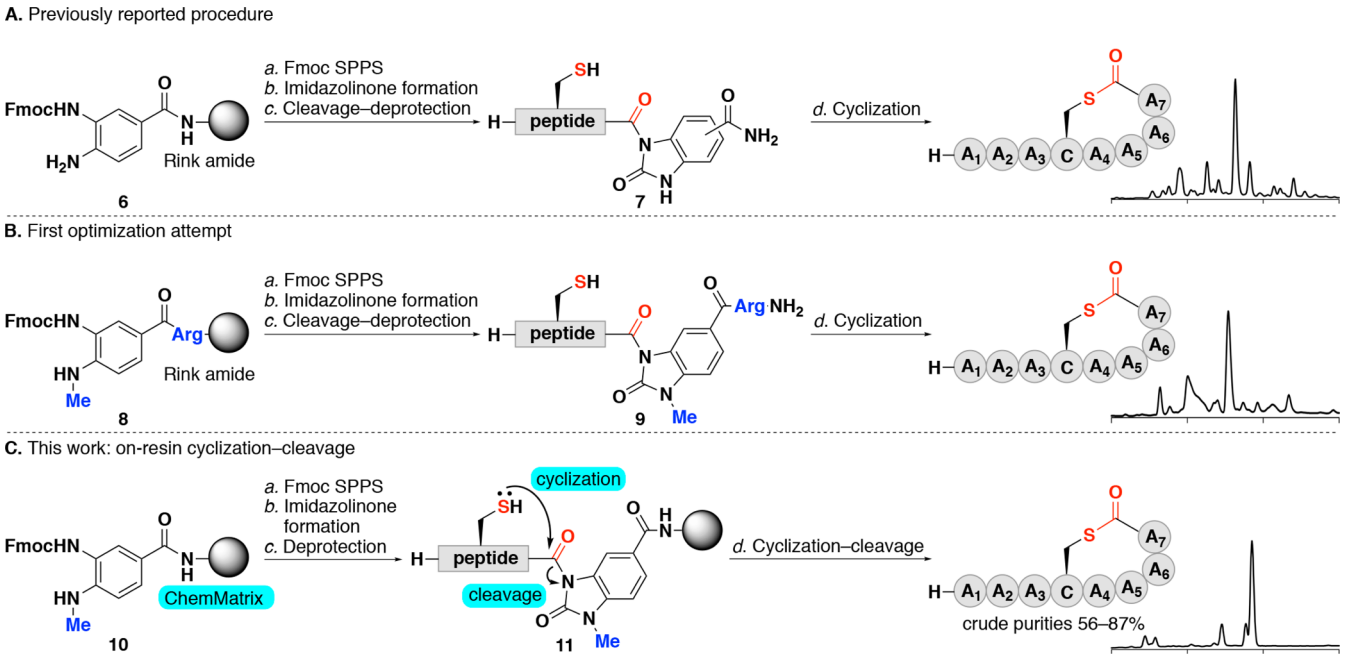

Figure 2. Preparation of thiolactone-containing peptides with chromatograms of crude AIP-I (**1**) inserted. (A) Previously reported strategy. (B) Initial attempt of optimization. (C) On-resin cyclization−cleavage strategy.

Table 1. Compound Yields and IC_{50} Values for Inhibition of AgrC-I

compound	sequence	yield (%)[a]	IC_{50} (nM)[b]
AIP-I (**1**)	YST-[CDFIM]	15	
AIP-II (**2**)	GVNA-[CSSLF]	8	12 ± 2.9
AIP-III (**3**)	IN-[CDFLL]	14	8 ± 1.1
AIP-III D4A (**3a**)	IN-[CAFLL]	33	0.16 ± 0.01
AIP-IV (**4**)	YST-[CYFIM]	21	
Ss-AIP (**5**)	YPF-[CIGYF]	37	0.31 ± 0.08
Ss-AIP Y1A (**12**)	**A**PF-[CIGYF]	37	1.0 ± 0.2
Ss-AIP P2A (**13**)	Y**A**F-[CIGYF]	21	0.8 ± 0.2
Ss-AIP F3A (**14**)	YP**A**-[CIGYF]	45	1.2 ± 0.2
Ss-AIP I5A (**15**)	YPF-[CA**G**YF]	27	1.1 ± 0.2
Ss-AIP G6A (**16**)	YPF-[CI**A**YF]	25	0.20 ± 0.02
Ss-AIP Y7A (**17**)	YPF-[CIGA**F**]	38	3.1 ± 0.9
Ss-AIP F8A (**18**)	YPF-[CIGY**A**]	39	1200 ± 240
tr*Ss*-AIP (**19**)	**Ac**-[CIGYF]	27	16.8 ± 0.6

[a]Isolated yield based on the resin loading. [b]Mean values ± standard error of the mean based on at least three individual assays performed in triplicate.

the *Ss*-AIP I5A (**15**) mutant did not result in significant improvement of potency as observed for AIP-I D5A[10b] and AIP-III D4A (**3a**).[10e] (*iii*) Removal of the exocyclic tail, to give truncated *Ss*-AIP **19**, resulted in a 56-fold decrease in inhibition. (*iv*) Perhaps most interestingly, equipotency was recorded for the *Ss*-AIP G6A (**16**) mutant, which is the only analogue that gains structural complexity. Since AIP-I, -III, and -IV (**1**, **3**, and **4**) have important hydrophobic residues in this position, it appears to be an obvious site for further modification. Furthermore, double mutants will be of interest for extended SAR studies to search for high potency compounds.

Finally, we tested *Ss*-AIP (**5**), its potent G6A mutant (**16**), and reported pan-group inhibitor **3a** for their ability to inhibit all specificity groups (*agr*-I−IV). Using fluorescence reporter strains and flow cytometry as previously described,[9d] we found that *Ss*-AIP (**5**) and G6A (**16**) inhibited *agr*-I−III, while no inhibition was observed for *agr*-IV. As expected, control compound **3a** exhibited pan-group inhibition (Figure S5). Based on previous experiments using *S. schleiferi* supernatant,[9d] it was surprising that **5** did not exhibit pan-group inhibition, which warrants further investigation.

The scaled syntheses allowed full characterization of *Ss*-AIP (**5**) by NMR spectroscopy, which revealed two spin systems in a ratio of 1:0.4 in intensity. Because the chromatography (HPLC and LC−MS) indicated the presence of a single compound and all proline-containing analogues in the alanine scan exhibited the same behavior, we presumed that this arose from *cis*−*trans* isomerism at Pro2. When performing NMR spectroscopy at varying temperatures up to 60 °C, we did not observe coalescence (Figure S2A). However, ^{1}H NMR spectra were recorded in DMSO-d_6, CD_3CN-d_3, and CD_3OH-d_3, respectively, and these revealed different ratios of the two spin systems, strongly suggesting the presence of rotamers (Figure S2B). The signal overlap of these two rotamers precluded determination of a high-resolution structure of either conformation based on NOE and *J*-coupling constants. To gather some structural insight related to the SAR, we therefore compared the changes in chemical shifts of all residues in the most abundant conformation of *Ss*-AIP (**5**) in response to each alanine mutation (Figures 3 and S3). Not surprisingly, the most affected residue was Tyr1 when its neighbor Pro2 was mutated (purple bar), and the same was the case for neighboring residues in other mutants. However, glycine to alanine substitution affected all residues in the macrocycle (blue bars). This strongly indicates that the presence of glycine provides a unique conformational space compared to previously investigated AIPs, and it will therefore be interesting to investigate these conformations further. Finally, removal of the exocyclic tail only affected the connecting cysteine residue significantly, indicating that the tail does not have strong interactions with the macrocycle in DMSO.

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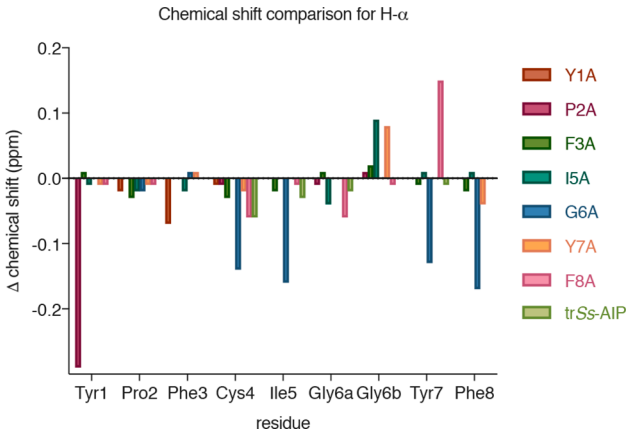

Figure 3. Chemical shift changes of α-protons observed in DMSO-d_6.

In summary, we have developed an efficient method for on-resin cyclization of thiodepsipeptides with concomitant cleavage from the solid support and applied this strategy to enable SAR investigation of an autoinducing peptide from *S. schleiferi*. This furnished the highly potent *agr*-I inhibitor *Ss*-AIP G6A (**16**), highlighting the potential of exploring nonhuman pathogens for identification of novel QS modulators. Finally, synthesis of microbe-specific signaling molecules (such as AIPs) will enable a deeper understanding of the intriguing cross-talk between pathogens of different species and their effects on colonization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.7b02550.

Supporting figures, experimental details, compound characterization data, and copies of HPLC traces and ^{1}H and ^{13}C NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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