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Non-Native *N*-Aroyl L-Homoserine Lactones Are Potent Modulators of the Quorum Sensing Receptor RpaR in *Rhodopseudomonas palustris*

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Abstract

Quorum sensing (QS) is a process by which bacteria use low molecular weight signaling molecules (or autoinducers) to assess their local population densities and alter gene expression levels at high cell numbers. Many Gram-negative bacteria use *N*-acyl L-homoserine lactones (AHLs) with aliphatic acyl groups as signaling molecules for QS. However, bacteria that utilize AHLs with aroyl acyl groups have been recently discovered, including the metabolically versatile soil bacterium *Rhodopseudomonas palustris*, which uses *p*-coumaroyl HL (*p*-cAHL) as its QS signal. This autoinducer is especially unusual because its acyl group is believed to originate from a monolignol (*i.e.*, *p*-coumarate) produced exogenously by plants in the *R. palustris* environment, rather than through the endogenous fatty acid biosynthesis pathway like other native AHLs. As such, *p*-cAHL could signal not only bacterial density but also the availability of an exogenous plant-derived substrate, and may even constitute an interkingdom signal. Similar to other Gram-negative bacteria, QS in *R. palustris* is controlled by the *p*-cAHL signal binding its cognate LuxR-type receptor, RpaR. We sought to determine if non-native aroyl HLs (ArHLs) could potentially activate or inhibit RpaR in *R. palustris*, and thereby modulate QS in this soil bacterium. Herein, we report the testing of a set of synthetic ArHLs for RpaR agonism and antagonism using a *R. palustris* reporter strain. Several potent non-native RpaR agonists and antagonists were identified. Additionally, the screening data revealed that lower concentrations of ArHL are required to strongly agonize RpaR relative to antagonizing RpaR. Structure-activity relationship (SAR) analyses of the active ArHLs indicated that potent RpaR agonists tend to have sterically small substituents on their aryl groups, most notably in the *ortho* position. In turn, the strong RpaR antagonists were based on either the phenylpropionyl HL (PPHL) or the phenoxyacetyl HL (POHL) scaffold, and many contained an electron-withdrawing group at either the *meta* or *para* positions of the aryl ring. To our knowledge, the compounds reported herein represent the first abiotic chemical modulators of RpaR, and more generally, the first abiotic ligands capable of intercepting QS in bacteria that utilize native ArHL signals. In view of the novel origins of the *p*-cAHL signal in *R. palustris*, the largely unknown role of QS in this bacterium, and *R. palustris*' unique environmental lifestyles, we anticipate that these compounds could be valuable as chemical probes to study QS in *R. palustris* in a range of fundamental and applied contexts.

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Keywords

N-Acyl homoserine lactone; Bacteria; Cell-cell signaling; LuxR-type receptor; Quorum sensing; *Rhodopseudomonas palustris*; RpaR

Introduction

Bacteria can sense and respond to their environment through a variety of pathways, including quorum sensing (QS).^[1] QS allows bacteria to assess their local population densities using small signal molecules (or autoinducers) and initiate specific group behaviors when a critical cell density is achieved. For example, many pathogens use QS to control virulence factor production and biofilm formation once they reach a threshold cell number on a eukaryotic host.^[2] In turn, symbionts often use QS to initiate mutually beneficial relationships with a host at high cell densities.^[3] Gram-negative bacteria have arguably the best-characterized QS systems, which are most commonly regulated by *N*-acyl L-homoserine lactone (AHL) signals.^[4] These cell permeable molecules are derived from the bacteria's endogenous fatty acid biosynthesis pathway and typically possess aliphatic acyl groups of varying lengths (4–18 carbons) and with different oxidation states at the 3-position.^[5] Selected naturally occurring AHLs are shown in Scheme 1A, including the signals used by the marine symbiont *Vibrio fischeri* (OHHL) and the opportunistic pathogen *Pseudomonas aeruginosa* (BHL and OdDHL). AHL-regulated QS circuits consist of a synthase (or LuxI-type) protein that produces the AHLs and a cytoplasmic receptor (or LuxR-type) protein that binds the AHLs and behaves as a transcriptional regulator. A threshold population of cells, and therefore local AHL concentration, is needed for productive AHL:receptor binding.^[4a, 4b] The AHL:LuxR-type receptor complex then commonly dimerizes, binds to specific QS promoters, and alters the transcription of genes associated with QS phenotypes.^[6]

Our laboratory and several others have been engaged in the design and development of non-native AHLs as chemical probes to study QS pathways in bacteria.^[5a, 7] The ability to modulate QS with spatial and temporal control using small molecules is of significant value for the elucidation of the role of this intercellular signaling pathway in a range of biologically relevant contexts.^[8] Over the past several years, we have synthesized a library of over 250 AHL analogues, the bulk of which consist of AHLs with modified acyl tails since the acyl tail generally imparts LuxR-type receptor selectivity for natural AHLs.^[7c, 7j, 7l, 9] We have examined these compounds in a range of Gram-negative bacteria and have uncovered non-native AHLs capable of strongly agonizing and antagonizing many LuxR-type receptors, and thereby, numerous QS-controlled phenotypes.

A major focus of our recent work has been to delineate the structure-activity relationships (SARs) for our AHL probes to determine which features are needed for agonism, antagonism, and receptor selectivity for LuxR-type proteins.^[7c, 7g, 7h, 7j, 7l] The majority of the most active non-native AHLs contain aroyl groups with varying substitution patterns on the phenyl ring and short “linkers” (1–3 atoms) between the aryl ring and the amide bond. These aroyl HL (ArHL) structures were assumed to be inaccessible in Nature as they are not derived from the fatty acid biosynthesis pathway, the source of all of the natural AHL acyl chains.^[10] Therefore, we were very interested to read the 2008 report by Harwood and co-workers^[11] of a Gram-negative bacterium (*Rhodopseudomonas palustris*) that uses a natural ArHL (*p*-coumaroyl-HL (*p*-cAHL); Scheme 1B) to control QS. Indeed, we had actually synthesized a derivative of *p*-cAHL in one of our original libraries of “non-native” AHLs and found that it possessed QS modulatory activity in other Gram-negative bacteria.^[7l] More recently, Greenberg and co-workers reported that a related ArHL, cinnamoyl HL

(**B10**); shown in Scheme 2), is used by the photosynthetic stem-nodulating *Bradyrhizobium* ORS278,^[12] suggesting that ArHLs could be more common QS signals than originally anticipated. Very little is known about the structural features of ArHLs necessary to activate the LuxR-type receptors in these Gram-negative bacteria. In the current study, we focus on ArHL-based QS signaling in *R. palustris*.

R. palustris is a purple, soil-dwelling bacterium unique in its ability to survive in a variety of environments by utilizing four distinct modes of metabolism: photosynthetic, photoheterotrophic, chemoautotrophic, and chemoheterotrophic.^[13] Under photosynthetic conditions, *R. palustris* can convert nitrogen gas into ammonia, a process known as nitrogen fixation.^[14] *R. palustris* can also degrade plant lignols,^[15] which presents a major hurdle in the chemical conversion of biomass.^[16] These valuable abilities, amongst others, have attracted considerable interest to this versatile bacterium. In 2008, Harwood and co-workers identified RpaI and RpaR as the LuxI/LuxR homologs in *R. palustris*, which act to synthesize and sense the *p*-cAHL signal, respectively.^[11] Interestingly, the acyl tail of *p*-cAHL is not derived from an endogenous metabolite in *R. palustris*, but rather originates from exogenous *p*-coumarate, a monolignol commonly found in the plant material on which *R. palustris* commonly lives, which is then processed by RpaI (as *p*-coumaryl coenzyme A (CoA)) to generate *p*-cAHL. Therefore, *p*-cAHL could actually provide dual cues for *R. palustris*, signaling both cell density and the presence of plant metabolites (and thereby, plant hosts) in its local environment. *Bradyrhizobium* ORS278 also lives in association with plants and uses an ArHL for QS (cinnamoyl HL, **B10**); however, in contrast to *R. palustris*, the cinnamic acid precursor for this ArHL appears to be produced by the bacterium as opposed to the plant host.^[12]

Numerous questions abound about the mechanisms by which *R. palustris* uses *p*-cAHL as an intercellular signal, and possibly even an interkingdom signal. For example, several of the genes regulated by RpaR appear to be involved with chemotaxis,^[11, 17] providing an interesting connection between this important motility mechanism and QS in *R. palustris*. However, no obvious phenotype is apparent in *R. palustris* QS mutants so far.^[18] Activation of RpaR by *p*-cAHL has also been shown to activate a novel *rpaR* antisense transcript, which could play a role in QS-controlled gene activation. Such small regulatory RNAs have been implicated in the control of QS in other bacteria.^[19] We sought to identify non-native ArHLs that eventually could be used as chemical probes to investigate these and other research questions in *R. palustris*. Toward this goal, we report herein the evaluation of a set of synthetic ArHLs for non-native RpaR agonists and antagonists using an RpaR bacterial reporter strain. Several potent, abiotic RpaR modulators were identified, and a set of SARs for ArHL-based signaling in *R. palustris* was generated. These compounds represent, to our knowledge, the first non-native ligands capable of intercepting QS in bacteria that utilize native ArHL signals.

Results and Discussion

AHL Library Selection

We selected a set of 41 AHLs from our previously reported AHL libraries for screening in RpaR (shown Scheme 2). We chose to screen predominantly ArHLs due to the structural resemblance of these compounds to *p*-cAHL (**B11** and **D5** were the only non-aroyl derivatives studied, yet still were structurally similar). The ArHLs contained aroyl group substituents of varying size and electronics to probe their potential effects on activity in RpaR, and the majority were either phenylacetyl HLs (PHLs), phenoxyacetyl HLs (POHLs), or phenylpropionyl HLs (PPHLs). Several of these compounds have previously been shown to have strong activities as LuxR-type receptor antagonists or agonists in other Gram-negative bacteria that use fatty acid-derived AHLs as their native QS signal;^[7j-1] we were

interested to determine if these activity profiles would be maintained in a bacterium that uses a native ArHL for QS.

We augmented this first set of AHLs with a new, focused library of cinnamoyl-type HLs (**1**–**19**) that was designed to very closely resemble *p*-cAHL and further explore the effects of varying substituents on the phenyl ring (Scheme 3). We included one non-cinnamoyl HL in this set (AHL **19**) that lacked the conjugated alkene, in order to probe the necessity for this functionality in the activity of *p*-cAHL. The library was synthesized in solution from readily available carboxylic acids and L-homoserine lactone using standard amide bond coupling reactions (see Experimental Section). The 19 cinnamoyl-type HLs were isolated on ~100 mg scale in 65–85% yields with purities of 90–95%.

Compound Screening

We evaluated the 60 ArHLs for agonistic and antagonistic activity in RpaR using a *R. palustris* reporter strain (CGA814). This strain lacks a functioning RpaI synthase, yet retains a functional RpaR receptor, and reports RpaR activity *via* β -galactosidase (see Experimental Section for strain and assay details).^[11] The ArHLs were tested alone in the RpaR agonism assays, and were tested against *p*-cAHL (at its EC₅₀ value of 1 nM) in the competitive RpaR antagonism assays. A preliminary screen was conducted using 10 μ M ArHL to identify both agonists and antagonists of RpaR. Over 75% of the ArHLs showed very strong activities in the preliminary screen, which provided initial data to support that RpaR can be both agonized and antagonized by non-native ArHLs (see Supporting Information for full data). In order to narrow this set of initial lead compounds, ArHLs exhibiting activities of greater than ~70% agonism or ~50% antagonism in the preliminary screen were rescreened at ten-fold lower concentration (1 μ M). The results of this primary screen are shown in Tables 1 and 2. Compounds with RpaR agonistic activities of greater than ~40% or RpaR antagonistic activities of greater than ~60% were subjected to dose response analysis using the same reporter gene assays, and EC₅₀ or IC₅₀ values were calculated, respectively (listed in Tables 1 and 2).

Biological Assay Results

Overall, we identified several potent RpaR agonists and antagonists in the *R. palustris* reporter gene assays. The majority of the RpaR agonists were found in the new cinnamoyl-type HL library, while the majority of the RpaR antagonists were from our set of in-house ArHLs. Each class of RpaR modulators is discussed in turn below.

Fluoro cinnamoyl HLs **1**, **2**, and **4**, 2-bromo cinnamoyl HL **10**, and cinnamoyl HL **B10** were the strongest RpaR agonists identified (Table 1). The 2-fluoro and 2,6-difluoro cinnamoyl HLs (**1** and **4**) were the most potent overall, with EC₅₀ values comparable to the native ligand for RpaR, *p*-cAHL (~1 nM). Few potent, non-native AHL-based agonists of LuxR-type receptors have been identified;^[7g, 7k] therefore, the discovery of **1** and **4** as strong RpaR agonists is notable. Moving the fluoro substituent to the 3-position (*i.e.*, in **2**) caused a 20-fold drop in agonistic activity relative to **1**, while the 4-fluoro cinnamoyl HL **3** was inactive at 1 μ M. The 2-bromo cinnamoyl HL (**10**) was also ~20-fold less active than its 2-fluoro cinnamoyl HL analog (**1**), and agonistic activity further decreased when an additional halogen substituent was added to the aryl group (*i.e.*, in **7** and **8**). These data indicate that RpaR is highly sensitive to AHL aryl group substitution patterns, and has a preference for sterically small halogens in the 2-position (or none at all, as in **B10**). Similar high sensitivities to halogen substituents have been observed for PHLs in other LuxR-type receptors (*e.g.*, LuxR, TraR, LasR, and ExpR1/ExpR2).^[7g, 7j–l] The PPHL analog of *p*-cAHL (**19**), only lacking the acyl chain double bond, had an EC₅₀ value 500-fold higher

than *p*-cAHL, indicating that the more rigid, conjugated acyl tail is important for agonistic activity in RpaR. We return to this hypothesis below.

Previous studies by Harwood and co-workers have shown that the *R. palustris* reporter strain (CGA814) can be weakly induced (10%) by closely related *p*-cAHL analogs such as cinnamoyl HL (**B10**), *ortho*-cAHL (**15**), *meta*-cAHL (**16**), and caffeoyl HL (**17**), with *meta*-cAHL (**16**) the most active overall (at 10%) relative to *p*-cAHL.^[11] The authors also observed the ferulic acid derivative (**18**) to be inactive as an RpaR agonist. However, these results were obtained by testing extracts isolated from wild-type *R. palustris* grown in media containing the requisite aromatic acid substrate for ArHL synthesis, as opposed to testing the purified ArHL directly, as we did in the current study. Thus, the former assay also measures the ability of the acid (as an acyl-CoA conjugate) to be processed by RpaI into the respective ArHL. Despite this difference in assay techniques, our agonism assay data largely corroborates this past report with the exception of cinnamoyl HL (**B10**), which we found to be considerably more active as a RpaR agonist (EC₅₀ value = 32.3 nM). We note that **B10** is also the native ArHL used by the closely related bacterium *Bradyrhizobium* ORS278 (see above).^[12]

Turning to the antagonism assay data (Table 2), the bulk of the active compounds identified in the primary screens were PPHL and POHLs, or very closely related derivatives. The majority of these compounds had halogens or nitro groups in the 4-position, with a smaller subset having these same substituents in the 3-position. Of these lead compounds, cyclohexyl HL **B11**, POHL **E14**, 3-NO₂ POHL **E25**, 4-F PPHL **E29**, 4-NO₂ PPHL **E37**, and 1,3-benzodioxole PPHL **E39** were the strongest antagonists of RpaR identified. These non-native AHLs were capable of inhibiting RpaR by 63–89% at 1 μM; we note, however, that their IC₅₀ values are ~200–400 times larger than the EC₅₀ value for *p*-cAHL (Table 2). This activity trend indicates that, for the AHLs tested in this study, RpaR antagonism requires higher concentrations of non-native AHL relative to RpaR agonism by either native or non-native AHL. Such a trend has previously been observed for non-AHL modulators of LasR in *P. aeruginosa*; Müh and co-workers identified triphenyl derivatives capable of strongly activating or moderately inhibiting LasR in reporter gene assays (TP-1 and TP-5, respectively),^[20] and Zou and Nair have provided a structural rationale for this divergent behavior.^[21] Similarly delineating the mechanisms of RpaR agonism and antagonism by ArHLs would also be interesting.

Structure-Activity Relationship (SAR) Synopsis

The SARs for active RpaR modulators identified in this study are relatively straightforward (shown schematically in Scheme 4). Strong RpaR agonists have cinnamoyl-type HL structural scaffolds and most have halogens at the 2-position on the aromatic ring (*e.g.*, **1**, **4**, **8**, and **10**). Activity increases with decreasing halogen size. Interestingly, the native ligand *p*-cAHL has a hydroxyl group in the 4-position as opposed to the 2-position on the aromatic ring, and placement of this hydroxyl group in any other position causes a significant reduction in activity (*i.e.*, ArHLs **15–17**; Table 1). Alternatively, strong RpaR antagonists are largely based on the PPHL or POHL scaffolds and most contain electron withdrawing substituents at either the 3- or 4-positions on the aromatic ring (*e.g.*, **E25**, **E29**, and **E37**). These SAR indicate that ArHLs that lack the conjugated alkene bridge exhibit heightened antagonistic activities against RpaR relative to cinnamoyl-type HLs. Assuming these non-native ArHLs can target the native ligand binding site in RpaR, one can then posit that their more flexible acyl groups are able to make contacts with RpaR that are antagonistic, while the more rigid cinnamoyl-type HLs are able to engage in interactions that are agonistic (with the exception of **9** and **14**). This hypothesis is further supported by the low agonistic activity of PPHL **19**, the analog of *p*-cAHL that only lacks the alkene bridge. We also note that

aromaticity is non-essential for RpaR antagonism by PPHLs, as cyclohexyl HL **B11**, a fully saturated version of PPHL **B9**, was one of the most potent RpaR antagonists uncovered in this study.

In view of the more moderate IC₅₀ values for the RpaR antagonists identified herein relative to the EC₅₀ values of the RpaR agonists, the SAR above will be useful for the design of next-generation AHLs with potentially improved antagonistic activities against RpaR. For example, evaluating ArHLs with longer and more flexible linkers (>2 atoms) between the aromatic group and the amide group could be productive. The inclusion of alternate heterocycles or carbocycles in the acyl group, or sterically larger substituents in the 3 or 4-position of the PPHL or POHL aromatic group, would also be valuable.

Multispecies Ligand Activity Trends

One advantage of screening AHLs from our in-house AHL library is that they have been screened previously for activity in a range of other LuxR-type proteins from Gram-negative bacteria including LuxR in *Vibrio fischeri*,^[7k, 7l] LasR and QscR in *Pseudomonas aeruginosa*,^[7i, 7j, 7l] TraR in *Agrobacterium tumefaciens*,^[7l] and ExpR1/ExpR2 in *Pectobacterium carotovora*.^[7g] Thus, we have been able to uncover structural features of specific AHLs that render them either broad-spectrum modulators, capable of activating or inhibiting many of the LuxR-type receptors, or those that render them selective for one particular receptor.^[7h] Comparing these previous activity trends with the results for RpaR in the current study revealed that RpaR shares many SAR for AHL-based modulation with LuxR from *V. fischeri*. Both receptors are inhibited by similar ArHLs; for example, **B11**, **E25**, **E29**, and **E37** were strong antagonists of both RpaR and LuxR.^[7j, 7l] Conversely, TraR, which we have shown is more selective for both antagonists and agonists relative to other LuxR-type receptors,^[7h, 7j] shares few active ligands with RpaR, suggesting that the ligand binding site in RpaR may be significantly different than the binding site in TraR. The X-ray crystal structure of TraR bound to its native ligand (OOHL, Scheme 1A) does reveal a ligand-binding site that tightly envelops OOHL in the TraR interior.^[22] Another discovery made through this comparative analysis of AHL activity trends was that POHL **E14** is a good antagonist in RpaR, yet is largely inactive in other LuxR-type receptors either as an agonist or antagonist.^[7j] Thus, **E14** represents a novel receptor-selective inhibitor active in *R. palustris*. The identification of such a selective ligand points towards possible future experiments to explore the role of QS in mixed microbial systems (*e.g.*, in the soil and on plant surfaces where *R. palustris* is found) using receptor selective modulators.

Summary and Conclusions

The unique structure and source of the *p*-cAHL signal used by *R. palustris* for QS make this compound and its underlying role in intercellular (and possibly interkingdom) signaling important targets for study. Herein we have reported our design, synthesis, and biological evaluation of a set of non-native ArHLs for agonism and antagonism of the LuxR-type QS receptor, RpaR, in *R. palustris*. The cell-based reporter gene assays revealed a suite of highly potent agonists and several moderately potent antagonists of RpaR. To our knowledge, these compounds represent the first abiotic chemical modulators of RpaR, and more generally, the first abiotic ligands capable of strongly intercepting QS in a bacterium that utilize a native ArHL signal. The screening data allowed for well-defined SARs to be established for both RpaR agonists and antagonists. The non-native RpaR agonists all had structures closely related to *p*-cAHL. Two *p*-cAHL analogs containing fluorine substitutions on the aryl ring were found to activate RpaR at single digit nanomolar concentrations, with the same potency as the native *p*-cAHL signal in *R. palustris*. Six other ArHLs were also able to activate RpaR at submicromolar concentrations. In turn, six RpaR antagonists were identified that could competitively inhibit RpaR at high nanomolar concentrations versus *p*-

cAHL at its EC₅₀ value. These non-native antagonists all contained acyl groups that were sterically larger than that of *p*-cAHL, and the majority contained electron withdrawing substituents on the aryl group. Additional research is necessary to delineate the molecular mechanisms by which these AHLs interact with RpaR; such biochemical and structural studies are ongoing in our laboratory with several LuxR-type receptors. In view of the novel origin of the *p*-cAHL signal in *R. palustris*, the ability of *R. palustris* to adopt a range of unique environmental lifestyles, and the largely unknown role of QS in this bacterium, we anticipate that the AHLs reported herein could be valuable as chemical tools to study QS in *R. palustris* in a variety of contexts.

Experimental Section

Synthesis of cinnamoyl-type HLs

For the synthesis of cinnamoyl-type HLs without phenol functionalities, the HBr salt of L-homoserine lactone (0.57 mmol) and triethylamine (0.57 mmol) were dissolved in methylene chloride (3 mL) and allowed to stir for 10 min at room temperature (rt). In a separate vial, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 0.95 mmol) and triethylamine (0.57 mmol) were dissolved in methylene chloride (3 mL), after which the carboxylic acid (0.48 mmol) was added and allowed to stir for 10 min at rt. The two solutions were then mixed together and allowed to react overnight (~16 h) at rt. The reaction mixture was evaporated to dryness and redissolved in ethyl acetate prior to being washed 1x each with 1 M citric acid, saturated sodium bicarbonate, and saturated sodium chloride. The organic fraction was dried over magnesium sulfate, filtered, and dried *in vacuo* to yield the products as white solids.

For the synthesis of cinnamoyl-type HLs with phenol functionalities, all reagents and equivalents were analogous to that for the synthesis the non-phenol derivatives above. All of the reagents were mixed simultaneously in a 10 mL microwave vial with 3 mL solvent (deionized water with enough acetonitrile to solubilize all reagents) and subjected to microwave irradiation in a monomodal microwave reactor for 30 min at 80 °C. The reaction mixture was then acidified with 1 M citric acid and extracted twice with ethyl acetate. The organic fractions were combined, dry loaded onto silica gel, and subjected to flash column chromatography (eluent 75% ethyl acetate/hexanes) to yield the products as white solids. See Supporting Information for full details of cinnamoyl-type HL synthesis and characterization data for all new compounds.

Bacterial reporter gene assays

Reporter gene assays were conducted as described previously in *R. palustris* CGA814, with minor modifications.^[11] This strain has a chromosomal *rpa::lacZ* mutation, and reports RpaR activity *via* the production of β-galactosidase. Standard Miller-type absorbance assays were used to measure β-galactosidase activity.^[23] See Supporting Information for full details of assay methods, primary assay data, and dose response curves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.

A) Representative natural AHLs used by *P. aeruginosa* (*N*-butanoyl HL (BHL) and *N*-(3-oxo)-dodecanoyl HL (OdDHL)), *V. fischeri* (*N*-(3-oxo)-hexanoyl HL (OHHL)), *Agrobacterium tumefaciens* (*N*-(3-oxo)-octanoyl HL (OOHHL), *Acinetobacter baumannii* ((2*S*,3'*R*)-*N*-(3'-hydroxy)-dodecanoyl-HL (3-OH-C12 HL)), *Rhizobium leguminosarum* ((2*S*,3'*R*,7'*Z*)-*N*-(3'-hydroxy)-7'-tetradecenoyl-HL (3-OH-C14:1 HL)), and other bacteria for QS.

B) The structure of *p*-cAHL used by *R. palustris* for QS.

Scheme 2.

Set of in-house AHLs selected for testing in RpaR. Compound numbering matches that used in our previous publications.^[7j, 7l]

- 1:** R = 2-F
2: R = 3-F
3: R = 4-F
4: R = 2,6-F
5: R = 3,5-F
6: R = F₅
7: R = 2-F, 4-Br
8: R = 2,4-Cl
9: R = 4-Cl
10: R = 2-Br

- 11:** R = 2-NO₂
12: R = 3-NO₂
13: R = 4-NO₂
14: R = 3-NO₂, 4-Cl
15: R = 2-OH
16: R = 3-OH
17: R = 3,4-OH
18: R = 3-OCH₃, 4-OH

Scheme 3.

Library of cinnamoyl-type HLs synthesized in the current study for testing in RpaR.

Scheme 4.

A) General structure for a strong RpaR agonist. X represents a halogen. B) General structure for a strong RpaR antagonist. X can be either C or O. EWG = electron withdrawing group.

Table 1

Selected agonism screening data for ArHLs in RpaR.

Compound	Percent activation [%] ^[a]	EC ₅₀ value [nM] ^[b]
1	99	3.4
2	84	65.6
3	1	--
4	101	4.8
7	13	--
8	68	103
10	118	77.1
11	15	--
12	7	--
15	59	1170
16	94	144
17	14	--
19	72	532
B10	65	32.3
E18	108	-- ^[c]
E27	2	--
E31	3	--
E33	49	2400
E36	38	4800

^[a] Percent activation measured at 1 μ M synthetic ligand and reported relative to *p*-cAHL at 1 μ M. Each compound tested in triplicate of triplicate; error < $\pm$ 10%.

^[b] Dose response curves obtained for compounds displaying > ~40% agonism at 1 μ M.

^[c] Not determined (ND).

Table 2

Selected antagonism screening data for ArHLs in RpaR.

Compound	Percent inhibition [%] ^[a]	IC ₅₀ value [nM] ^[b]
9	30	--
14	9	--
B7	35	--
B11	72	227
D2	43	--
D18	1	--
E2	-22	--
E14	80	388
E19	12	--
E20	12	--
E21	14	--
E22	17	--
E24	7	--
E25	63	238
E26	36	--
E28	27	--
E29	70	300
E30	29	--
E32	44	--
E34	30	--
E35	26	--
E37	89	235
E38	70	-- ^[c]
E39	63	228

^[a] Percent inhibition measured at 1 μ M synthetic ligand against *p*-cAHL at its EC₅₀ value (1 nM). Negative values indicate agonistic responses. Each compound tested in triplicate of triplicate; error < $\pm$ 10%.

^[b] Dose responses obtained for compounds displaying > ~60% antagonism at 1 μ M.

^[c] ND.