COMMUNICATION

Potent pan-group quorum sensing inhibitors in *Staphylococcus aureus* revealed by N-terminal tailoring of peptidomimetics

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The pathogen *Staphylococcus aureus* uses small peptides to assess its population densisty (i.e., quorum sensing) and regulate virulence at high cell number. Here, we report the design and synthesis of peptidomimetics based on these native signals that strongly block this communication pathway in all four specificity groups of *S. aureus*.

Staphylococcus aureus is a Gram-positive bacterial pathogen and frequent cause of deadly hospital-acquired infections. The rapid emergence of multi-drug resistant strains *S. aureus* makes the identification of alternate pathways to block infections by this common pathogen not only attractive, but also urgent.[1] *S. aureus* regulates many aspects of virulence via the chemical signalling process called quorum sensing (QS),[2] which has received considerable attention as a target for infection control.[3] Chemical and biological agents capable of interfering with QS pathways in *S. aureus* and related bacteria have been identified with increasing frequency over the past two decades, and these molecules (and macromolecules) represent valuable tools to understand the role QS in a range of disease types.[4, 5]

S. aureus uses the accessory gene regulator (*agr*) system for QS (Fig. 1A), which controls over 100 virulence factors.[6] The chemical QS signal, a macrocyclic peptide thioester called the autoinducing peptide (AIP), is produced from the AgrD propeptide. AgrD is cyclized via AgrB, further tailored outside of the cell, and gradually increases in local concentration with cell number. Once a quorate cell density (and thus signal level) is reached, the AIP ligand will bind the extracellular domain of the transmembrane histidine kinase, AgrC. AgrC subsequently activates its cognate response regulator, AgrA, which then upregulates both the *agrBDCA* operon and an arsenal of virulence factors via RNAIII.[6] To date, most efforts to intercept the *agr* system, and thereby QS, in *S. aureus* have been focused on blocking AIP-AgrC interactions, with AgrA inhibition being a secondary target.[4] *S. aureus* has evolved into four *agr* specificity groups (I-IV), each group with a unique AIP signal-AgrC pair (Fig. 1B) and often propensity in disease types.[7] Both universal inhibitors active in all four groups (i.e., pan-group inhibitors) and group-selective inhibitors represent valuable tools to study the mechanisms of QS in *S. aureus* and their role in infections.[4]

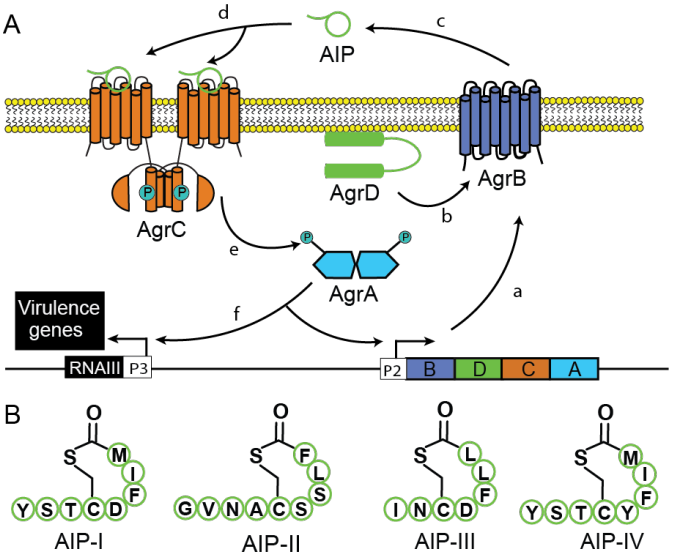

Fig. 1: (A) Schematic of the *agr* system in *S. aureus.* (a) AgrA-D are produced. (b) The precursor of AIP, AgrD, is processed by AgrB (c) to release the mature AIP outside the cell. (d) Binding of AIP to AgrC causes autophosphorylation of AgrC dimer. (e) Phospho-transfer from AgrC to AgrA, followed by AgrA dimerization. (f) AgrA dimer binds to promoters to upregulate gene transcription. (B) Structures of the group I-IV AIP signals.

Relatively early research on the *agr* system revealed that the native *S. aureus* AIPs can cross-inhibit non-cognate AgrC receptors (e.g., AIP-II inhibits AgrC-I, -III and -IV).[5, 6] Based on this observation, the first synthetic *agr* inhibitors were derived from native AIP scaffolds. Examples of these inhibitors include AIP-I-D5A, truncated AIP-II (t-AIP-II), and AIP-III-D4A (Fig. 2A), each of which are pan-group inhibitors.[8, 9] Our laboratory has been interested in developing *agr* inhibitors based on peptidomimetics and small molecules, with the intent of identifying scaffolds with improved stability, solubility, and

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synthetic accessibility relative to peptides.[10-12] Using t-AIP-II as a starting point (due to its already minimized structure), our lab developed the peptidomimetic compound n7OFF, which only contains the two Phe residues determined essential for activity (Fig. 2B) and had moderate inhibitory potency in *agr* (in groups I-III; as determined in cell-based reporter assays).[11] We later made a series of structural modifications to n7OFF in order to enhance its potency and efficacy across all four groups of *S. aureus.* These studies demonstrated that replacement of the n7OFF acetyl tail with a benzyl tail (i.e., Bn-n7OFF, Fig. 2B) increased its potency around 10-fold in all four groups.[12] Additional modifications to the two Phe sidechains in Bn-n7OFF led to the discovery of Bnc3 (Fig. 2B), which has IC_{50} values ranging from high picomolar to single digit nanomolar values in *S. aureus* groups I–IV. In view of the promise and tunability of the n7OFF scaffold, additional studies are warranted to enhance its activity profile and expand its potential utility as a chemical probe to study QS in *S. aureus.*

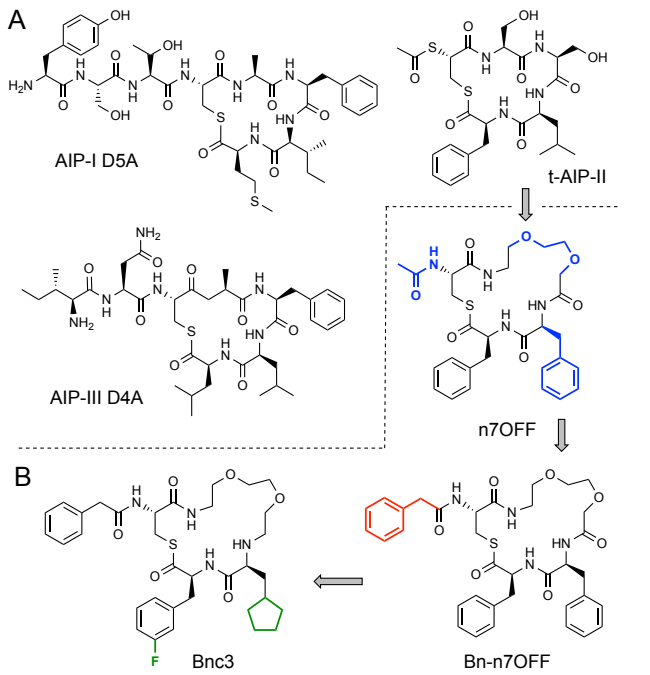

Fig. 2: (A) Peptide *agr* inhibitors developed from native AIPs. (B) Peptidomimetic *agr* inhibitors based on t-AIP-II. Colours indicate structural changes relative to precursor compound; grey arrows indicate flow of discovery.

In the current study, we investigated the role of the N-terminal Bn tail on the *agr* inhibitory activity of n7OFF derivatives through the introduction of a range of substituents with differing electronics and sterics. We then combined the N-terminal tail features yielding improved potency with the two hydrophobic amino acids present in Bnc3 to provide the most potent peptidomimetic-type *agr* inhibitor reported to date in groups-I, -II, and -IV, PhPr(3Br)-Bnc3. These studies also revealed that the coumaryl group is well tolerated as a tail in both Bnc3 and n7OFF scaffolds, providing new fluorescent probe molecules to explore QS signaling in *S. aureus*.

To start, we designed and synthesized a small collection of n7OFF analogues with different N-terminal benzyl-derived tails (Fig. 3). Linear peptide precursors were made via solid-phase peptide synthesis and capped at the N-termini with various phenylacetic and phenylpropionic (PhPr) acids, along with napthleneacetic (Nap), phenylbutyric (PhBu), and 7-hydroxycoumarin-4-acetic acid. The precursors were then cleaved, subjected to our reported solution-phase thioester macrocyclization protocol,[13] purified to homogeneity using reverse-phase HPLC, and characterized by MS (see Table S1). The *agr* inhibitory activity of each compound was tested in the four groups of *S. aureus* using cell-based reporter strains. These reporter strains produce yellow fluorescent protein (YFP) when the native AIP binds to AgrC and activates the QS system, which can be quantified using fluorescence (see SI). Exogenously added compounds that interfere with this binding and activation event will result in a decrease in fluorescence. The efficacy (% inhibition) and potency (IC_{50} value) of each compound was determined by testing across a range of concentrations. The IC_{50} values for representative compounds are listed in Table 1.; additional IC_{50}s are listed in Table S2.

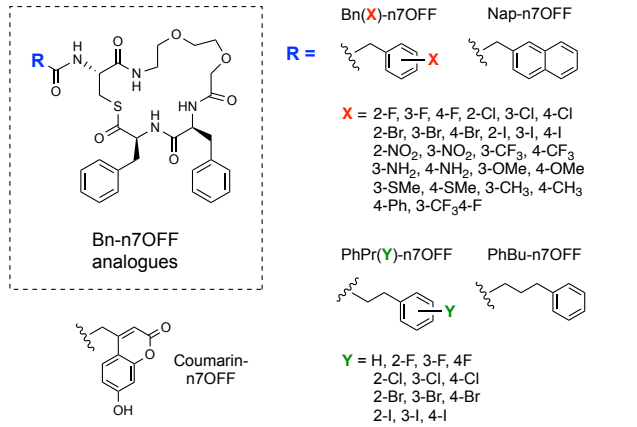

Fig. 3: Bn-n7OFF derivatives examined in this study. None affected *S. aureus* growth.

To explore the role of electronics on Bn-n7OFF activity, we attached electron donating groups (EDGs) and electron withdrawing groups (EWGs) at the ortho (2-), meta (3-), and para (4-) positions of the Bn tail of Bn-n7OFF (Fig. 3). We named these compounds Bn(#X)-n7OFF, where X indicates the substituent and # indicates its position. Initial investigations showed that the introduction of 3- and 4-Cl yielded comparable increases in potency in groups-I and -II, while only 3-Cl increased the potency in group-IV (Table 1). Both the 3- and 4-Cl derivatives showed decreased potency in group-III, and the 2-Cl substituent caused an overall loss of potency relative to Bn-n7OFF in all four groups. Turning to smaller and more electronegative fluorine, the 3-F analogue was the most potent compared to the 2-F and 4-F analogues, but none of the F analogues were more potent than Cl analogues (Table S2).

The larger halogens had more dramatic effects on *agr* inhibitory activity. The installation of 3-Br had 6-, 2-, and 6-fold potency increases in group-I, -II, and -IV, respectfully, relative to Bn-n7OFF (Table 1). The 4-Br analogue was not as potent as 3-Br but still demonstrated increased potency in groups -I, -II, and -IV, while 2-Br, like 2-Cl and 2-F, showed decreased potency

overall relative to Bn-n7OFF. The addition of iodine at all three positions gave rise to the most potent *agr* antagonists in the halogen series in group -II, III, and -IV, with comparable potency to the Br analogues in group-I. Overall, the best pan-group inhibitor in this series was Bn(3I)-n7OFF, which was 6-, 4-, and 60- fold more potent in groups-I, -II, -IV and had the similar potency in group-III compared to Bn-n7OFF (Table 1).

Next, we installed CF_3 and NO_2 groups on the Bn tail to explore the effects of other EWGs on Bn-n7OFF, and we observed potency enhancements in certain groups. Both the 3-CF_3 and 4-CF_3 analogues had analogous *agr* inhibitory potencies and were more potent in group-II and group-IV, while displaying similar potency in group-I and 10-fold lower potency in group-III, relative to Bn-n7OFF (Table 1). The 2-NO_2 derivative was slightly more potent in group-I and was around 5-fold more potent in group-IV compared to Bn-n7OFF. However, the potency decreased more than 2-fold in groups-II and -III. The 3-NO_2 analogue had similar potency compared to 2-NO_2 in group-IV but was less active in the other groups. In contrast to these enhancements for CF_3 and NO_2, adding the electron donating CH_3 group (at either -3 or -4) yielded derivatives that were all 5- to 10-fold less potent in groups-I, -II and -IV compared to Bn-n7OFF. Similar results were found for the OMe analogues. These results, in combination with the halogen results above, support the conclusion that the addition of EWGs to the Bn group generally increases the potency of Bn-n7OFF as an *agr* inhibitor (in all groups but group-III).

We generated additional analogues to further explore the effects of EDGs vs. EWGs on Bn-n7OFF potency. Surprisingly, installing SMe at the 3- and 4-positions increased potency compared with Bn-n7OFF in group-I and was more potent compared to other analogues with EDGs in groups -I, -II, and -III. Among all the EDGs tested, SMe has the largest steric size. Therefore, we reasoned that both the electronics and size of the Bn-substituent affect the potency of the Bn-n7OFF scaffold; this hypothesis is congruent with the trends in the halogen series, where the iodo analogues were the most potent overall.

To explore our hypotheses on substituent effects, we tested a Bn-n7OFF derivative with a sterically large, electron withdrawing phenyl substituent. Since only the 4-Ph analogue was readily accessible via synthesis, we compared this compound with the 4-I analogue. Although the phenyl group is larger than iodine, the phenyl analogue was only slightly more potent in group-I while retaining similar potency in the other groups relative to the 4-I analogue (Table 1). This result suggests that there can be size limitations to enhancing the potency of Bn-n7OFF at the 4-position.

We also examined a 3-CF_3 4-F analogue to explore whether the effects of EWGs on potency were additive. Bn(3-CF_3 4-F)-n7OFF had similar potency compared to the 3-I analogue in groups-I, -II, and -IV, and most notably, had a 2-fold increase in potency in group-III compared to Bn-n7OFF (Table 1). This compound was the first Bn-n7OFF analogue in this study to have an increased potency in group-III. This result is further congruent with the hypothesis that an electron deficient Bn group can enhance *agr* inhibitory potency for the Bn-n7OFF compound class (and now expands this to all specificity groups).

Table 1: IC_{50} values for Bn-n7OFF and Bnc3 analogues in group I–IV *S. aureus agr* reporter strains. All units in nM. 95% confidence intervals indicated in parentheses.[a]

compound	group-I	group-II	group-III	group-IV
Bn-n7OFF	15.9 (13.0-19.4)	51.6 (47.1-56.6)	14.5 (11.7-17.8)	1890[b] (>1020)
Bn(2Cl)-n7OFF	48.0 (36.7-65.1)	120 (86.7-176)	33.3 (23.7-46.4)	3190[b] (>1500)
Bn(3Cl)-n7OFF	6.27 (5.51-7.13)	33.8 (25.3-45.7)	58.5 (45.8-75.0)	111 (99.0-124)
Bn(4Cl)-n7OFF	8.31 (6.45-10.6)	22.6 (19.1-26.6)	53.4 (43.2-66.9)	3450[b] (>885)
Bn(3Br)-n7OFF	2.06 (1.72-2.45)	23.0 (21.0-25.1)	45.1 (41.7-48.7)	242 (230-255)
Bn(3I)-n7OFF	2.85 (2.34-3.44)	16.0 (13.8-18.5)	12.7 (8.16-19)	31.7 (27.5-36.8)
Bn(3CF_3)-n7OFF	8.35 (6.74-10.3)	14.3 (12.2-16.8)	103 (84.7-125)	136 (115-163)
Bn(2NO_2)-n7OFF	6.35 (5.32-7.50)	120 (93.7-158)	28.3 (21.9-36.4)	271 (222-331)
Bn(4Me)-n7OFF	53.4 (48.1-58.9)	78.0 (59.5-103)	38.6 (32.1-46.1)	4080[b] (>1500)
Bn(4SMe)-n7OFF	4.52 (3.35-6.01)	40.2 (35.4-45.6)	32.4 (20.8-49.0)	>1120[b]
Bn(4Ph)-n7OFF	1.41 (1.19-1.65)	15.5 (13.8-17.5)	46.5 (37.1-57.8)	147 (126-171)
Bn(4I)-n7OFF	3.36 (2.81-4.00)	14.0 (11.8-15.9)	31.5 (25.2-39.0)	136 (102-184)
Bn(3$CF_3$4F)-n7OFF	1.35 (1.01-1.73)	10.2 (8.29-12.7)	6.57 (5.50-7.76)	22.3 (18.7-26.5)
PhPr-n7OFF	1.42 (1.17-1.72)	21.0 (19.1-23.6)	149 (123-175)	525[b] (430-647)
PhBu-n7OFF	0.68 (0.56-0.83)	16.0 (13.8-18.6)	4.87 (4.16-5.57)	54.1 (44.2-67.1)
PhPr(2F)-n7OFF	0.50 (0.40-0.60)	17.9 (14.7-21.7)	9.50 (7.82-11.2)	53.7 (44.8-64.4)
PhPr(3F)-n7OFF	0.74 (0.62-0.88)	15.7 (13.3-18.6)	11.2 (9.06-13.9)	44.0 (35.5-52.8)
PhPr(3Cl)-n7OFF	0.90 (0.63-1.26)	12.8 (11.1-14.9)	19.5 (16.1-23.5)	49.9 (45.2-55.1)
PhPr(4Cl)-n7OFF	1.00 (0.88-1.14)	9.40 (7.04-9.96)	6.94 (6.26-7.68)	21.3 (19.0-23.9)
PhPr(3Br)-n7OFF	0.91 (0.79-1.01)	5.06 (4.01-6.44)	16.5 (15.0-18.2)	32.3 (28.2-36.7)
PhPr(4Br)-n7OFF	0.52 (0.44-0.62)	6.15 (4.86-7.77)	16.2 (14.0-18.6)	14.0 (12.7-15.3)
PhPr(3I)-n7OFF	1.83 (1.45-2.31)	4.49 (4.01-5.03)	8.35 (7.28-9.55)	20.8 (18.8-23.1)
Bnc3	2.10 (1.83-2.37)	8.30 (6.50-10.9)	0.30 (0.26-0.33)	8.37[b] (6.37-11.0)
PhPr(3Br)-Bnc3	0.36 (0.32-0.40)	2.00 (1.80-2.16)	0.39 (0.34-0.56)	0.56 (0.50-0.63)
Nap-n7OFF	9.24 (7.93-10.7)	22.3 (19.5-25.2)	19.1 (14.6-24.8)	297 (238-369)
Coumarin-n7OFF	3.08 (2.63-3.60)	38.1 (32.7-44.4)	69.8 (53.4-86.6)	95.7 (125-163)
Coumarin-Bnc3	1.15 (0.95-1.38)	20.0 (17.3-21.6)	0.59 (0.52-0.66)	4.01 (3.68-4.35)

[a]Borders in table group data by analogue type for clarity. IC_{50} values for additional compounds are listed in Table S2. Full details of reporter assay protocol in SI. [b]Compound unable to fully inhibit *agr* over conc. range tested.

We next investigated the effect of adding additional atoms between the phenyl and carbonyl group of the Bn tail, and synthesized homologs with two (PhPr-n7OFF) or three (PhBu-n7OFF) carbon linkages (Fig. 3). When n=2, the compound was more potent in group-I and group-IV compared to Bn-n7OFF. When n=3, compound potency increased in all groups relative to Bn-n7OFF. Notably, PhBu-n7OFF had a sub-nanomolar IC_{50} in group-I and a 3-fold potency increase in group-III. This result indicates that longer aliphatic chains can result in enhanced inhibitory potency within this compounds series.

To compare to the *agr* inhibitory trends observed for the Bn analogues above, we explored the effects of altering the tail group electronics on the activity of the extended analogue PhPr-n7OFF. We attached F, Cl, Br, and I at the 2-, 3-, or 4-positions of the Ph group; compounds in this category were named as PhPr(#Y)-n7OFF, where Y indicates the substituent and # indicates its position (Fig. 3.). All these derivatives were more potent than Bn-n7OFF, but no clear structure-activity trends were observed across the groups. For example, the IC_{50} values of 2-F, 3-F, and 4-Br analogues were all at high picomolar levels in group-I. However, iodo substituents in any position decreased potency relative to PhPr-n7OFF, suggesting a steric size limit on activity in group-I. In group-II, potency trended higher with the size of the halogen substituent, and except for Cl, the substituents were more potent in the 3-position. The 3-Br, 4-Br and 3-I substituents were the most potent and all had single nanomolar IC_{50}s. In group-III, all the compounds were around 10-fold more potent compared to PhPr-n7OFF. In group-IV, while only slight inhibitory activity was seen in the 2-Cl, 2-Br, and 2-I analogues, the other analogues all saw more than 5-fold potency increases compared to PhPr-n7OFF. These results indicate that Ph group substituents can alter the activity of the PhPr-n7OFF scaffold across *S. aureus agr* groups, but the activity trends are less obvious than those for Bn-n7OFF.

We were interested to combine one of the most potent N-terminal tails uncovered above with the related yet intrinsically more potent peptidomimetic scaffold—Bnc3 (Fig. 2B)—to determine whether this alteration could improve Bnc3's inhibitory profile. To do so, we installed the PhPr(3Br) group onto Bnc3 to generate PhPr(3Br)-Bnc3. This hybrid analogue maintained the potency of Bnc3 in group-III. More notably however, PhPr(3Br)-Bnc3 was 6-, 4-, and 10- fold more potent than Bnc3 and 30-, 25-, 3800- fold more potent than Bn-n7OFF in groups-I, -II, and -IV (Fig. 4). Further, this compound displayed dramatically improved efficacy in group-IV compared to Bnc3, capable of 95% vs. 65% inhibition, and was non-toxic in mammalian cells (Fig. S5). To our knowledge, PhPr(3Br)-Bnc3 represents the most potent peptidomimetic-type *agr* inhibitor across all four *S. aureus* groups reported to date.

Finally, we sought to install a sterically large aromatic group onto the tail of the Bn-n7OFF scaffold to determine if dye-type groups could be accommodated without loss of activity. We first replaced the phenyl ring with a naphthyl group (Nap-n7OFF, Fig. 3) and saw similar potency compared to its parent in groups-I and -III and increased potency in groups-II and -IV (Table 1). Observing tolerance for the naphthyl group, we replaced the Bn-tails of Bn-n7OFF and Bnc3 with a coumaryl group. Both compounds were inhibitory in all four groups (Table 1). Coumarin-n7OFF had improved potency in groups-I and -II, and a marked potency improvement in group-IV relative to Bn-n7OFF. Coumarin-Bnc3 also had improved potency in group-I and -IV relative to Bnc3. These fluorescently labeled compounds (spectra in Fig. S6) could be useful for biochemical studies of AgrC (assuming they interact with these receptors directly).

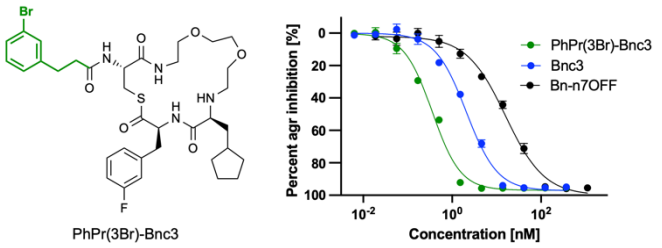

Fig 4: Structure of the most potent *agr* inhibitor identified (left) and its dose-response inhibition curve in comparison to Bnc3 and Bn-n7OFF in group-I *S. aureus* (right).

In summary, we have identified highly potent peptidomimetic inhibitors of the *agr* QS system in the formidable pathogen *S. aureus*. These compounds provide new bridges between peptide to non-peptide derived *agr* modulators, the latter of which are rare.[4] Studies aimed at further enhancing the potency of these peptidomimetics and understanding their mechanisms of action are ongoing and will be reported in due course.

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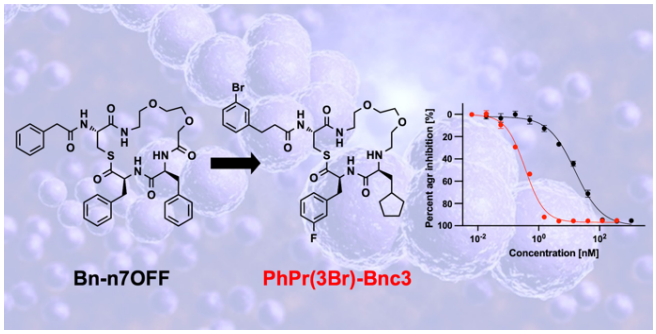
There are no conflicts to declare.

Notes and references

1. N. A. Turner, B. K. Sharma-Kuinkel, S. A. Maskarinec, E. M. Eichenberger, P. P. Shah, M. Carugati, T. L. Holland and V. G. Fowler, Jr., *Nat. Rev. Microbiol.*, 2019, **17**, 203.
2. C. Jenul and A. R. Horswill, *Microbiol. Spectr.*, 2019, **7**, GPP3-0031-2018.
3. O. Fleitas Martinez, M. H. Cardoso, S. M. Ribeiro and O. L. Franco, *Front. Cell. Infect. Microbiol.*, 2019, **9**, 74.
4. A. R. Horswill and C. P. Gordon, *J. Med. Chem.*, 2019, **63**, 2705.
5. B. Wang and T. W. Muir, *Cell Chem. Biol.*, 2016, **23**, 214.
6. M. Thoendel, J. S. Kavanaugh, C. E. Flack and A. R. Horswill, *Chem. Rev.*, 2011, **111**, 117.
7. S. Jarraud, C. Mougel, J. Thioulouse, G. Lina, H. Meugnier, F. Forey, X. Nesme, J. Etienne and F. Vandenesch, *Infect. Immun.*, 2002, **70**, 631.
8. G. J. Lyon, J. S. Wright, T. W. Muir and R. P. Novick, *Biochemistry*, 2002, **41**, 10095.
9. Y. Tal-Gan, D. M. Stacy, M. K. Foegen, D. W. Koenig and H. E. Blackwell, *J. Am. Chem. Soc.*, 2013, **135**, 7869.
10. Y. Tal-Gan, M. Ivancic, G. Cornilescu, T. Yang and H. E. Blackwell, *Angew. Chem. Int. Ed.*, 2016, **55**, 8913.
11. J. K. Vasquez, Y. Tal-Gan, G. Cornilescu, K. A. Tyler and H. E. Blackwell, *ChemBioChem*, 2017, **18**, 413.
12. J. K. Vasquez and H. E. Blackwell, *ACS Infect. Dis.*, 2019, **5**, 484.
13. Y. Tal-Gan, M. Ivancic, G. Cornilescu, C. C. Cornilescu and H. E. Blackwell, *J. Am. Chem. Soc.*, 2013, **135**, 18436.

TOC graphic



TOC text: Mimetics of a natural quorum sensing (QS) signal are shown to inhibit *agr*-type QS in *S. aureus*. These compounds include the most potent peptidomimetic-based QS inhibitor reported in *S. aureus*, remain active when tagged with a fluorescent label, and block *agr* in all four specificity groups.