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Synthetic ligands that activate and inhibit a quorum-sensing regulator in *Pseudomonas aeruginosa*

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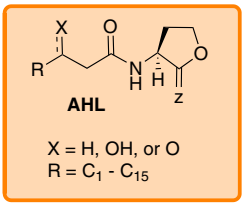
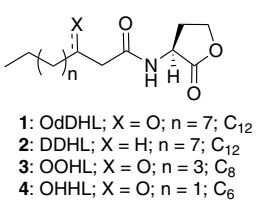
Abstract—The transcription factor QscR is a regulator of quorum sensing in *Pseudomonas aeruginosa* and plays a role in controlling virulence in this prevalent opportunistic pathogen. This study outlines the discovery of a set of synthetic *N*-acylated homoserine lactones that are capable of either activating or strongly inhibiting QscR in a cell-based reporter gene assay. We demonstrate that the synthetic antagonists inhibit ligand-dependent QscR binding to DNA. Several of these ligands can selectively modulate QscR instead of LasR, or modulate the activity of both receptors, and represent new chemical tools to study the hierarchy of quorum-sensing signaling in *P. aeruginosa*.

Bacteria use a set of simple signal molecules and their cognate protein receptors to monitor their local population densities in a process termed quorum sensing (QS).[1,2] Once they reach a sufficiently high population density, many bacteria undergo a lifestyle switch from that of solitary cells to that of a multicellular group. As a group, bacteria initiate processes that benefit the growing colony, and these behaviors can have significant impacts on their eukaryotic hosts. For example, virulence factor production and biofilm formation are under the control of QS in numerous clinically relevant pathogens, including *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[3] Mutants lacking functional QS systems have been shown to exhibit significantly reduced virulence both in vitro and in vivo. Therefore, QS has emerged as an attractive target for the development of new anti-infective therapies.[4,5] As QS is under the control of low molecular weight molecules and peptides, one strategy to attenuate QS-controlled behaviors is to design non-native molecules[6,7] or biomacromolecules[8] that can intercept or block native QS signals. Here, we report the discovery of a set of small molecules capable of either activating or inhibiting a key receptor regulating QS in *P. aeruginosa*, QscR.

QS in Proteobacteria relies on LuxI-type proteins that synthesize *N*-acylated L-homoserine lactone signals (AHLs, Fig. 1), and LuxR-type signal receptors.[1,2,9] LuxR-type receptor genes are often genetically linked to their associated LuxI-type synthases. Upon binding their cognate AHL ligand, LuxR-type receptors control the expression of genes involved in bacterial group behaviors. Interception of AHL:LuxR-type receptor





1: OdDHL; X = O; n = 7; C_{12}
2: DDHL; X = H; n = 7; C_{12}
3: OOHL; X = O; n = 3; C_8
4: OHHL; X = O; n = 1; C_6

Figure 1. Generic structure for *N*-acylated-L-homoserine lactones (AHLs; left) and structures of selected naturally occurring AHLs (**1**–**4**; right). Acyl chain carbon numbers are shown for clarity.

Keywords: *N*-acylated homoserine lactone; QscR; Quorum sensing.
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binding with a non-native ligand represents an approach to directly modulate QS-controlled processes, and has been the focus of considerable recent research.[5,6] Much of this work has focused on the LasR QS circuit in *P. aeruginosa*, which is regulated by *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL, **1**; Fig. 1) and, together with an additional LuxR-type receptor, RhlR, plays a principal role in pathogenesis.[10]

In 2001, Chugani et al. reported the discovery of a gene for a third LuxR-type protein in *P. aeruginosa* that is not genetically linked to an AHL synthase.[11] This 'orphan receptor,' termed QscR (quorum sensing control repressor), was later shown to respond to the LasR ligand, OdDHL (**1**), and control a set of genes unique from those controlled by LasR and RhlR.[12,13] Mutants with inactivated QscR were hypervirulent in an insect infection model.[11] These data suggest that QscR represents a novel target for the modulation of QS-controlled genes in *P. aeruginosa*.[14] To probe this hypothesis, we sought to identify synthetic ligands that either activated or inhibited QscR. Such ligands would represent chemical tools to study the molecular mechanisms of QS gene control by QscR and their role in pathogenesis.

AHLs with aliphatic acyl chain structures closely related to that of OdDHL (**1**) (e.g., dodecanoyl HL (DDHL, **2**; Fig. 1)) have previously been shown to activate QscR to similar, if not slightly higher, levels as **1** in cell-based reporter gene assays.[12] This expanded signal specificity has prompted the suggestion that QscR might also respond to AHL signals used by other bacteria that coexist with *P. aeruginosa*.[14] Such hypotheses have been put forth for other orphan LuxR-type receptors, such as SdiA from *Escherichia coli* and *Salmonella enterica*, along with certain LuxR-type receptors that have cognate signals.[2,9] We reasoned that QscR could be susceptible to modulation by other, non-native AHLs, and therefore focused on this ligand class in the present study.

We recently reported an efficient, solid-phase synthetic route to AHLs and the design and synthesis of several focused libraries of AHLs.[15] These libraries contained AHLs with differing acyl chain length, lactone stereochemistry, and functional groups in the acyl chain.[16] They also included several naturally occurring AHLs, such as **1**–**4** (Fig. 1). Evaluation of these ~90 compounds in a range of Gram-negative bacteria (e.g., *P. aeruginosa*, *Agrobacterium tumefaciens*, and *Vibrio fischeri*) revealed several AHLs with activities ranging from that of potent inhibitors to a super-activator of LuxR-type protein function.[16,17] Accordingly, these AHL libraries represented a logical set of compounds with which to start our search for synthetic, AHL-derived QscR activators and inhibitors.

We screened our AHL libraries for agonistic and antagonistic activity against QscR using a previously reported, recombinant *E. coli* strain that reports QscR activity *via* the production of β-galactosidase (β-gal) from a promoter fusion.[18] OdDHL (**1**) and DDHL (**2**) served as positive controls for these assays. All AHLs were examined at 5 μM concentrations. Agonism assays contained AHL library member alone, while competitive antagonism assays were performed against DDHL (**2**, at 10 nM).[19] [DDHL (**2**) was found to activate QscR at lower concentrations relative to OdDHL (**1**), and thus provided a more stringent control for these antagonism assays.] We observed that 11% of the AHL library was able to activate QscR to ⩾50%. In turn, 6% of the library was capable of *inhibiting* QscR by ⩾75% in these reporter gene assays. Identifying such a large percentage of active compounds further underscores the value of screening these previously validated AHL libraries against LuxR-type receptors. We focused on these most active QscR agonists and antagonists for the remainder of this study.[20]

The structures of the eight most active QscR agonists are shown in Figure 2, and their primary assay data are listed in Table 1. No AHLs were identified in the primary assay with agonistic activities that surpassed that of controls OdDHL (**1**) or DDHL (**2**). Not surprisingly, many of these compounds had structures closely related to that of OdDHL (**1**), e.g., **A3**, **A4**, **A8**, **A16**, and OOHL (**3**), corroborating data reported by Lee et al. in this same reporter strain.[12] However, the remaining four AHLs (**B2**, **B7**, **C22**, and **D7**) had structures that diverged from those of **1** or **2**, most notably **B2**, which had D-stereochemistry. All four AHLs contained aromatic acyl groups. We previously observed that **A4**, **A8**, **B2**, and **C22**, but not **A16** and **D7**, are also weak to strong activators of LasR.[16,17] More notably, however, the other three QscR activators, OOHL (**3**), **A3**, and **B7**, are also capable of *inhibiting* LasR (by ⩾50%). These screening data suggest that activation of QscR by AHLs is not restricted to unbranched, aliphatic AHLs. Moreover, they indicate that the development of ligands that specifically modulate QscR instead of LasR, or that activate or inhibit both receptors, will be feasible. A recent report by Müh et al. of a non-AHL ligand (termed TP-1) that activates LasR, yet does not activate QscR, provides further support for these data.[21]

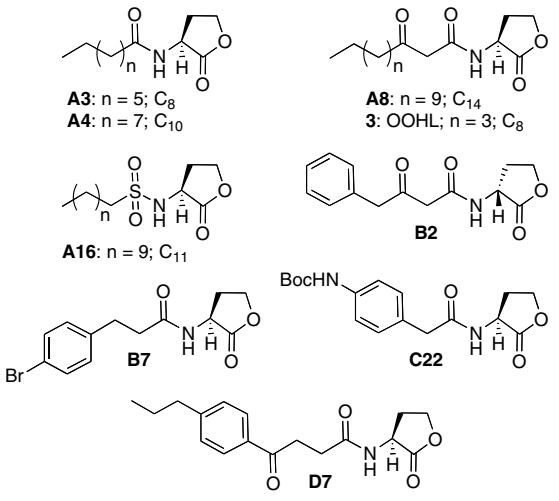

Figure 2. Structures of the synthetic QscR agonists identified in this study. Selected acyl chain carbon numbers are shown for clarity.

Table 1. Percent activation data and EC_{50} values for QscR agonists[a]

Compound	Activation (%)	EC_{50}[b] (nM)
1: OdDHL	109	22
2: DDHL	100	5
3: OOHL	74	11[c]
A3	59	—[d]
A4	104	6
A8	78	56[c]
A16	51	—
B2	59	—
B7	64	—
C22	61	—
D7	54	—

[a] For strain and assay information, see Refs. 18 and 19. Compounds evaluated at 5 μM. Activity for DDHL (**2**) set to 100%. All assays performed in triplicate. Error = ±8%. Shaded compounds are controls.
[b] Determined by testing AHLs over a range of concentrations (0.02–2×10^5 nM).
[c] Dose–response curve reached a plateau over the concentrations tested, yet the level of maximal induction was ~25% lower than that for OdDHL (**1**); EC_{50} value calculated from this dose–response curve. See Figure S-3 in Supp. data.
[d] Not determined.

Table 2. Percent inhibition data and IC_{50} values for QscR antagonists[a]

Compound	Inhibition (%)	IC_{50}[b] (nM)
B6	78	180[c]
C6	73	—[d]
C8	71	—
C11	70	—
C14	71	—
C18	88	30
C25	75	160[c]
D12	77	200[c]
D13	76	130

[a] For strain and assay information, see Refs. 18 and 19. Compounds evaluated at 5 μM against DDHL (**2**) at 10 nM. Error = ±8%.
[b] Determined by testing AHLs over a range of concentrations (0.02–2×10^5 nM) against **2** at 10 nM.
[c] Antagonism dose–response curve upturned at higher concentrations; see Supp. data.
[d] Not determined.

We performed dose–response agonism assays on the most active AHLs in this set and OdDHL (**1**) and DDHL (**2**) using the β-gal reporter strain, and determined their EC_{50} values (Table 1). These assays revealed that the control DDHL (**2**) and **A4** exhibited the lowest EC_{50} values in this study (i.e., ~5 nM), with OOHL (**3**) having the next lowest value; these trends were congruent with our primary agonism assay data.

The antagonism assay of the AHL library revealed five non-native AHLs capable of inhibiting QscR by ⩾75% (shown in Fig. 3). Their IC_{50} values are listed in Table 2. Four phenylacetanoyl HLs (PHLs) were uncovered as slightly weaker QscR antagonists (~70%) and are also included here in our data analysis.

Like the QscR agonists, the acyl chain structures of these QscR antagonists ranged from relatively compact (**B6**) to long and lipophilic (**D13**), making the development of specific structure–activity relationships (SARs) for AHL-mediated QscR inhibition challenging. Some

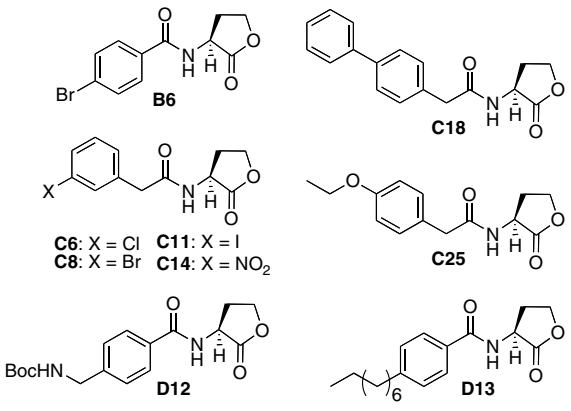

Figure 3. Structures of nine QscR antagonists identified in this study.

trends were clear, however. All of the QscR antagonists contained aromatic acyl groups and native (L) lactone stereochemistry. Three of the most effective inhibitors had benzoyl groups (**B6**, **D12**, and **D13**); potent LuxR-type receptor inhibitors with this type of acyl chain are yet to be reported.[16,17] PHLs were also strong antagonists of QscR (i.e., the **C#** AHLs), with **C18** the most active QscR antagonist uncovered in this study (IC_{50} = 30 nM; Table 2). This PHL is only a weak LasR inhibitor (20%); however, PHLs **C6**, **C8**, **C11**, and **C14** with *meta*-halogen or nitro groups are also moderate antagonists of LasR (~50%).[16,17] None of the other QscR antagonists displayed appreciable inhibitory activity against LasR. Interestingly, the other active PHL identified in this study, **C22**, is an *activator* of QscR at the concentration tested (Table 1), and further highlights PHLs as a versatile structure class for the development of both inhibitors and activators of LuxR-type receptors.[15b]

We sought to obtain further insights into the mechanism of QscR modulation by non-native AHLs. Previous work by Lee et al. has shown that QscR specifically binds the promoter region of the PA1897 gene in the presence of OdDHL (**1**).[12] In view of the reporter gene assay data outlined above, we reasoned that our QscR antagonists could competitively inhibit the binding of QscR to DNA in the presence of OdDHL (**1**). We therefore performed electromobility shift assays (EMSA) on purified QscR and radiolabeled PA1897 promoter in the presence of selected QscR antagonists and **1**.[19] The results of these assays are listed in Table 3.

The EMSAs revealed that all five AHLs (**B6**, **C18**, **C25**, **D12**, and **D13**) were capable of inhibiting OdDHL (**1**)-dependent QscR:DNA binding. At a 100:1 ratio with OdDHL (**1**), PHL **C18** inhibited OdDHL-dependent binding by 85%, while **D12** was 26% inhibitive (Table 3). This activity trend correlated well with the cell-based reporter assays (Table 2), where **C18** had a sixfold lower IC_{50} relative to **D12**. All of the antagonists could further retard QscR:DNA binding at 1000:1 ratios versus OdDHL (**1**), with **C18** and **D13** reducing binding by 100%. These data suggest that the mechanism of QscR antag-

Table 3. EMSA data for QscR binding the PA1897 promoter in the presence of OdDHL (**1**) and antagonists[a]

Compound	% Inhibition with 100× ligand[b]	% Inhibition with 1000× ligand
B6	29 ± 12	71 ± 16.3
C18	85 ± 29	100 ± 7
C25	47 ± 1	82 ± 9
D12	26 ± 8	59 ± 1
D13	59 ± 6.3	100 ± 4.8

[a] His-tagged QscR was expressed and purified as previously reported; see Ref. 12. Competition experiments performed in the presence of 2 μM OdDHL (**1**) and either 200 μM or 2 mM ligand.

[b] Results are presented as % inhibition of OdDHL (**1**)-dependent DNA shift. OdDHL (**1**) at 2 μM shifts DNA by 72 ± 10% and no OdDHL (**1**) shifts DNA by 38 ± 7.4%.

onism by these synthetic AHLs is inhibition of OdDHL (**1**)-dependent QscR:DNA binding. We speculate that these ligands directly bind QscR and/or displace OdDHL (**1**), yielding a QscR:AHL complex with lowered affinity for the PA1897 promoter sequence. In turn, we hypothesize that our AHL activators bind QscR and promote QscR:DNA binding; EMSAs on selected synthetic QscR agonists are ongoing to probe this model.

In summary, we have identified a series of AHLs that are capable of activating or inhibiting the LuxR-type receptor, QscR, from *P. aeruginosa*. Several of the antagonists were capable of inhibiting OdDHL (**1**)-dependent QscR:DNA binding. These ligands represent the first non-native small molecule modulators of QscR. We have also discovered synthetic AHLs that either selectively activate or inhibit QscR instead of LasR, or activate or inhibit both receptors. The most interesting non-native ligand in this class is perhaps AHL **B7**, as it can moderately activate QscR and has previously been shown to strongly inhibit LasR.[16,17] This bifunctional compound represents a unique tool to probe the role of QscR as a negative regulator of QS pathways in *P. aeruginosa*. Activating QscR, while simultaneously repressing LasR, could have a synergistic effect on virulence inhibition in *P. aeruginosa*. Likewise, synthetic ligands that activate or inhibit both QscR and LasR (e.g., **B2** and **C11**), or modulate QscR specifically (e.g., **D7** and **C18**), could prove valuable in delineating the hierarchy of these two receptors in QS control.

Ongoing studies are focused on the development of more quantitative assays to characterize the interactions between these synthetic AHLs and QscR, and the design of second-generation ligands for QscR according to SARs delineated in this study. This work will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.11.095.

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20. Compound numbering matches that used in earlier publications reporting these libraries. See Refs. 16 and 17 and Supplementary data.
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