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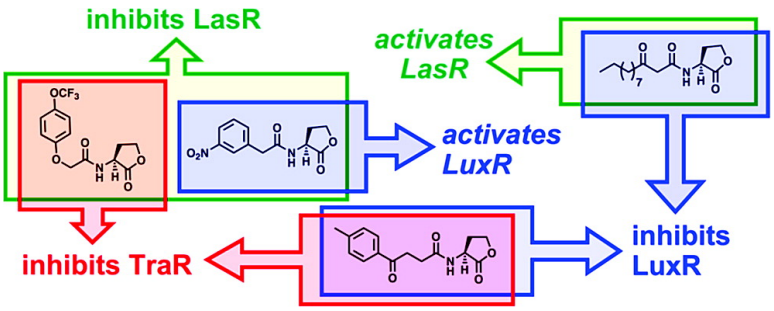

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Modulation of Bacterial Quorum Sensing with Synthetic Ligands: Systematic Evaluation of *N*-Acylated Homoserine Lactones in Multiple Species and New Insights into Their Mechanisms of Action

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Abstract: Bacteria use a language of low molecular weight ligands to assess their population densities in a process called quorum sensing. This chemical signaling process plays a pivotal role both in the pathogenesis of infectious disease and in beneficial symbioses. There is intense interest in the development of synthetic ligands that can intercept quorum-sensing signals and attenuate these divergent outcomes. Both broad-spectrum and species-selective modulators of quorum sensing hold significant value as small-molecule tools for fundamental studies of this complex cell−cell signaling process and for future biomedical and environmental applications. Here, we report the design and synthesis of focused collections of non-native *N*-acylated homoserine lactones and the systematic evaluation of these ~90 ligands across three Gram-negative bacterial species: the pathogens *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*; the model symbiont *Vibrio fischeri*. This study is the first to report and compare the activities of a set of ligands across multiple species and has revealed some of the most potent synthetic modulators of quorum sensing to date. Moreover, several of these ligands exhibit agonistic or antagonistic activity in all three species, while other ligands are only active in one or two species. Analysis of the screening data revealed that at least a subset of these ligands modulate quorum sensing via a partial agonism mechanism. We also demonstrate that selected ligands can either inhibit or promote the production of elastase B, a key virulence factor in wild-type *P. aeruginosa*, depending on their concentrations. Overall, this work provides broad insights into the molecular features required for small-molecule inhibition or activation of quorum sensing in Gram-negative bacteria. In addition, this study has supplied an expansive set of chemical tools for the further investigation of quorum-sensing pathways and responses.

Introduction

Bacteria produce and monitor low molecular weight molecules (autoinducers) to assess their population densities in a behavior called quorum sensing.[1−3] The concentration of these signaling molecules in a given environment is proportional to the bacterial cell density. When bacteria reach a sufficiently high population density, they will alter gene expression so as to carry out a range of processes that require the cooperation of a large number of cells, including secretion of virulence factors, biofilm formation, antibiotic production, bioluminescence, sporulation, and conjugation. These diverse processes have widespread and often devastating effects on human health, agriculture, and the environment. In the case of pathogenic bacteria, quorum sensing allows the bacteria to amass in sufficiently high densities before launching a coordinated attack on a host and overwhelming its defenses.[4−6] Symbiotic bacteria, in contrast, have co-opted quorum-sensing pathways to commence mutually beneficial relationships with their hosts at high cell densities.[2,7,8] As interception of quorum sensing represents a strategy to possibly control both pathogenesis and symbiosis, there is significant interest in the development of non-native ligands that can block or mimic native autoinducer signals and attenuate quorum-sensing outcomes.[9,10] Such molecules would represent tools to study the molecular mechanisms of quorum sensing and probe its validity as an anti-infective target.[4,5]

Quorum sensing is best characterized in the Gram-negative proteobacteria, which use *N*-acylated-L-homoserine lactones

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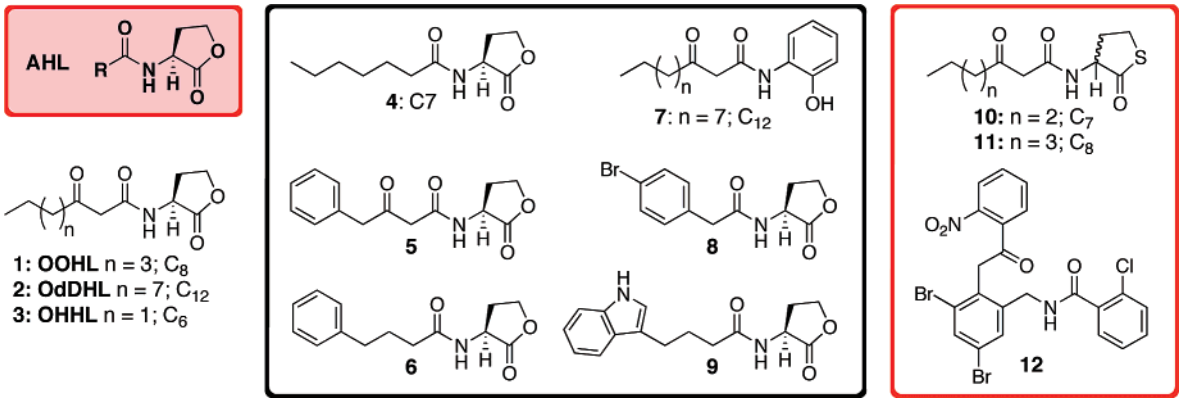

Figure 1. Generic structure for an *N*-acylated-L-homoserine lactone (AHL), selected native AHL ligands (**1**−**3**), known synthetic antagonists of R protein function (**4**−**9**), and known synthetic super-activators of R protein function (**10**−**12**). The numbers of carbons (C) in selected aliphatic acyl groups are indicated for clarity.

(AHLs, Figure 1) and their cognate cytoplasmic receptors (R proteins) for intercellular signaling.[11−13] The AHL ligand is generated by inducer synthases (I proteins) at low basal levels, and high cell densities are required to achieve a sufficient intracellular concentration of ligand for R protein binding. Thereafter, the AHL:R protein complex most often homodimerizes and activates transcription of target genes required for bacterial group behavior. A schematic of this process is shown in Figure 2. Thus, through quorum sensing, bacterial populations can efficiently couple gene expression to fluctuations in cell density. To date, this signaling process has been extensively studied in three Gram-negative bacteria: *Agrobacterium tumefaciens*; *Pseudomonas aeruginosa*; *Vibrio fischeri*. As such, these three species represent excellent model organisms for intervention with synthetic quorum-sensing modulators and are the focus of this study.

A. tumefaciens, *P. aeruginosa*, and *V. fischeri* each utilize quorum sensing for remarkably different purposes. *A. tumefaciens* is a widespread plant pathogen and uses quorum sensing in its induction of crown gall tumors on plant hosts under the control of *N*-(3-oxooctanoyl)-L-homoserine lactone (OOHL, **1**; Figure 1) and its receptor, TraR.[14] TraR is the only R protein for which a three-dimensional structure of the receptor bound to ligand and DNA has been determined by X-ray crystallography.[15,16] This X-ray structure revealed that the OOHL (**1**): TraR complex binds DNA as a homodimer and that OOHL (**1**) is completely engulfed in a hydrophobic site on TraR upon DNA binding.

P. aeruginosa is both a plant and animal pathogen and uses two AHL signaling molecules, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL, **2**; Figure 1) and *N*-butanoyl-L-homoserine lactone, and two R proteins, LasR and RhlR, respectively, to control the expression of an arsenal of virulence factors that cause extensive tissue damage during infection.[17,18]

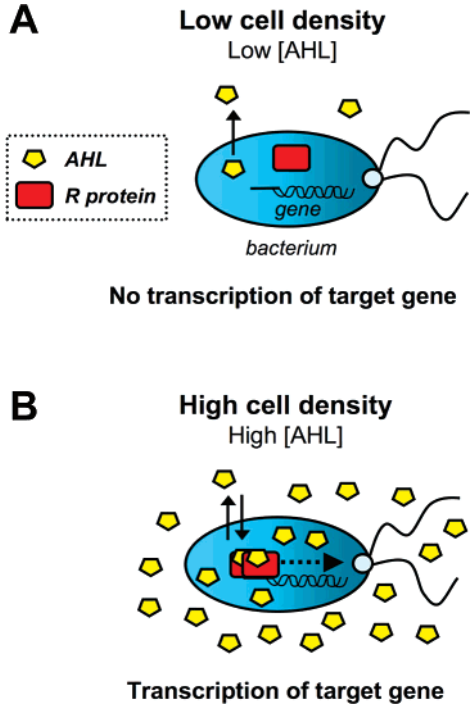

Figure 2. Schematic of quorum sensing in Gram-negative bacteria. Transcriptional activation shown. (A) Bacteria constitutively produce small amounts AHL synthase (I protein) and therefore AHL. These ligands are capable of diffusing out of the cell and into other cells by either passive or active diffusion processes. (B) At a high cell density, the concentration of AHL reaches a threshold level within the cell, and the AHL binds its cognate receptor (an R protein). The AHL:R protein complex then binds a target promoter sequence as a homodimer (or higher order multimers) and activates transcription. AHL = *N*-acylated-L-homoserine lactone.

There is tremendous interest in AHL-mediated quorum sensing in *P. aeruginosa* due to the prevalence of this opportunistic bacterium in life-threatening hospital-acquired infections[18] and in chronic lung infections associated with cystic fibrosis.[19] An X-ray structure of the N-terminal ligand-binding domain of LasR complexed to OdDHL (**2**) was recently reported and exhibited a structure highly homologous to that of TraR, albeit with a slightly expanded ligand-binding pocket to accommodate its larger cognate ligand.[20] Similar to OOHL (**1**) in TraR, OdDHL

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(**2**) was shown to be completely engulfed in the LasR ligand-binding site. Biochemical experiments with both TraR and LasR suggest that native ligand is required for the folding of these proteins into their mature tertiary structures in vitro and ligand is bound almost irreversibly.[21,22]

In contrast to these two bacterial pathogens, *V. fischeri* uses quorum sensing to mediate a beneficial symbiosis. This marine bacterium colonizes the light-producing organs of certain marine fish and squids and uses quorum sensing to initiate bioluminescence and other mutually beneficial processes at high cell densities.[8,11,23] Quorum sensing is mediated in part by *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL, **3**; Figure 1) and its cognate receptor, LuxR.[24] The LuxR protein has been shown to bind OHHL (**3**) reversibly in vitro, suggesting that its ligand-binding site is more accessible than both TraR and LasR;[25] structural data have not been reported, however, to support this hypothesis. As AHL-mediated quorum sensing was first characterized in *V. fischeri*, the LuxR system represents the canonical quorum sensing circuit in Gram-negative bacteria.[11]

Considerable research efforts over the past 20 years have focused on the design and synthesis of ligands that can disrupt AHL−R protein binding and inhibit quorum-sensing outcomes in these three bacterial species.[26−43] However, synthetic antagonists of quorum sensing remain scarce. The known antagonists are mainly structural mimics of native AHLs, and four of the most active R protein antagonists are shown in Figure 1: *N*-heptanoyl-L-homoserine lactone (**4**) reported by Zhu et al. and active against TraR;[30] *N*-(3-oxophenylbutanoyl)- and *N*-(phenylbutanoyl)-L-homoserine lactones (**5** and **6**) reported by Reverchon et al. and active against LuxR;[32] the 2-aminophenol analog of OdDHL (**7**) reported by Smith et al. and active against LasR.[39] Likewise, compounds exhibiting heightened activities relative to native AHLs (i.e., "super-activators" of quorum sensing) are also of significant interest, as they could potentially initiate bacterial group behaviors at lower cell densities than required in natural environments. However, only three such super-activators of quorum sensing have been reported to date. These three ligands are shown in Figure 1: *N*-(3-oxoheptanoyl)- and *N*-(3-oxooctanoyl)-DL-homoserine thiolactones (**10** and **11**) capable of super-activating the LuxR homolog SdiA in *Salmonella enterica*;[44,45] and the triphenyl signal mimic (**12**) capable of super-activating LasR.[46]

Clearly, new synthesis and design strategies are needed to expand the current set of quorum-sensing modulators active in Gram-negative bacteria. Unfortunately, the structures of the few known antagonists and agonists vary considerably and their mechanisms of action are unclear;[9,10,13,37] thus, no obvious rationales have emerged for new ligand design. Moreover, to our knowledge, the known antagonists and agonists of quorum sensing have been examined primarily in one bacterial species. Therefore, we currently do not know whether these compounds target one R protein selectively or if they can modulate the functions of a range of different R proteins. As such, the molecular features that confer selectivity or broad-range activity to synthetic quorum-sensing modulators in Gram-negative bacteria remain unknown. The moderate sequence homology in the putative ligand-binding sites of the ∼50 known R proteins (70−80%) suggests that if non-native ligands target these sites, both R protein-selective and broad-spectrum ligands potentially could be developed.[11,12] Ligands with either of these activity profiles would be of significant value as chemical probes to study quorum sensing, most notably in natural environments harboring multiple species.

To address these challenges, we have been engaged in the design of focused, combinatorial libraries of synthetic ligands for the modulation of quorum sensing in a range of different bacteria.[47−50] Our preliminary work has resulted in the identification of five potent modulators of R protein function in either *A. tumefaciens*, *P. aeruginosa*, or *V. fischeri*, including the antagonists *N*-(4-bromophenylacetanoyl)-L-homoserine lactone (4-bromo PHL **8**) and indole AHL (**9**) (Figure 1).[47,50] These initial studies surveyed a limited set of non-native AHLs and were primarily focused on the discovery of R protein antagonists in one bacterial species. Here, we report the design and synthesis of four focused libraries of non-native AHLs, the parallel evaluation of these ∼90 compounds for R protein antagonism and agonism in all three species (*A. tumefaciens*, *P. aeruginosa*, and *V. fischeri*), and a detailed analysis of these comparative screening data. Each of the libraries was designed to probe the role of key features of AHL structure on quorum-sensing activity, including acyl chain length, lactone stereochemistry, and functionality on the acyl group. These studies represent the

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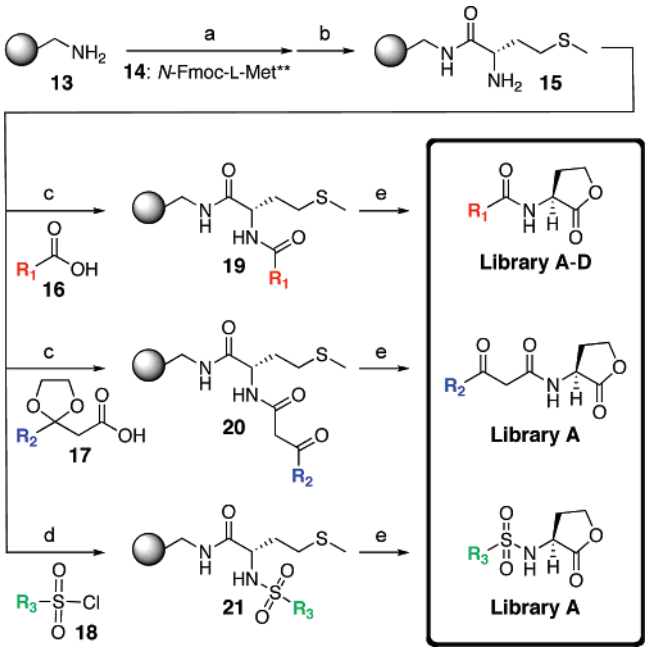
Scheme 1. Solid-phase Synthetic Route to AHL Libraries A−D[a]



[a] Reagents and Conditions: (a) DIC, HOBt, $CHCl_3$/DMF, μW 50 °C, 10 min; (b) DMF, μW 150 °C, 7 min; (c) DIC, $CHCl_3$/DMF, μW 50 °C, 10 min; (d) DMAP, $CHCl_3$, μW 50 °C, 10 min; (e) CNBr, TFA, $CHCl_3$/H_2O, RT, 24 h. Notes and abbreviations: (**) *N*-Fmoc-D-Met (**14**) used in the construction of compounds **B1**−**B5**; DIC = *N*,*N*′-diisopropylcarbodiimide; HOBt = *N*-hydroxybenzotriazole; DMF = *N*,*N*-dimethylformamide; Fmoc = 9-fluorenylmethoxycarbonyl; Met = methionine; DMAP = 4-(dimethylamino)pyridine; μW = temperature-controlled microwave irradiation.

first comparative investigation of non-native AHL function across multiple Gram-negative bacteria. They have revealed an expansive new set of synthetic R protein modulators and the most comprehensive set of structure−activity relationships (SARs) for non-native AHL ligands reported to date. Furthermore, we have identified quorum-sensing modulators that are either selective for one or two species or are active in all three species. Several of these ligands are among the most potent modulators of R protein function known, with the ability to inhibit or even super-activate R protein function at 10-fold lower concentrations than the native AHL ligand. We present our current rationales for the mechanisms of R protein modulation by these non-native AHLs, most notably by a partial agonism pathway. Together, the ligands described herein have the potential to significantly broaden the current understanding of quorum sensing and its roles in host−bacteria interactions.

Experimental Section

Chemistry. All reagents and solvents were purchased from commercial sources and used without further purification, with the exception of dichloromethane (CH_2Cl_2), which was distilled over calcium hydride. All solid-phase syntheses were performed using aminomethyl polystyrene resin (EMD Biosciences, 100−200 mesh; loading 1.1−1.2 mmol/g). Microwave-assisted solid-phase reactions were carried out using either Milestone or CEM commercial microwave (μW) reactors under temperature control. Full details of the instrumentation and analytical methods used in this work can be found in the Supporting Information.

Ligand Synthesis. AHL libraries A−D, OOHL (**1**), OdDHL (**2**), and OHHL (**3**), and the control compounds **4**−**6**, **8**, and **9** were prepared according to Scheme 1 using reported methods on a 20 mg scale,[47] except the final cyclization−cleavage step was performed at RT (room temperature) for 24 h. The 1,3-dioxolane protected β-keto acids building blocks (**17**) were prepared via a modified version of the methods reported by Barnick and Rathke.[51,52] Sulfonyl chloride building blocks (**18**) were prepared according to the method reported by Castang et al.[33] Control compound **7** was prepared in solution according to our previously reported method.[47] Purities and isolated yields for libraries A−D, the native ligands, and the control compounds were 90−99% and 55−75%, respectively. Compounds were submitted to biological assays following resin cleavage and an aqueous workup without further purification. Full characterization data for the active compounds in Tables 1 and 2 can be found in the Supporting Information.

Compound Handling. Stock solutions of synthetic compounds (10 mM) were prepared in DMSO and stored at room temperature in sealed vials. The amount of DMSO used in small molecule screens did not exceed 2% (by volume). Solvent resistant polypropylene or polystyrene 96-well multititer plates were used when appropriate for small molecule screening. The concentrations of synthetic AHL ligand used in the primary antagonism and agonism assays and the relative ratios of synthetic ligand to native ligand (1:1 to ~100:1) in the antagonism assays were chosen to provide the most obvious differences between inhibitors and activators for each bacterial reporter strain. The concentration of native ligand used in the antagonism assays was approximately equal to its EC_{50} value in each bacterial reporter strain.

Bacteriology. All biological reagents were purchased from Fisher Scientific and used according to enclosed instructions. Agrobacterium (AB) minimal medium was prepared as previously reported.[47] Luria−Bertani (LB) and LB salt media (LBS) were prepared as instructed with pH = 7.5 (LBS contained an additional 1.5% NaCl, 0.3% glycerol, and 50 mM Tris-HCl).[24] Buffers and solutions (Z buffer, 0.1% aqueous SDS, and phosphate buffer) for Miller absorbance assays in *A. tumefaciens* and *E. coli* were prepared as described.[53] The three bacterial reporter strains used in this study were: *A. tumefaciens* WCF47 (Δ-*traI*) harboring a plasmid-born P*traI*-*lacZ* fusion (pCF372);[30] *E. coli* DH5α harboring the LasR expression vector pJN105L and a plasmid-born P*lasI*-*lacZ* fusion (pSC11);[54] *V. fischeri* ES114 (Δ-*luxI*).[24] *P. aeruginosa* PAO1 was used in elastase B production assays.[55] All bacteria were grown in a standard laboratory incubator with shaking (200 rpm) unless noted otherwise. Absorbance and luminescence measurements were obtained using a Perkin-Elmer Wallac 2100 EnVision multilabel plate reader using Wallac Manager v1.03 software. All bacteriological assays were performed in triplicate.

***A. tumefaciens* Reporter Gene Assay Protocols.** For primary TraR agonism assays, an appropriate amount of concentrated control or AHL stock solution (to give a final concentration of 10 μM) was added to wells in a 96-well multititer plate. An overnight culture of *A. tumefaciens* WCF47 (pCF372) was diluted 1:10 with fresh AB minimal medium containing 400 μg/mL octopine and 50 μg/mL streptomycin. A 200 μL portion of the diluted culture was added to each well of the multititer plate containing AHLs. Plates were incubated at 28 °C for 18−24 h. The cultures were then assayed for β-galactosidase activity following the Miller assay method.[53] Briefly, 200 μL aliquots of bacteria from each of the wells were added to wells of a 96-well multititer plate, and the OD_{600} of each well was recorded. Next, 50 μL aliquots from each well were transferred to a solvent resistant 96-well multititer plate containing 200 μL Z buffer, 8 μL $CHCl_3$, and 4 μL 0.1% aqueous SDS. This suspension was mixed via repetitive pipetting, after which the $CHCl_3$ was allowed to settle. A 100 μL aliquot from each well was transferred to a fresh 96-well multititer plate, and 20 μL of substrate, *o*-nitrophenyl-β-D-galactopyranoside (ONPG, 4 μg/mL in phosphate

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(55) Stover, C. K.; et al. *Nature* **2000**, *406*, 959−964.

buffer), was added at time zero. After the development of appropriate yellow color (ca. 20−30 min), the reaction was terminated by the addition of 50 μL of 1 M Na_2CO_3. Absorbances at 420 and 550 nm were measured for each well using a plate reader, and Miller units were calculated according to standard methods.[53] Primary TraR antagonism assays were performed in a similar manner except the AHL or control was screened at 10 μM against 100 nM OOHL (**1**).

***E. coli* LasR Reporter Gene Assay Protocols.** For primary LasR agonism assays, an appropriate amount of concentrated control or AHL stock solution (to give a final concentration of 5 μM) was added to wells in a 96-well multititer plate. An overnight culture of *E. coli* DH5α (pJN105L pSC11) was diluted 1:10 with fresh LB medium containing 100 μg/mL ampicillin and 15 μg/mL gentamicin. This subculture was incubated at 37 °C until OD_{600} = 0.3 (4−6 h). Arabinose (4 mg/mL) was then added to induce the LasR promoter, and a 200 μL portion of this culture was added to each well of the multititer plate containing AHLs. Plates were incubated at 37 °C until OD_{600} = 0.45 (4−6 h). The cultures were then assayed for LasR activity by following the identical β-galactosidase assay protocols used in the *A. tumefaciens* reporter gene assays (see above). Primary LasR antagonism assays were performed in a similar manner except the AHL or control was screened at 5 μM against 7.5 nM OdDHL (**2**).

***V. fischeri* Reporter Gene Assay Protocols.** For primary LuxR agonism assays, an appropriate amount of concentrated control or AHL stock solution (to give a final concentration of 200 μM) was added to wells in a 96-well multititer plate. An overnight culture of *V. fischeri* ES114 (Δ-*luxI*) was diluted 1:10 with LBS medium. A 200 μL portion of the diluted culture was added to each well of the multititer plate. Plates were incubated at RT until the OD_{600} = 0.35−0.4 (4−6 h). Luminescence then was measured and normalized to cell density/well. Primary LuxR antagonism assays were performed in a similar manner except the AHL or control was screened at 5 μM against 5 μM OHHL (**3**).

Dose Response Reporter Gene Assays. The dose response reporter gene assays were performed according to the protocols outlined above, except the concentrations of control compounds and AHLs were varied between 2×10^{-2} and 2×10^{5} nM. IC_{50} and EC_{50} values were calculated using GraphPad Prism software (v. 4.0) using a sigmoidal curve fit.

Elastase B Production Assay in *P. aeruginosa*. Elastase B activity in *P. aeruginosa* was measured according to a previously reported method,[38] with the following modifications: *P. aeruginosa* PAO1 was grown overnight at 37 °C and then diluted 1:10 with fresh LB medium. Portions (2 mL) of this culture were added to test tubes containing synthetic compounds to give final compound concentrations of 20 μM or 200 μM. The tubes were incubated for 12−14 h at 37 °C. The OD_{600} was measured for each tube, after which the contents of the tubes were filtered through a 0.2 μm Whatman filter to remove all cellular matter. A 100 μL aliquot of the supernatant was added to 900 μL of an elastin−Congo red solution (5 mg of elastin−Congo red substrate/1 mL of buffer (100 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.2)) and incubated for 12 h at 37 °C with 250 rpm shaking. The contents of these tubes were then filtered to remove unreacted elastin−Congo red substrate, and the supernatant containing cleaved Congo red was isolated. A 200 μL aliquot of the supernatant was added to a 96-well multititer plate, and the OD_{492} was measured. Elastase B activity was calculated by dividing the absorbance of the cleaved Congo red solution (OD_{492}) by the cell density (OD_{600} of the cells before first filtration).

Background: AHL Library Design and Biological Assay Formats

General Considerations for Ligand Design. AHLs bearing non-native acyl chains represent the most extensively studied structure class of synthetic quorum-sensing modulators in *A. tumefaciens*, *P. aeruginosa*, and *V. fischeri*.[26−30,32−34,41,47,50] Modifications to the lactone ring of AHLs, including inversion of stereochemistry[31] and replacement of the lactone with different carbocyclic or heterocyclic functionalities,[26−28,38−41] have been examined to a lesser extent. Clear SARs for quorum-sensing modulators are yet to be established due to the relatively limited set of ligands examined to date. The use of different bacterial reporter strains and assay procedures to assess agonistic or antagonistic activities against the same R protein has further hindered comparison between past studies. Our analysis of this prior work, however, revealed the following broad trends for synthetic R protein modulators in *A. tumefaciens*, *P. aeruginosa*, and *V. fischeri*: (1) Changing the number of carbons in the acyl chain relative to the native AHL by 1−3 carbons can weaken a ligand's agonistic activity and/or convert the ligand into a weak antagonist.[26−28,30,33,34] (2) Inversion of lactone stereochemistry (L to D) nearly abolishes agonistic and antagonistic activities for AHLs with native[31] and non-native acyl chains.[41] (3) Introduction of terminal phenyl moieties on the acyl group can result in compounds with antagonistic activities.[27,32−34,47,50]

These broad trends did not provide us with an obvious strategy for the rational design of new AHLs that modulate quorum sensing in these three bacteria. However, they did offer a foundation on which to design focused, combinatorial libraries of non-native AHL ligands to systematically examine the structural features required for agonistic or antagonistic activity across the three species. In this study, we sought to investigate three key structural features of AHLs: (1) acyl chain length; (2) lactone stereochemistry; (3) functional group diversity in the acyl chain. We designed four focused libraries of AHLs (A−D) that allowed us to probe each of these features individually and in tandem (shown in Figures 3 and 4). The X-ray crystal structure of TraR (i.e., the ligand-binding site) was also used to guide our initial ligand design.[15,47]

Design of AHL Libraries A−D. Library A was designed to test the effects of different aliphatic acyl, 3-oxoacyl, and sulfonyl groups on AHL ligand activity in the three bacterial species (Figure 3A). This library contained the most structurally simple AHL derivatives examined in this study, and several of these ligands have been shown to modulate R protein function previously (albeit largely in different bacterial strains than those utilized in this study).[26−28,30,33] Therefore, library A was also designed to provide critical benchmark R protein activation and inhibition data. Library B was designed to investigate the roles of the following AHL structural features: (1) lactone stereochemistry; (2) acyl group aromaticity; (3) alkyl "spacer" length between aromatic groups and the HL ring (Figure 3B). We examined these three features by perturbing the structures of known active compounds: the native agonist OOHL (**1**); the control antagonists of Reverchon et al. (**5** and **6**);[32] our previously reported antagonists, 4-bromo PHL **8** and indole AHL **9** (Figure 1).

Library C consisted entirely of PHLs and was designed to systematically examine the role of phenylacetanoyl group substituents on R protein antagonism and agonism (Figure 3C). This library was inspired in part by the strong antagonistic activity of 4-bromo PHL **8** toward TraR and LasR reported previously by our laboratory.[47] Further, we recently examined a subset of the PHLs in library C in LuxR antagonism and agonism assays and identified several potent inhibitors and

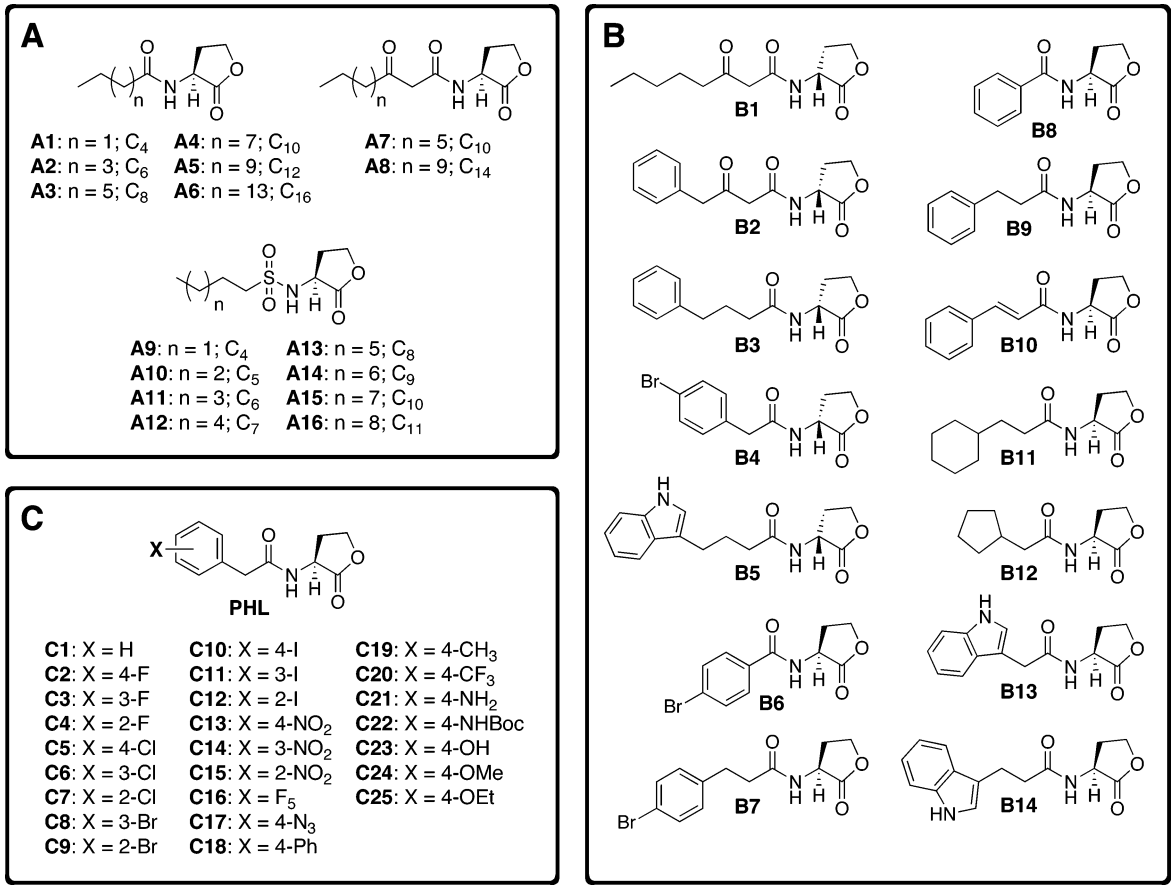

Figure 3. Structures of AHL libraries A−C. (A) Library A. General structural features tested: (1) aliphatic acyl group length; (2) replacement of acyl carbonyl with a sulfonyl group. The numbers of carbons (C) in aliphatic acyl groups are indicated for clarity. (B) Library B. General structural features tested: (1) lactone stereochemistry in OOHL (**1**) and control compounds **5**, **6**, **8**, and **9**; (2) acyl chain alkyl spacer length and aromaticity in control compounds **8** and **9**. (C) Library C. PHL = *N*-(phenylacetanoyl)-L-homoserine lactone. General structural features tested: (1) different substituents; (2) their placement on the acyl group phenyl ring.

activators of LuxR;[50] we sought to build on these initial findings in this study. Last, library D contained the most structurally diverse set of non-native AHLs synthesized to date (shown in Figure 4) and was designed to broadly examine the influence of a range of different acyl groups on AHL-mediated R protein antagonism and agonism. These acyl substituents differed significantly in terms of overall size and the type and placement of functional groups. However, as many of the known active, non-native AHLs contain aromatic groups (see Figure 1), we deliberately installed aromatic functionality (or at least one π-system) in the acyl chains of the majority of library D.

Quorum-Sensing Reporter Gene Assays. The low stability of most R proteins in vitro has precluded the development of routine protein−ligand binding assays.[11−13] As such, non-native ligands have been most commonly assessed for R protein antagonism and agonism in cell-based assays using bacterial reporter strains.[10,13,37] These reporter strains lack their AHL synthase (I) genes but retain their native R genes. In the presence of exogenously added AHL ligand, the AHL:R protein complex will activate transcription of a promoter that controls reporter gene expression. Therefore, R protein activity, and consequently ligand activity, can be measured using standard reporter gene read-outs, such as absorbance, luminescence, or fluorescence. This method provides a straightforward and high-throughput assay for small molecules that either agonize or antagonize (when examined in competition with native AHL ligand) R protein function.

We selected three bacterial reporter strains for the R protein antagonism and agonism assays in this study: *A. tumefaciens* WCF47 (pCF372);[30] *E. coli* DH5α (pJN105L pSC11);[54] *V. fischeri* ES114 (Δ-*luxI*) (see Experimental Section).[24] This *A. tumefaciens* strain produces the enzyme β-galactosidase upon TraR activation, and ligand activity can be measured using standard Miller absorbance assays in the presence of a colored enzyme substrate.[53] The *E. coli* strain harbors LasR from *P. aeruginosa* and also reports LasR activity by β-galactosidase production. We initially examined a Δ-*lasI* Δ-*rhlI* derivative of *P. aeruginosa* with a green fluorescent protein reporter gene in these primary assays,[39,47] as we sought to evaluate our synthetic ligands in the native backgrounds for each of the three R proteins. However, unacceptably large error values in the assay data (due in part to inconsistent cell growth) forced us to seek this alternate strain (data not shown). We found that the heterologous *E. coli* DH5α system provided reproducible data, although the differences between active and inactive LasR antagonists were somewhat muted relative to the other two strains.[56] Finally, the *V. fischeri* reporter strain retains its native *lux* operon (yet lacks a functional *luxI*), which allows LuxR activation or inhibition to be measured by luminescence. We recently found that this strain, while not typically used to assess

(56) We speculate that these muted effects may be due to high LasR protein levels in this strain, as has been reported for an *A. tumefaciens* reporter strain producing significantly higher levels of TraR relative to wild-type. See ref 30.

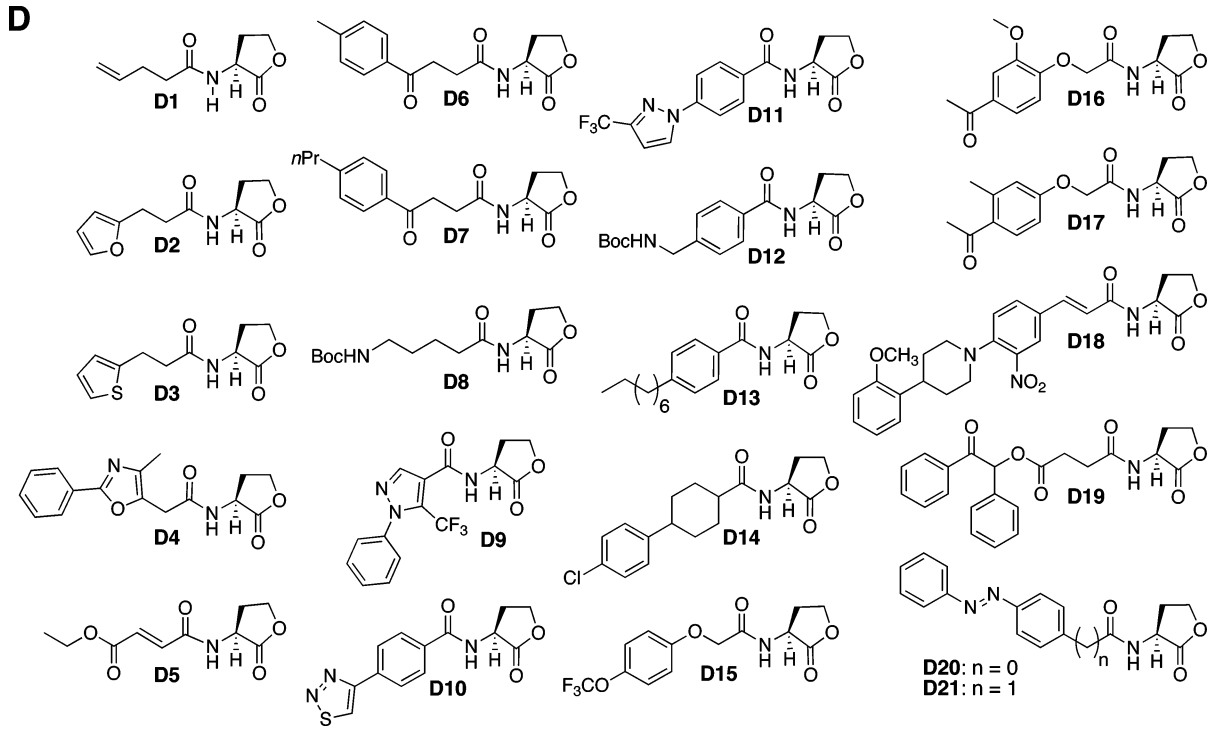

Figure 4. Structures of AHL library D. General structural features tested: (1) varying functionalities on the acyl group; (2) varying acyl group sizes. Boc = *tert*-butyloxycarbonyl.

the activity of non-native AHL ligands against LuxR, is straightforward to manipulate and provides highly reliable small molecule screening data.[50]

Results and Discussion

Library Synthesis. AHL libraries A−D were synthesized rapidly using a microwave-assisted, solid-phase route to AHLs previously reported by our laboratory (Scheme 1).[47] This route allowed for the straightforward construction of either L- or D-lactones through the use of either *N*-Fmoc-L- or D-methionine (Met, **14**) in the initial acylation step and the introduction of a wide variety of acyl groups, including simple alkyl, 3-oxoalkyl, and sulfonyl moieties (**16**−**18**). The ∼90 AHLs were isolated in moderate to good yields (55−75%), with excellent purities (90−99%) and in sufficient quantities (∼20 mg/compound) for full compound characterization and multiple biological experiments.

Reporter Gene Assays Results. Libraries A−D were screened in competitive R protein antagonism and agonism assays in the three bacterial reporter strains introduced above. Competitive antagonism assays were performed with synthetic ligand in the presence of native AHL ligand (at its approximate EC_{50} value) at ratios ranging from 1:1 to 100:1 (synthetic vs native AHL). Agonism assays were performed with synthetic ligand alone. The native ligands OOHL (**1**), OdDHL (**2**), and OHHL (**3**) and the known R protein antagonists **4**−**9** served as controls for these experiments (Figure 1).[57] Agonistic activity for each of the native ligands (**1**−**3**) was set to 100% in its corresponding strain for comparison. As expected, all of the control antagonists showed inhibitory activity in the three strains, albeit at varied levels (18−93%), with the exception of 2-aminophenol **7**, which was surprisingly inactive (see Table S-2; Supporting Information).[58] This latter result contrasted with previous reports that **7** is a strong inhibitor of LasR activity in similar assays; however, these studies involved a different LasR reporter strain.[39] Heptanoyl HL (**4**), phenylbutanoyl HL (**6**), and 4-bromo PHL (**8**) were the most active control antagonists across all three strains; these three ligands exhibited similar levels of activity against each strain (∼90% in TraR, 25% in LasR, and 76% in LuxR). Notably, neither the control compounds nor the library members were observed to be insoluble or affect bacterial growth over the time course of these assays (4−24 h). Further, no ligand was found to degrade (by lactonolysis, proteolysis or reaction with biological reagents) over the time course of these assays (as determined by LC-MS or GC-MS; data not shown).

The reporter gene assays of libraries A−D revealed a set of highly potent quorum-sensing antagonists and agonists, along with several prominent trends in ligand activity within and between strains that could be correlated to structure. A detailed analysis of the SAR trends in the primary assay data for libraries A−D will be reported elsewhere. Here, we focus on the most active R protein antagonists and agonists identified in libraries A−D, which corresponded to 37 compounds. A total of 31 ligands were identified that displayed inhibitory activities of >80% against TraR, >35% against LasR, and/or >75% against LuxR. In turn, 14 ligands were identified as either LasR or LuxR agonists, with activities of >20% in LasR and/or >60% in LuxR. (No TraR agonists were identified in the four libraries.) Interestingly, several of the ligands were observed to be antagonists in one strain yet were agonists in another. To obtain more quantitative data about the activity of these synthetic R protein antagonists and agonists, we performed dose response

(57) We did not examine super-activators **10**−**12** as controls in these assays because we either found the compound unstable or inactive under our assays conditions (**10** and **11**) or did not have access to a sample of the compound (**12**).

(58) None of the control antagonists displayed appreciable agonistic activity in any of the strains.

Table 1. IC_{50} Values for Most Active Antagonists across the Three Strains[a]

entry	compd	*A. tumefaciens* TraR (μM)[b,e]	*P. aeruginosa* LasR (μM)[c,e]	*V. fischeri* LuxR (μM)[d,e]
1	**1** (OOHL)		0.11[f,g]	
2	**2** (OdDHL)			0.40
3	**4**	0.69[f]		1.36[f]
4	**5**	0.83[f]		
5	**6**	1.12[f]		
6	**8**	4.73	3.89	3.70
7	**9**		8.38	
8	**A3**	0.77[f]	1.75[f]	0.77[f]
9	**A4**	1.05	0.25[f,g]	0.40
10	**A8**			0.74
11	**A9**	0.61[f]		
12	**A11**	0.83[f]		
13	**A12**	3.49		
14	**A13**			1.43
15	**A14**			1.03
16	**A15**			1.39
17	**B7**	0.92[f]	0.34[f,g]	1.35
18	**B11**		1.75[f,g]	2.69[f]
19	**B14**		0.83[f,g]	
20	**C5**			4.13
21	**C6**		3.97[f,g]	
22	**C8**		4.06[f,g]	
23	**C10**	1.25[f]	1.72[f,g]	0.86[f]
24	**C11**		4.63	
25	**C13**	2.25[f]		0.96[f]
26	**C14**		0.61[f,g]	
27	**C18**			1.06[f]
28	**C20**	0.81[f]		0.61
29	**D6**	0.57[f]		
30	**D15**	0.46[f]	4.67[f,g]	
31	**D17**	1.40[f]		

[a] See Experimental Section for details of reporter strains. IC_{50} values determined by testing compounds over a range of concentrations (2×10^{-2}–2×10^{5} nM) against native AHL ligand in each reporter strain. All assays performed in triplicate. See Supporting Information for plots of full antagonism dose response curves. [b] Determined against 100 nM OOHL (**1**). [c] Determined against 7.5 nM OdDHL (**2**). [d] Determined against 5 μM OHHL (**3**). [e] A missing entry means not determined. [f] Antagonism dose response curve upturned at higher concentrations. See text. [g] Dose response curve did not reach 100% inhibition over the concentrations tested (prior to upturn); IC_{50} value calculated from the partial antagonism dose response curve reported.

assays in the three reporter strains and determined either IC_{50} or EC_{50} values for the 37 ligands. The calculated IC_{50} and EC_{50} values for these compounds are listed in Tables 1 and 2, respectively.

IC_{50} Values for Synthetic AHL Antagonists. The IC_{50} values for the antagonists ranged from high nanomolar to low micromolar concentrations, and over 60% of these ligands (20 compounds) had lower IC_{50} values than those for the control antagonists reported in this study (Table 1). This high percentage of hits is significant (25% of the total library), as few potent synthetic antagonists of R proteins have been reported. Moreover, several new antagonists were identified that either selectively inhibited one or two R proteins or inhibited all three R proteins. The structures of the most active antagonists are shown in Figure 5A, and their observed selectivities for R protein targets are illustrated using a Venn diagram for clarity. Butane sulfonyl HL **A9**, bulky AHL **D6**, and phenyl ether AHL **D15** were the most active inhibitors of TraR, with each inhibiting TraR by 50% at ~5.5:1 against native ligand OOHL (**1**) (entries 11, 29, and 30, respectively; Table 1). Notably, **A9** and **D6** were highly selective for TraR over LuxR and LasR, while **D15** exhibited moderate cross-inhibitory activity against LasR.

Five ligands were identified with IC_{50} values against LasR that were 1 order of magnitude lower than control antagonists **8** and **9** (Table 1). Interestingly, the most active inhibitor of LasR identified in this study was OOHL (**1**), the native AHL ligand from *A. tumefaciens*, which inhibited LasR by 50% at ~15:1 against OdDHL (**2**) (entry 1). The second most active antagonist of LasR in this study was the C_{10} AHL **A4**, inhibiting by 50% at ~30:1 against OdDHL (**2**) (entry 9). In contrast, previous studies by Passador et al., using an alternate *E. coli* reporter stain, revealed neither OOHL (**1**) nor AHL **A4** as active inhibitors of LasR.[28] AHL **B7** (the one-carbon longer homolog of control antagonist 4-bromo PHL **8**), AHL **B14** (the one-carbon shorter homolog of control antagonist indole AHL **9**), and 3-nitro PHL **C14** were the next most active inhibitors of LasR identified (entries 17, 19, and 26). In terms of selectivity for R protein, AHL **B14** and 3-nitro PHL **C14** were most selective for LasR, while OOHL (**1**) inhibited LasR and, to a lesser degree, LuxR (see Table S-2; Supporting Information). Conversely, AHLs **A4** and **B7** were moderate to strong antagonists of all three R proteins (entries 9 and 17).

The most potent antagonists identified overall were active against LuxR. Remarkably, these ligands were capable of inhibiting LuxR *by 50% at ~10-fold lower concentrations relative to its native ligand OHHL (**3**)*. Here, two aliphatic AHLs and one PHL were identified as the most potent antagonists: the native ligand for *P. aeruginosa*, OdDHL (**2**); C_{10} AHL **A4**; 4-trifluoromethyl PHL **C20** (Figure 5A). Both OdDHL (**2**) and **A4** were capable of inhibiting LuxR activity by 50% at a ~1:12.5 ratio against OHHL (**3**) (entries 2 and 9, Table 1). This result corroborated previous work by Schaefer et al. that indicated OdDHL (**2**) and **A4** can inhibit LuxR, albeit to a significantly lesser degree (again, this work was performed in an alternate LuxR reporter strain).[27] The non-native, 4-trifluoromethyl PHL **C20** displayed a similarly high level of antagonistic activity against LuxR at a ~1:8 ratio against OHHL (**3**) (entry 28). AHL **A8**, the C_{14} analog of OdDHL (**2**), was the next most potent inhibitor of LuxR (entry 10). These four ligands varied significantly in terms of their selectivities; both OdDHL (**2**) and **A8** were highly selective for LuxR, while PHL **C20** was a potent inhibitor of LuxR and TraR and **A4** again inhibited all three R proteins (see above).

EC_{50} Values for Synthetic AHL Agonists. It is obvious from Figure 5B that far fewer R protein agonists were identified in our primary screens relative to antagonists (14 compounds) and that these agonists show exquisite selectivity for individual R proteins. The agonism dose response studies revealed several ligands with EC_{50} values against LasR comparable to the native ligand OdDHL (**2**) (entry 2, Table 2). The C_{12} AHL **A5** and 3-oxo C_{14} AHL **A8** displayed the lowest EC_{50} values (40 and 10 nM, respectively), and these values supported activity data previously reported by Passador et al. for these two compounds.[28] Not surprisingly, these two LasR activators were similar in structure to OdDHL (**2**) (Figure 5B). D-AHL **B2** was far less structurally analogous and exhibited a 50-fold higher EC_{50} value relative to OdDHL (**2**) (entry 7, Table 2). This synthetic LasR activator is noteworthy, however, as it represents, to our knowledge, the most active D-AHL reported to date. Interestingly, the L-stereoisomer of **B2**, AHL **5**, is virtually inactive against LasR (see Table S-2; Supporting Information). This trend is opposite to what has been observed for native AHL

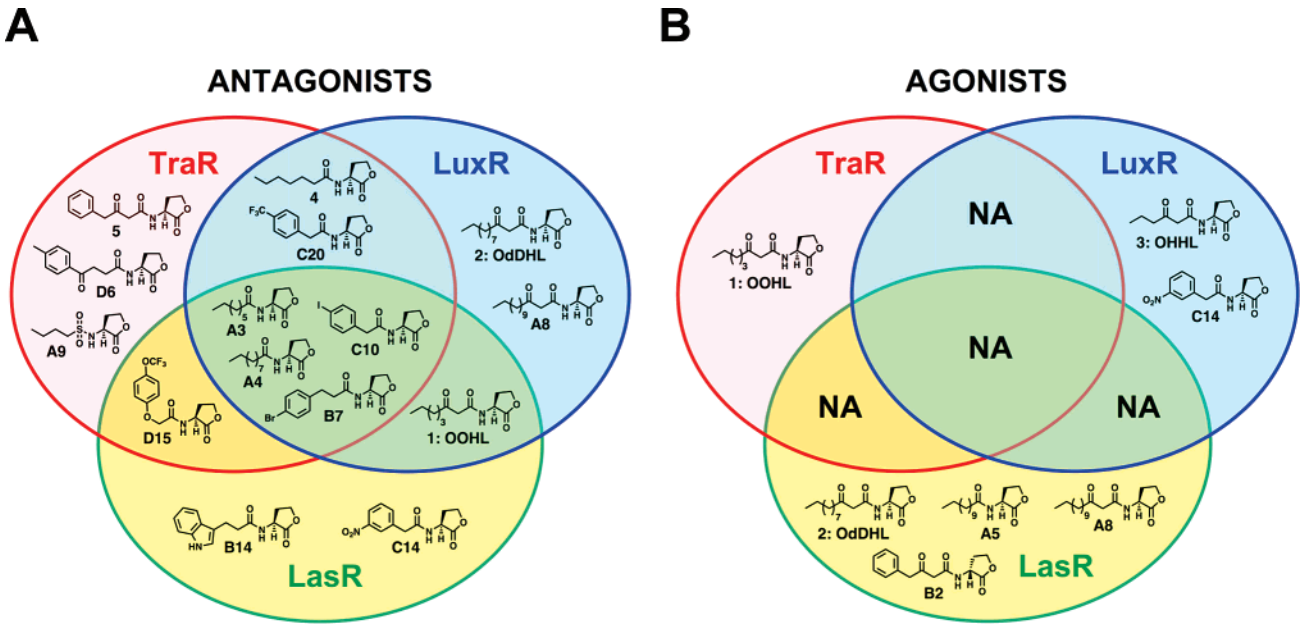

Figure 5. Venn diagrams showing the structures of most potent R protein antagonists and agonists identified and their selectivities for different R proteins over the concentrations tested in this study. Ligands in the intersections of the circles have significant selectivity for two or more R proteins. (A) Diagram of the 13 most active antagonists from libraries A−D and the two most active control antagonists (**4** and **5**). (B) Diagram of the four most active agonists from libraries A−D and the three native AHL ligands (**1**−**3**). NA = no applicable ligands identified.

Table 2. EC_{50} Values for Most Active Agonists across the Three Strains[a]

entry	compd	*A. tumefaciens* TraR (μM)[b]	*P. aeruginosa* LasR (μM)[b]	*V. fischeri* LuxR (μM)[b]
1	**1** (OOHL)	0.20		
2	**2** (OdDHL)		0.01	
3	**3** (OHHL)			3.00
4	**A4**		>200[c]	
5	**A5**		0.04	
6	**A8**		0.01	
7	**B2**		0.54	
8	**C6**			>200[c]
9	**C8**			>50[c]
10	**C14**			0.35
11	**C22**		>200[c]	
12	**D14**		1.62 (36%)[d]	
13	**D15**		6.28 (30%)[d]	
14	**D18**		0.47 (32%)[d]	

[a] See Experimental Section for details of reporter strains. EC_{50} values determined by testing compounds over a range of concentrations (2×10^{-2}−2×10^{5} nM) in each reporter strain. All assays performed in triplicate. See Supporting Information for plots of full agonism dose response curves.[b] A missing entry means not determined. [c] Dose response curve did not fully plateau over the concentrations tested. [d] Dose response curve reached a plateau over the concentrations tested, yet the level of maximal induction was lower than that for the natural ligand OdDHL (**2**); EC_{50} value calculated from this dose response curve. Value in parentheses equals the maximum induction value achievable (at 200 μM ligand) relative to OHHL (**3**).

ligands, where the L-stereoisomer is an active R protein agonist and the D-stereoisomer is almost inactive.[31]

The most remarkable outcome of our agonism dose response studies was in *V. fischeri*. Here, PHLs with substituents in the 3-position on the phenyl ring were the only non-native ligands displaying appreciable agonistic activity (**C6**, **C8**, and **C14**; Table 2). Most notably, we determined an EC_{50} value for 3-nitro PHL **C14** against LuxR that was *~10-fold lower than that for its native ligand OHHL (**3**)* (0.35 vs 3.0 μM). Additional studies of **C14** in our laboratory have demonstrated that this PHL also exerts it super-agonistic activity in wild-type *V. fischeri*.[50] This result was extraordinary, as there are only three other reported synthetic super-activators of R proteins (see above)[44,46] and no known super-activators of the model symbiont *V. fischeri*. PHL **C14** is structurally dissimilar to these other three ligands (**10**−**12**; Figure 1) and represents a powerful new tool to probe the roles of quorum sensing in beneficial bacterial symbioses. Intriguingly, PHL **C14** was also identified as a potent *antagonist* of LasR (see above; Figure 5A), indicating a complex activity profile for PHLs as R protein modulators.

Examination of Synthetic LasR Antagonists in a Virulence Factor Production Assay. As the primary assays for synthetic LasR modulators were performed in a heterologous *E. coli* reporter strain, we sought to determine if the active ligands identified in these screens were also active against LasR in *P. aeruginosa*. The metalloprotease elastase B is a virulence factor that is produced and excreted by *P. aeruginosa* under the control of LasR. Synthetic ligands that inhibit LasR should therefore also inhibit the production of elastase B, and this can be measured by a standard enzymatic assay in the presence of an elastase B substrate (elastin). We examined a set of LasR antagonists identified in the primary assays of libraries A−D (**B7**, **C11**, **C14**, and **D15**), along with selected controls (**8**, **9**, and **C18**), using a previously reported colometric assay for elastase B in *P. aeruginosa* (PAO1) that utilizes an elastin−Congo red substrate.[38] Notably, controls **8** and **9** had been previously shown to inhibit LasR in a *P. aeruginosa* strain.[47] PHL **C18** was chosen as an additional control for this assay, as this ligand only exhibited weak LasR inhibition in the *E. coli* reporter strain (see Table S-2; Supporting Information), and we sought to determine if it would also show weak activity in the elastase B production assay.

The assay revealed several potent synthetic inhibitors of elastase B production and, thus, LasR in wild-type *P. aeruginosa* (Figure 6). As expected, the 4-bromo PHL **8** and indole AHL **9** controls were strong inhibitors of elastase B production in this assay, inhibiting enzyme activity by 77% and 66% at 200 μM, respectively, while PHL **C18** poorly inhibited elastase B

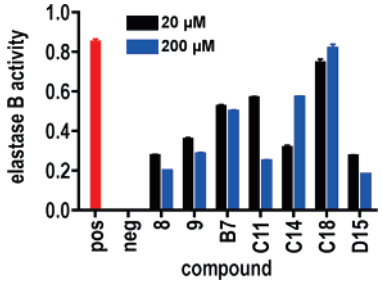

Figure 6. Elastase B production inhibition assay in *P. aeruginosa* PAO1. Selected controls and synthetic ligands in libraries A−D examined for inhibitory activity at two concentrations. Positive control (pos) = activity of elastase B produced by *P. aeruginosa* in the absence of compound. Negative control (neg) = growth media. Error bars = standard deviation of the means of triplicate samples.

production (4% at 200 μM). We were pleased to observe that all of the new LasR antagonists (**B7**, **C11**, **C14**, and **D15**) were moderate to strong inhibitors of elastase B production at 200 μM (up to 79% for phenyl ether AHL **D15**). Interestingly, 3-nitro PHL **C14** displayed *increased* inhibitory activity at *10-fold lower* concentration (33% inhibition at 200 μM vs 63% at 20 μM; Figure 6), while the inhibitory activities of the other three ligands (**B7**, **C11**, and **D15**) were either comparable or slightly stronger at 200 μM relative to 20 μM. The reasons behind this concentration-dependent change in activity for PHL **C14** were unclear, and we return to this observation below. Nevertheless, this virulence factor production assay demonstrated that the active inhibitors we uncovered in the *E. coli* LasR reporter strain assays are indeed inhibitors of LasR in *P. aeruginosa* and served to validate the use of this reporter strain for the primary screening of synthetic modulators of LasR.

SAR Analysis of Active Ligands. The dose response analyses above identified the most active R protein modulators in libraries A−D (Figure 5), and we carefully inspected these structures in an attempt to determine SARs that conveyed R protein selectivity or activity across the three species. Such study revealed that subtle structural differences tuned ligand selectivity and activity. A list of the eight most prominent SAR trends and R protein characteristics influencing antagonistic and agonistic activities for libraries A−D is provided as follows: (1) In general, AHLs with acyl groups of moderate size (up to eight atoms long) and containing either aromatic functionality with electron-withdrawing groups or straight chain aliphatic functionality can antagonize TraR, LasR, and LuxR. AHL **B7** exemplifies such a broad-spectrum antagonist. (2) The PHL appears to be a "privileged" scaffold for R protein modulation, as these ligands display a wide range of antagonistic and agonistic activities across all three R proteins in this study. Ligand activity is highly dependent on the structure and position of substituents on the phenyl group. Specifically, PHLs with electron-withdrawing and lipophilic substituents in the 4-position on the phenyl group display the strongest antagonistic activities against TraR and LuxR. The same trend holds true in LasR for PHLs with substituents in the 3-position. (3) Of the AHLs structurally related to control 4-bromo PHL **8**, a flexible carbon spacer of at least one carbon between the lactone ring and an aromatic acyl group and a 4-bromo substituent on the phenyl group engender the strongest antagonistic activity, with AHL **B7** being the most active inhibitor in this structure class across the three R proteins. (4) A three-carbon spacer between the lactone ring and an aromatic acyl group is optimal for inhibition in ligands structurally related to control indole AHL **9**. This trend is most apparent for LasR (i.e., AHL **B14**). (5) Sulfonyl groups can replace carbonyl groups on aliphatic AHL TraR and LuxR antagonists without significant loss in activity. The sulfonyl HLs in this study were most active against TraR (e.g., **A9**), yet virtually inactive against LasR. (6) TraR is the most sensitive to the length of the acyl group on AHLs, as inhibitory activity drops off dramatically for AHLs with acyl tails longer than eight atoms. This observation is in accord with both its native ligand, OOHL **(1)**, which contains an octanoyl group, and the sterically constricted ligand-binding site of TraR as revealed by X-ray crystallography (assuming the synthetic ligands target the same site; see below). (7) LasR is the most tolerant of varying functionality on the AHL acyl chain, acyl chain size, and the stereochemistry of the homoserine lactone ring, suggesting that it has a larger ligand-binding site than TraR. This result is in accord with the X-ray crystal structure of LasR, assuming that synthetic AHLs target the same binding site on LasR (as with TraR, see below).[20] (8) LuxR is most strongly inhibited by AHLs with medium to long (6−14 carbons), 3-oxo-aliphatic acyl groups and most strongly *activated* by PHL ligands with electron-withdrawing substituents in the 3-position.

Targets of Synthetic AHL Antagonists and Agonists. In view of the structural similarities of the synthetic AHLs in libraries A−D to native AHLs and the subtle SARs described above, we hypothesize that these ligands target R protein ligand-binding sites and that inhibition or activation is based on the specific binding mode and, therefore, affinity of the ligand. Further, we do not believe that these changes in antagonistic or agonistic activity simply reflect the different *chemical* properties of the synthetic AHLs.[13] This assertion is supported by several observations. First, the percentage of lactone hydrolysis (which abolishes activity for native AHLs)[41] for the synthetic ligands was minimal and identical with that of the native ligands over the time course of the reporter gene assays (see above). Second, higher ligand lipophilicity and, therefore, higher potential cell permeability did not correlate with enhanced antagonistic or agonistic activity (Tables 1 and 2). This was further exemplified by the D-AHLs **B1**−**B5**, which have lipophilicities identical with those of OOHL (**1**) and control antagonists **5**, **6**, **8**, and **9**, respectively, yet exhibit markedly different activities (see Table S-2; Supporting Information). Third, as we previously reported, selected PHLs (e.g., PHL **C14**) failed to exhibit any activity in a Δ-*luxR* derivative of *V. fischeri* ES114, suggesting that these ligands exert their activity through the LuxR protein.[50]

To further test our hypothesis that these ligands target R protein ligand-binding sites, we performed molecular modeling studies of several of the most active synthetic AHLs docked into the ligand-binding sites of TraR and LasR (using the X-ray crystal structures)[15,16,20] and the putative ligand-binding site of LuxR (built in silico from TraR by homology modeling;[33,34,36] see Figures S-22−S-33; Supporting Information). The results of these studies suggest that all three ligand-binding sites can readily accommodate the synthetic AHLs, and that activation or inhibition of the R protein may depend on the subtle balance of favorable hydrogen bonding and unfavorable steric interactions within the binding pocket. The LasR ligand-binding site appears to be the most accommodating in terms of ligand size, while the TraR ligand-binding site appears the most restrictive and the LuxR ligand-binding site falls between these two

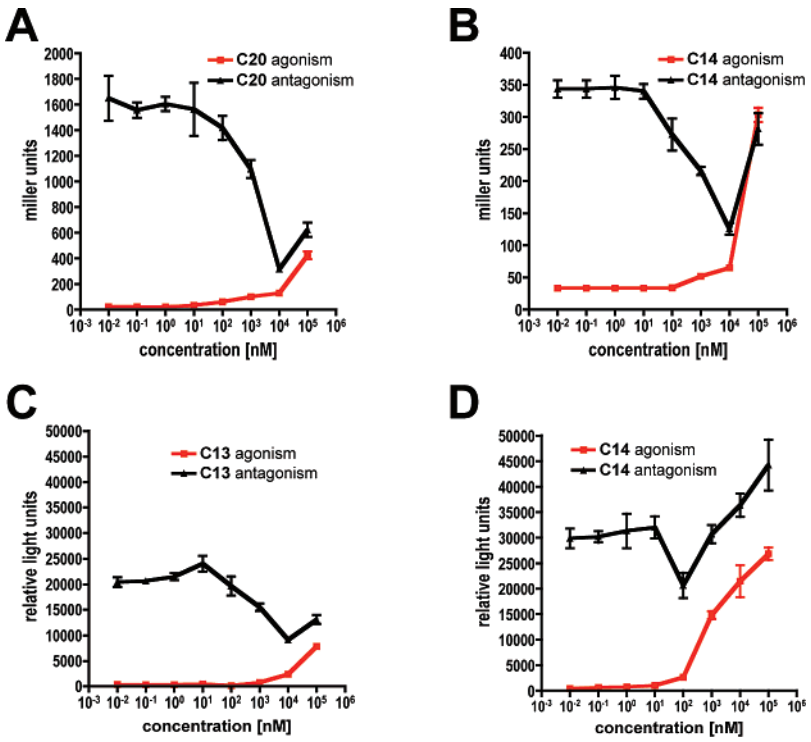

Figure 7. Representative dose response curves for selected synthetic ligands identified in this study. For strains, see the Experimental Section. (A) PHL **C20** screened in *A. tumefaciens*. Antagonism dose response assay performed in the presence of 100 nM OOHL (**1**). Miller units report relative absorbance. (B) PHL **C14** screened in *E. coli* (LasR reporter). The antagonism dose response assay is performed in the presence of 7.5 nM OdDHL (**2**). (C) PHL **C13** screened in *V. fischeri*. The antagonism dose response assay is performed in the presence of 3 μM OHHL (**3**). Relative light units report luminescence. (D) PHL **C14** screened in *V. fischeri*. The antagonism dose response assay is performed in the presence of 3 μM OHHL (**3**).

extremes; these observations match the general SAR trends delineated above. While additional biochemical and structural experiments are needed to further test our hypothesis, these computational experiments further support the supposition that the synthetic AHLs identified in this study target R proteins.

Mechanistic Insights into R Protein Modulation by Synthetic Ligands. Further insights into how our synthetic ligands modulate R protein function were acquired through additional scrutiny of the primary screening data and dose response studies outlined above. Several ligands displayed unexpected activity trends in these assays. For example, AHLs **D14**, **D15**, and **D18** inhibited LasR in the presence of native ligand OHHL (**3**) in antagonism assays, yet *activated* LasR to the same level in agonism assays (~35%; see Table S-2; Supporting Information). More strikingly, we observed that over 60% of the AHL antagonists identified in this study exhibited antagonism dose response curves that started to slope back up at higher concentrations (see Table 1), indicating that these ligands were also capable of *activating R proteins at higher concentrations*. This trend could not be correlated with specific structural features yet loosely correlated with ligand activity (i.e., those with the lowest IC_{50} values) in TraR and LasR. Representative antagonism dose response curves demonstrating this upturn are shown in Figure 7A−C (PHLs **C20** in TraR, **C14** in LasR, and **C13** in LuxR). Agonism dose response studies of these compounds revealed that they were in fact capable of activating R proteins at higher concentration, in some cases quite strongly (i.e., **C14** in LasR). Indeed, the agonism dose response curves began to curve up at precisely the concentration where their respective antagonism dose response curves began to upturn (see Figure 7A−C). The 3-nitro PHL **C14** exhibited this dual behavior in two other instances in this study. First, similar antagonism and agonism dose response curves could be generated for PHL **C14** in *V. fischeri*, where this compound can behave as a super-activator (Figure 7D). Second, PHL **C14**, initially identified as a LasR antagonist, was also capable of activating elastase B production in *P. aeruginosa* at high concentrations (see Figure 6). These observations suggested that defining these AHLs exclusively as R protein antagonists or agonists, as we had up until this point, was incorrect.

This duality of ligand activity suggests that these "antagonists" (such as PHLs **C13** in LuxR, **D14**, **D15**, **D18**, and **C14** in LasR and **C20** in TraR) are actually best described as partial agonists. Partial agonists have properties of agonists and antagonists and are broadly characterized by three phenomena: (1) At middle-range concentrations, partial agonists act as antagonists. (2) The maximum response (efficacy) of a partial agonist is lower than that of the natural ligand for a target receptor. (3) In dose response analyses against variable concentrations of the native ligand, the baseline activity increases with the concentration of partial agonist such that, at high concentrations, activity is equal to the partial agonist's efficacy.[59−61] Our primary antagonism assay data for the AHLs that displayed this dual activity were in accord with the first characteristic of partial agonism (e.g., see Table 1 and Figure 7A−C).

To examine if these AHLs also exhibit the two other defining characteristics of partial agonism, we carried out additional experiments with selected AHLs (**C8** and **C13** in LuxR and **D14**, **D15**, and **D18** in LasR). We performed agonism dose

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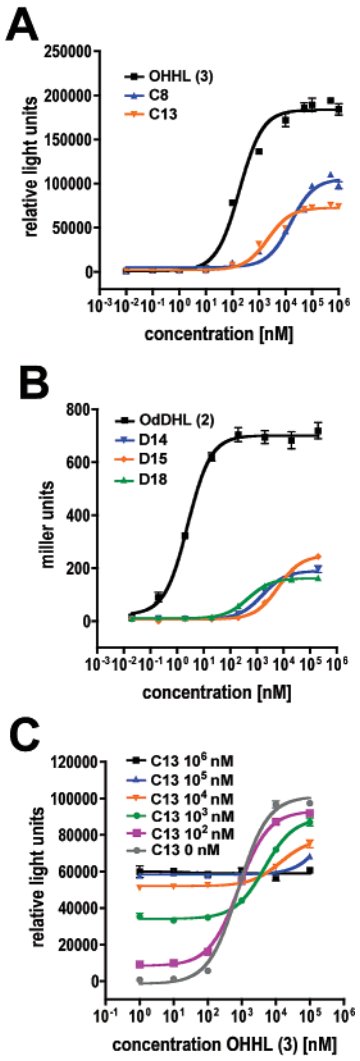

Figure 8. Representative agonism dose response curves for selected synthetic ligands identified in this study. For strains, see the Experimental Section. (A) OHHL (**3**) and PHLs **C8** and **C13** screened in *V. fischeri*. Relative light units report luminescence. (B) OdDHL (**2**) and AHLs **D14**, **D15**, and **D18** screened in *E. coli* (LasR reporter). Miller units report relative absorbance. (C) PHL **C13** screened in *V. fischeri* against OHHL (**3**) over varying concentrations in a two-dimensional dose response format.

response studies of the five AHLs over a broader range of concentrations in the LasR and LuxR reporter strains and determined that these ligands show lower agonism levels, or efficacies, than the native ligands OdDHL (**2**) and OHHL (**3**) (Figure 8A,B). Indeed, the maximal responses of these compounds were up to 4-fold lower than the maximal response of the corresponding native ligand. Next, we performed dose response studies on PHL **C13** in *V. fischeri* against varying concentrations of OHHL (**3**) to test the third distinguishing characteristic of partial agonism. We found that AHL **C13** exhibited an increase in baseline activity that is expected for a partial agonist (Figure 8C). Again, since the efficacy of a partial agonist is lower than that of the native ligand, the baseline dose did not reach a maximal response but instead reached a plateau at the efficacy level for **C13**. Similar competitive dose response data also were obtained for **C8**, **D14**, **D15**, and **D18** (data not shown). Together, these experiments provide strong support for a partial agonism mechanism for R protein modulation by these non-native AHLs.

The origins of this partial agonism by synthetic AHLs could be multifold, including lowered affinity of the ligand for the R protein, impeded folding (or destabilization) of the R protein upon ligand binding, lowered affinity of the R protein:ligand complex for homodimerization, formation of heterodimers with the R protein:native ligand complex, and/or lowered affinity of these homodimers or heterodimers for DNA. All such pathways would be in accord with the currently accepted mechanisms of action for native AHL ligands (see above).[21,22,25] In turn, this partial agonism model also helps to explain the mechanism of super-agonism for PHL **C14** in LuxR; here, this ligand may be capable of stabilizing LuxR *to a greater extent relative* to OHHL (**2**). Additional biochemical experiments are required to test these hypotheses and are ongoing in our laboratory.

To our knowledge, these data represent the first definitive report of synthetic AHL ligands behaving as partial R protein agonists and lead to many new and important questions. For example, do all of the ligands identified as agonists and antagonists in this study behave through a similar mechanism? If so, is this phenomenon dependent on the ligands having a HL head group? How do the SARs delineated above dictate partial agonist activity? What are the mechanisms of action of other reported AHL and non-AHL modulators of R protein function? We are actively seeking answers to these broad questions to fully understand the biochemical mechanisms of action of these synthetic ligands. In lieu of these answers, however, the activity trends for the synthetic AHLs identified in this study indicate that the mechanisms of small-molecule modulation of R protein function are more complex than perhaps we originally anticipated.

Summary and Conclusions

We have designed and synthesized four focused collections of synthetic AHL ligands and systematically examined these ~90 compounds in three bacterial reporter strains to determine their abilities to modulate R protein function. These studies have revealed some of the most potent synthetic antagonists and agonists of the well-characterized R proteins TraR, LasR, and LuxR reported to date. These ligands include AHL **A4**, 4-bromophenylpropionyl HL **B7**, 4-iodo PHL **C10**, and 3-nitro PHL **C14**. Several of the LasR antagonists (most notably AHL **D15**) were capable of strongly inhibiting virulence factor production in *P. aeruginosa* that is essential for pathogenesis. In addition, we have identified critical structural features that confer antagonistic and agonistic activities to these synthetic AHL ligands against the three R proteins. In general, the AHLs modulating TraR and LuxR were sterically more compact and less lipophilic than those for LasR, with TraR being the most discriminatory in terms of ligand size. These data are in accord with the ligand-binding sites for TraR and LasR as indicated by recent X-ray crystal structures.[15,16,20] Subtle alterations to substituents and their placement on the AHL acyl group dramatically influenced ligand activity. This effect was most remarkable in the PHL library (library C), where these structural changes (e.g., shifting substituents from the 4- to the 3-position on the phenyl ring) did not simply abolish activity, but rather converted potent antagonists (or partial agonists) into agonists or even a super-activator (i.e., **C14**). In addition, we also discovered that the synthetic D-AHL **B2** is capable of strongly activating LasR. This ligand represents, to our knowledge, the

first reported D-AHL capable of significantly modulating R protein activity.

Overall, the most significant outcome of this work is the identification of sets of ligands that selectively modulate one, two, or all three of the R proteins in this study (Figure 5). This result fulfilled the overarching goal of this study—to identify both selective and multispecies modulators of R proteins and, therefore, quorum-sensing responses in Gram-negative bacteria. A second major outcome of this work was our discovery that many of the most potent R protein "antagonists" identified in the reporter gene assays exert their activities through a partial agonism mechanism. This represents a new paradigm for ligand activity against R proteins. Further, such partial agonists could hold significant promise for the exploration of the medicinal outcomes of quorum-sensing modulation.[61] Last, a third key outcome is the identification of such a large set of potent ligands through the synthesis and screening of a relatively limited set of AHLs (~90 compounds). This result suggests that we have only scratched the surface of the pool of non-native modulators of R protein function and further underscores the utility of focused combinatorial libraries for the identification of such compounds. The design and examination of expanded compound libraries, containing AHL and non-AHL structures classes, is clearly warranted to further probe the features of chemical space essential for small-molecule-mediated R protein activation and inactivation. The new SARs and mechanistic insights delineated in this study will shape the design of a such next-generation quorum-sensing modulators.

In closing, we report that the synthetic AHLs identified herein represent a new and expansive set of chemical tools for the study of quorum sensing in Gram-negative bacteria and could, with further development, provide broad insights into bacterial pathogenesis and beneficial symbioses. We are actively engaged in such experiments to examine the scope and limitations of these compounds in vitro and in vivo. Preliminary work in invertebrate model systems indicates that several of these ligands are well tolerated and can modulate quorum-sensing responses in vivo. These data, along with the mechanistic studies introduced above, will be reported in due course.

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Supporting Information Available: General experimental information, purity and mass spectral data for all library compounds, selected primary assay data, full characterization data and dose response curves for compounds in Tables 1 and 2, molecular modeling data, and complete refs 35 and 55. This material is available free of charge via the Internet at http://pubs.acs.org.

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