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Letter

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Truncated Autoinducing Peptide Conjugates Selectively Recognize and Kill *Staphylococcus aureus*

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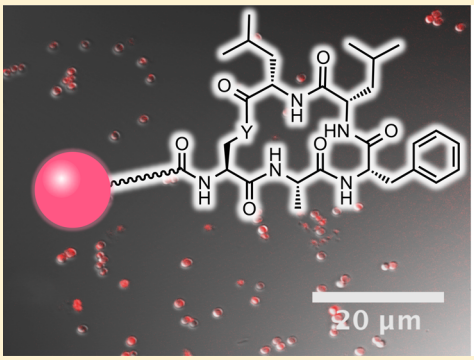
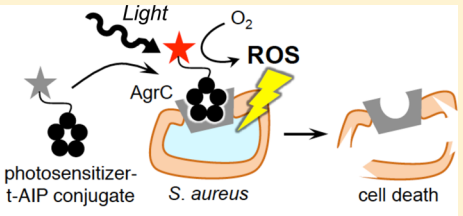
Supporting Information





Compound	MIC (nM) + Light	MIC (nM) – Light
t-AIP conjugate	80	> 20000
Non-targeting control	1250	> 20000

ABSTRACT: The accessory gene regulator (*agr*) of *Staphylococcus aureus* coordinates various pathogenic events and is recognized as a promising therapeutic target for virulence control. *S. aureus* utilizes autoinducing peptides (AIPs), cyclic-peptide signaling molecules, to mediate the *agr* system. Despite the high potency of synthetic AIP analogues in *agr* inhibition, the potential of AIP molecules as a delivery vehicle for antibacterial agents remains unexplored. Herein, we report that truncated AIP scaffolds can be fused with fluorophore and cytotoxic photosensitizer molecules without compromising their high *agr* inhibitory activity, binding affinity to the receptor AgrC, or cell specificity. Strikingly, a photosensitizer-AIP conjugate exhibited 16-fold greater efficacy in a *S. aureus* cell-killing assay than a nontargeting analogue. These findings highlight the potential of truncated AIP conjugates as useful chemical tools for in-depth biological studies and as effective anti-*S. aureus* agents.

KEYWORDS: *autoinducing peptides, conjugation, drug delivery, photodynamic therapy, quorum sensing, Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive opportunistic pathogen that often causes serious acute and chronic infections in community and clinical sites worldwide.[1] Antibiotics have been major treatment agents for infections caused by *S. aureus*. However, many *S. aureus* strains have developed resistance mechanisms against a variety of antibiotics, leading to complication of treatment.[2,3] Thus, establishment of novel strategies for virulence control and treatment is an urgent task for humanity to secure more clinical options for this threatening pathogen.

As is the case with other Gram-positive bacteria, *S. aureus* populations sense cell density via a quorum sensing system controlled by the accessory gene regulator (*agr*). This gene regulator is responsible for pathogenic events including virulence factor production and biofilm formation.[4,5] As such, modulation of this system has emerged as a potential strategy for controlling the virulence of *S. aureus*.[6−8] The *agr* system of *S. aureus* is mediated by thiolactone peptide signaling molecules, termed autoinducing peptides (AIPs) (Figure S1), that coordinate gene expressions in a cell density-dependent manner. In addition to genetic[9,10] and structural studies,[11] various synthetic AIP analogues have been prepared and tested to garner insights into structure−activity relationships within AIPs and the receptors AgrCs, resulting in highly potent AIP-based *agr* modulators.[12−14]

Despite the prominent potency and specificity of the AIP analogues in *agr* modulation revealed in the previous studies, the potential of AIP molecules as a delivery vehicle for antibacterial agents remains unexplored. Given that *agr* gene expression in *S. aureus* is likely to be associated with acute infection,[4,6] that *agr* inhibition resulted in attenuated virulence and enhanced clearance in in vivo studies,[15] and that no resistance was developed after continual chemical *agr* inhibition either in vitro or in vivo,[15] it is logical to envisage that AgrC-targeted delivery of antibacterial agents could be a potential strategy to augment current clinical options. Herein, we present a molecular design and synthetic strategy enabling successful conjugation of inhibitory AIP analogues and another functional molecule. Truncated AIP-III analogues can be fused with a fluorophore or a bactericidal photosensitizer without compromising their high *agr* inhibitory activity, binding affinity to the cell, or specificity to *S. aureus*.

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S. aureus strains use one of the four AIPs (AIP-I, -II, -III, and -IV) depending on the *agr* gene type (*agr*-I, -II, -III, and -IV) (Figure S1). Each AIP activates its cognate AgrC receptor, but competitively inhibits the other three noncognate AgrC receptors. Several AIP analogues with single or multiple amino acid mutations within their endocyclic sequences function as potent pan-*agr* inhibitors.[12,14,16] Among the potent AIP-based pan-*agr* inhibitors previously reported, we chose AIP-III D4A as a starting structure for further chemical modification (Figure 1). A recent seminal study proved that

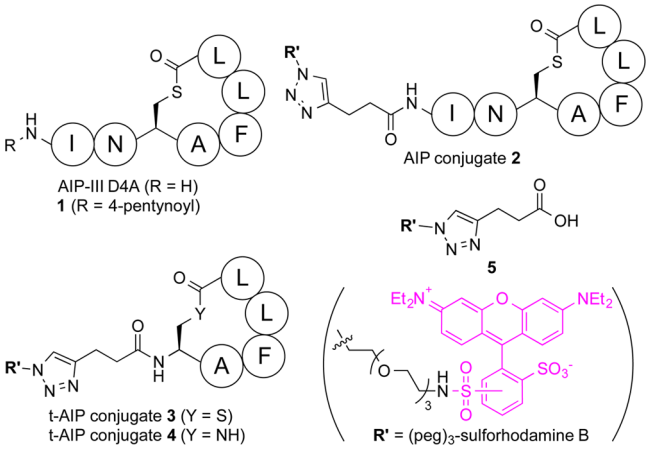

Figure 1. Structures of AIP-III D4A, fluorescent AIP conjugates, and control compounds synthesized in this study.

stable variants of this peptide containing a lactam bridge instead of thiolactone also functioned as pan-*agr* inhibitors with comparable potency,[17] which is advantageous from a drug development standpoint. To investigate how further structural modification of this scaffold affects the *agr* inhibitory activity, we synthesized fluorescent AIP-III D4A conjugates containing a full/truncated peptide sequence and a thiolactone/lactam bridge within the cyclic scaffolds (Figure 1). All of the peptide backbones were constructed by standard solid-phase peptide synthesis, and the N-termini were capped with pentynoic acid by amide coupling. The resulting linear peptides were cyclized in liquid phase. Finally, the AIP alkynes were coupled with an azide-functionalized sulforhodamine B by copper-catalyzed azide−alkyne 1,3-cycloaddition to afford rhodamine-AIP conjugates **2**−**4** (see the Supporting Information (SI) for synthesis details). Control **5** was also prepared in the same manner for fluorescent labeling studies (vide infra).

The obtained AIP alkyne **1** and conjugates **2**−**4** were evaluated for inhibition of *agr*-based quorum sensing in *S. aureus* (*agr*-I−IV) using established fluorescent reporter strains (Tables 1 and Figure S2).[9] These methicillin-resistant *S. aureus* (MRSA) strains harbor the native *agr* gene cluster and P3-*yfp* plasmids, which enables quantification of the endogenous AIP-dependent *agr* expression level based on fluorescence intensity. Cultures were incubated in the presence of each AIP analogue at various concentrations (0.064 nM−1 μM). No cytotoxicity was observed in compounds tested at 1 μM (Figure S2). Surprisingly, alkyne **1**, an AIP-III D4A congener with a small alkyne handle on its exocyclic tail, exhibited no inhibitory activity in all four *agr*-type strains, whereas the parent AIP-III D4A exerted low nanomolar to subnanomolar potency as previously reported.[14] Rhodamine conjugate **2** derivatized from alkyne **1** also showed a

Table 1. *agr* Inhibition by AIP Conjugates[a]

	IC_{50}[b] (nM)			
compound	*agr*-I	*agr*-II	*agr*-III	*ag*-IV
AIP-III D4A	2.9[c]	3.5[c]	0.35[c]	0.75[c]
alkyne **1**	>1000[d]	>1000[d]	>1000[d]	>1000[d]
rhod-AIP **2**	>500[e]	>500[e]	251	>500[e]
rhod-t-AIP **3**	7.6	2.1	1.1	4.5
rhod-t-NH-AIP **4**	3.5	3.0	0.87	1.1

[a]All assays were performed in triplicate. Rhod, sulforhodamine B, t, truncated. [b]All IC_{50} values were calculated by fitting dose response curves (see the SI). [c]Values were determined under our assay conditions. For values obtained under different conditions, see ref 14. [d]No inhibition was observed at 1 μM. [e]Inhibition was observed at 1 μM, but IC_{50} values could not be calculated due to unusual Hill's slopes.

significantly attenuated antagonist effect. In contrast, conjugates **3** and **4**, where rhodamine and AIP moieties are connected without an exocyclic peptide tail, functioned as inhibitors in all *agr* types with potency comparable to that of AIP-III D4A.

Previous studies have demonstrated that the exocyclic tail of the AIP core is essential for *agr* activation in all *agr* groups of *S. aureus*. Muir and Novick reported that installing fluorescein or biotin moieties on the N-terminus of AIP-I resulted in only moderate effects on its activity.[11,16] However, in many cases, a single mutation or deletion of the peptide tails of native AIPs significantly reduces the agonistic activity for their cognate AgrCs, or rather converts them into pan-*agr* inhibitors.[14,16] On the basis of these observations, one may assume that an AIP analogue that has been already converted into a potent pan-*agr* inhibitor can be conjugated with various functional groups on its N-terminus without reducing the potency. However, our finding clearly illustrates that the tail structure can be an influential factor for *agr* inhibitory potency even after such a conversion. On the basis of the 3D solution structures of AIP-III analogues revealed by Blackwell and co-workers,[17,18] we surmise that the drastic reduction of *agr* inhibitory activity in the inactive compounds **1** and **2** is due to the direction of the N-terminus of AIP-III D4A. The exocyclic tail is directed toward the hydrophobic surface composed of the three endocyclic side chains, a critical component for *agr* inhibition, whereas its truncated variants project their N-termini toward the opposite side of the hydrophobic surface (Figure S3). Consequently, installation of a functional group on the N-terminus of AIP-III D4A could alter proper structural arrangement of the essential side chains. Such a detrimental event is unlikely to occur in the case of its truncated variants. Further structure−activity relationship studies are necessary to determine whether the trend observed in this study holds true only for the AIP-III D4A scaffold or for other AIP-based *agr* inhibitors as well.

As expected, lactam conjugate **4** was extremely stable under physiological conditions; no significant degradation was observed in PBS (pH 7.4) and in human plasma at 37 °C after 4 days. In contrast, the half-lives of thiolactone conjugate **3** were about 3 days in PBS and 30 min in human plasma (Figure S4). Taking into account future applications, we decided to use the stable lactam conjugate for the subsequent studies.

Encouraged by the remarkable pan-*agr* inhibitory activity and plasma stability of truncated AIP conjugate **4**, we sought to investigate its binding to and specificity for AgrC of *S. aureus*. An overnight culture of the *S. aureus* strain AH1677 (*agr*-I) was

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treated with 1 μM of conjugate **4** or control **5** and then visualized using a confocal microscope. Overnight incubation of *S. aureus* strains allows for full induction of AgrC expression. Gratifyingly, the *S. aureus* cells treated with conjugate **4** were fluorescently labeled, whereas no significant fluorescent signal was detected from those treated with control **5**, demonstrating that the truncated AIP conjugate can bind to the cell surface of *S. aureus* expressing AgrC (Figure S5).

Next, to gain further insight into receptor binding and cell specificity, we performed flow cytometry-based analysis using these compounds. Overnight cultures of *S. aureus* (*agr*-I–IV), *Escherichia coli* (wild type) and the human epithelial cell line A431 were labeled with various concentrations of fluorescent conjugate **4** or control **5** and monitored using a flow cytometer (Figure 2 and Figures S6–S10). As anticipated from the results

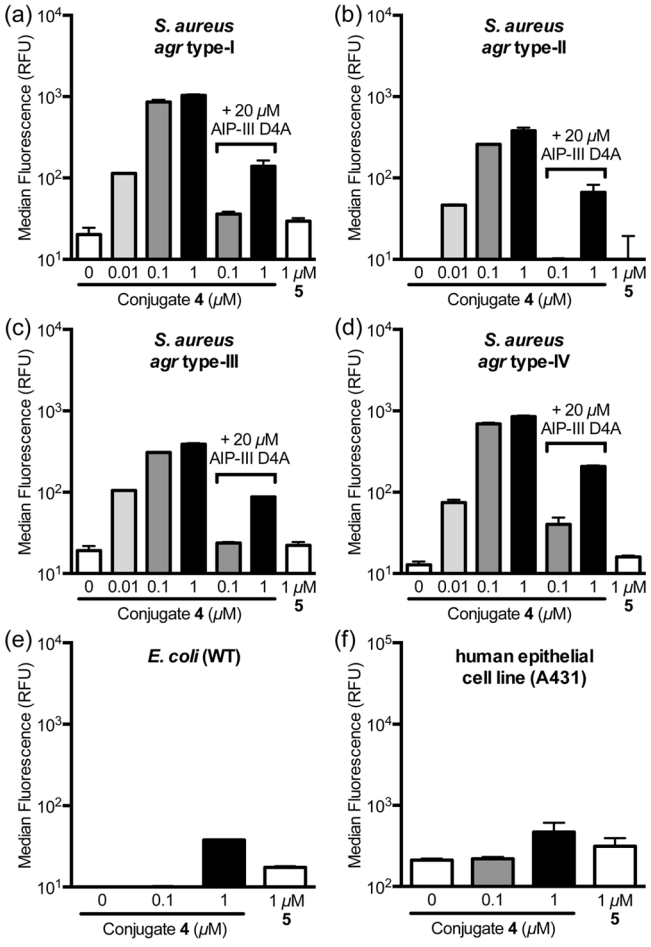

Figure 2. Flow cytometry-based analysis of binding properties of lactam AIP conjugate **4** and negative control **5** in the *S. aureus* (a) AH1677 (*agr*-I), (b) AH430 (*agr*-II), (c) AH1747 (*agr*-III), and (d) AH1872 (*agr*-IV) strains, (e) the *E. coli* K-12 (wild type), and (f) the human epithelial cell line A431. All assays were performed in duplicate, and error bars represent the SEM.

of the microscopic analysis, 1 μM of conjugate **4** was capable of fluorescently labeling all four *agr*-type *S. aureus* reporter strains, whereas negative control **5** showed minimal nonspecific binding at 1 μM (Figure 2a–d). The clinical MRSA strain USA300-0114 was also efficiently labeled (Figure S6c). The same trends in dose dependency were observed in all four *agr*-type strains; the labeling efficiency of conjugate **4** was comparable at 0.1 μM and decreased by 40–50% at 0.01 μM. In addition, the labeling was competitively inhibited by 20- and 200-fold AIP-III D4A, suggesting that conjugate **4** and AIP-III D4A share the same binding pocket within AgrC. Conjugate **4** did not show a log increase in median fluorescence intensity in the wild-type *E. coli* or the human epithelial cell line, even at 1 μM (Figure 2e,f). Furthermore, the labeling efficiency markedly decreased in *S. aureus* (*agr*-I) cells, where *agr* expression was chemically inhibited with 200 nM AIP-III D4A prior to the fluorescent labeling (Figure S6d). Collectively, these results illustrate that AIP-based probe **4** is highly specific to *S. aureus* cells expressing AgrC. The fluorescence intensities of the four *S. aureus* strains when labeled with 1 μM conjugate **4** were in the order *agr* type-I > -IV > -II ~ -III. Given that the AgrC receptors expressed should be mostly labeled at 1 μM (vide supra), this order likely reflects the level of AgrC expression in each strain. Thus, we speculate that the *agr* type-I strain has the highest expression of *agr* genes among the four used in this study.

Finally, we set out to test our hypothesis that AIP can be used as an efficient delivery vehicle for antibacterial agents. We incorporated an iodinated BODIPY, a photosensitizer that efficiently generates cytotoxic reactive oxygen species (ROS) upon visible light irradiation,[19] into the truncated AIP conjugate scaffold. We envisaged that a photosensitizer-t-AIP conjugate could bind to AgrC and accumulate on the cell surface of *S. aureus*, leading to enhanced cell-killing effectiveness due to the increased local concentration (Figure 3a). The advantage of this modality is that the conjugate does

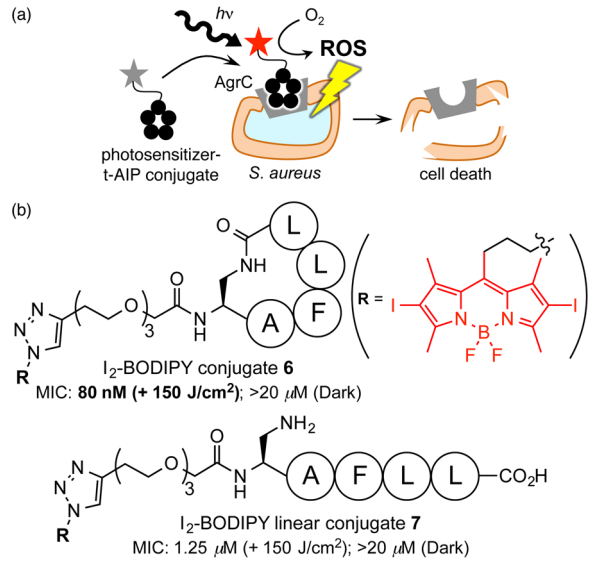

Figure 3. (a) Proposed mechanism of AgrC-targeted photoinactivation of *S. aureus* using a photosensitizer-t-AIP conjugate; (b) structures and MIC values of photosensitizer-t-AIP conjugate **6** and nontargeting control **7** in a photoinactivation assay using the MRSA AH1677 (*agr*-I). ROS, reactive oxygen species; MIC, minimum inhibitory concentration.

not have to get into the target bacterial cell. We obtained striking results that strongly supported our hypothesis; iodinated BODIPY-t-AIP conjugate **6** showed a minimum inhibitory concentration (MIC) value of 80 nM in a photoinactivation assay using an overnight (stationary-phase) culture of the MRSA strain AH1677 (*agr*-I). This MIC indicates 16-fold greater potency than that of linear control **7** (Figure 3b and Figure S11). The observed phototoxicity of

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nontargeting control **7** is probably due to nonspecific adsorption of the hydrophobic iodinated BODIPY moiety to the bacterial cell wall. Both compounds were nontoxic without light irradiation at 20 μM. Taking into account that both conjugates possess similar ROS generation properties under light irradiation (Figure S12) and that conjugate **6** functioned as a potent *agr* inhibitor, whereas linear variant **7** was inactive in the reporter strain assay (Figure S2), the enhanced antibacterial efficacy observed in conjugate **6** is highly likely due to t-AIP–AgrC interactions. Indeed, the MIC value of conjugate **6** increased in an *agr*-underexpressed culture (Figure S11).

In conclusion, we have shown that truncating the AIP analogue scaffold is a key for successful conjugation with fluorophore and photosensitizer molecules retaining high pan-*agr* inhibitory activity, binding affinity, and specificity to *S. aureus*. Flow cytometry-based analysis using our fluorescent t-AIP conjugate enables measurement of expression level of AgrC, which we believe will be a valuable approach for future in-depth studies of the biochemical and pathophysiological roles of the *agr* system in *S. aureus* infection. Our data demonstrate that the truncated AIP scaffold can efficiently guide a conjugated photosensitizer to the cell surface of MRSA expressing AgrC, resulting in an enhanced cell-killing effect. Photoinactivation is recognized as a potential therapeutic strategy for difficult-to-treat skin infections caused by drug-resistant bacteria because no development of bacterial resistance to this modality has been reported to date.[20,21] In addition, viable but nonreplicating MRSA in the stationary phase can exhibit higher antibiotic resistance than actively growing MRSA.[22] Thus, coupled with the potent pan-*agr* inhibitory activity, photosensitizer-t-AIP conjugates may lead to promising anti-MRSA therapies, reinforcing current antibiotic-based treatment regimens. We are currently working on refinement and detailed evaluation of this novel class of antibacterial agents, and our effort will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00013.

Synthetic protocols, characterization of new compounds, additional structural figures, biological assay protocols, and assay data (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

agr, accessory gene regulator; AIPs, autoinducing peptides; Ala, alanine; Cys, cysteine; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; PBS, phosphate-buffered saline; PEG, polyethylene glycol; rhod, sulforhodamine B; ROS, reactive oxygen species; t, truncated

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