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# A Comparative Analysis of Synthetic Quorum Sensing Modulators in *Pseudomonas aeruginosa*: New Insights into Mechanism, Active Efflux Susceptibility, Phenotypic Response, and Next-Generation Ligand Design

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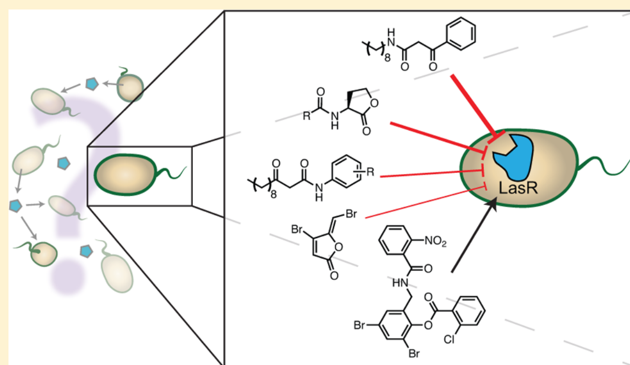
*Supporting Information*




**ABSTRACT:** Quorum sensing (QS) is a chemical signaling mechanism that allows bacterial populations to coordinate gene expression in response to social and environmental cues. Many bacterial pathogens use QS to initiate infection at high cell densities. Over the past two decades, chemical antagonists of QS in pathogenic bacteria have attracted substantial interest for use both as tools to further elucidate QS mechanisms and, with further development, potential anti-infective agents. Considerable recent research has been devoted to the design of small molecules capable of modulating the LasR QS receptor in the opportunistic pathogen *Pseudomonas aeruginosa*. These molecules hold significant promise in a range of contexts; however, as most compounds have been developed independently, comparative activity data for these compounds are scarce. Moreover, the mechanisms by which the bulk of these compounds act are largely unknown. This paucity of data has stalled the choice of an optimal chemical scaffold for further advancement. Herein, we submit the best-characterized LasR modulators to standardized cell-based reporter and QS phenotypic assays in *P. aeruginosa*, and we report the first comprehensive set of comparative LasR activity data for these compounds. Our experiments uncovered multiple interesting mechanistic phenomena (including a potential alternative QS-modulatory ligand binding site/partner) that provide new, and unexpected, insights into the modes by which many of these LasR ligands act. The lead compounds, data trends, and mechanistic insights reported here will significantly aid the design of new small molecule QS inhibitors and activators in *P. aeruginosa*, and in other bacteria, with enhanced potencies and defined modes of action.

## ■ INTRODUCTION

Many common bacteria use an intercellular chemical signaling process termed quorum sensing (QS) to coordinate local population density with group-beneficial behaviors.[1] In Gram-negative bacteria, QS is largely mediated by *N*-acylated L-homoserine lactone (AHL) signals, which are produced by LuxI-type enzymes and sensed by intracellular LuxR-type receptors (Figure 1).[2] The AHL ligands passively diffuse out of the cell and into neighboring cells; some bacteria also use active efflux to facilitate AHL dissemination.[3,4] As the bacterial population grows within the confines of a particular environment, the local concentration of AHL signal likewise increases. Once the AHL concentration reaches a threshold intracellular level (corresponding to a "quorate" bacterial population), productive binding of the AHL to its target LuxR-type receptor occurs. This binding event typically induces receptor dimerization, DNA binding, and subsequent transcriptional activation of QS target genes.

Numerous bacterial pathogens use QS to regulate the timing and extent of virulence factor production, thereby allowing them to amass until a sufficient population has been achieved to overwhelm a host immune response.[2] As QS is dependent on small molecule signals and the relative concentration thereof, there is substantial interest in the development of chemical strategies that disable QS signaling networks and thus stem or even prevent virulence. Such "anti-virulence" approaches could provide novel pathways to mitigate bacterial infection in humans, animals, and plants.[5−8] More fundamentally, chemical interventions could provide new insights into the mechanisms

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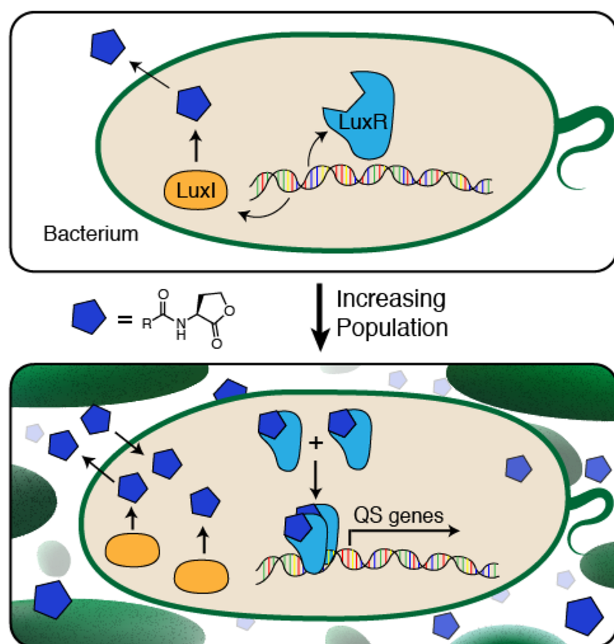


**Figure 1.** Simplified QS circuit in Gram-negative bacteria. LuxI-type synthases produce AHLs (blue pentagons) that can bind to cognate LuxR-type receptors. At high cell densities, activated receptors induce transcription of QS genes.

by which bacteria use QS to their advantage, insights that might not be readily elucidated using traditional genetic methods.[9−12]

The most common Gram-negative bacterium found in hospital-acquired infections, *Pseudomonas aeruginosa*, uses QS to regulate the production of numerous extracellular proteases, biofilm maturation factors, and toxins.[5] This opportunistic pathogen has become increasingly resistant to most current antibiotic therapies, so the need for the development of new approaches to treat *P. aeruginosa* infections is urgent.[13] Accordingly, chemical strategies to inhibit QS in *P. aeruginosa* have received significant recent attention. Such nonbactericidal, antivirulence approaches could also be particularly robust to resistance development, further enhancing their potential utility.[14,15]

*P. aeruginosa* has a relatively complex QS network that includes (at least) two LuxI/LuxR pairs: LasI/LasR and RhlI/RhlR. The *las* subnetwork utilizes *N*-(3-oxododecanoyl) L-homoserine lactone (OdDHL, **1**; Figure 2) as its signal, while the *rhl* subnetwork uses *N*-butyryl L-homoserine lactone (BHL). LasR and RhlR each activate discrete regulons involved in virulence; however, as LasR activates the *rhl* system, LasR has been a principle target of study for the development of small molecule QS modulators in *P. aeruginosa*.[16] The selection of LasR for investigation is further supported by the observation that *P. aeruginosa* LasR mutants have dramatically attenuated virulence and invasiveness in certain in vivo infection models.[17] Over the past ~20 years, campaigns of rational design,[18−25] high-throughput screening,[26−28] and computational modeling[29,30] have revealed a large number of compounds reported to modulate LasR transcriptional activity; the bulk of these ligands are anticipated to directly compete with OdDHL for binding to LasR (albeit definitive mechanistic data is scarce; see below). Several of these compounds, both AHL-derived and otherwise, have been shown to modulate important QS-dependent virulence phenotypes in *P. aeruginosa* and certainly constitute chemical tools to study QS pathways in this pathogen.

That said, there remain significant challenges for the further design and application of non-native LasR ligands. The following three issues are perhaps most urgent: First and foremost, the majority of these compounds have been tested for activity in LasR using *widely variable* biological assays (see below). Furthermore, any systematic side-by-side comparisons of known LasR modulators have been extremely limited, typically comparing, at maximum, 2−4 control compounds to new ligands of interest.[11,21,24,25,31] Second, for the compounds for which LasR $IC_{50}$ values have been calculated in *P. aeruginosa*, these values are typically only low-micromolar (in cell-based assays). Molecules with heightened potencies would undoubtedly be of value for both fundamental and applied QS research. Third, the scientific community has virtually no mechanistic information about how the known synthetic LasR ligands interact with the receptor (if they do so directly) and modulate its function. Slowing such studies is the fact that LasR, similar to many other LuxR-type receptors, is relatively unstable in the absence of native ligand (OdDHL), which has prevented the use of in vitro assays to directly assess small molecule antagonism. Collectively, these challenges preclude (i) the selection of a lead LasR ligand scaffold for advancement as a robust chemical probe, and (ii) the cultivation of new and informed ligand design strategies.

To date, the activities of reported LasR modulators typically have been measured using cell-based assays reliant on a genetically engineered reporter. Reporter gene assays have been performed in a wide array of *P. aeruginosa* and heterologous (*E. coli*) LasR-producing strains using many different reporter constructs and conditions, resulting in a broad range of reported ligand activities for LasR activation or inhibition.[32] Numerous research groups have also advanced lead compounds into *P. aeruginosa* bioassays that measure attenuation of QS-controlled virulence phenotypes,[16] but these studies are equally disparate in the phenotypes studied and in the experimental conditions used (for a listing, see Table S2). Confounding such assays is the fact that attenuating wild-type *P. aeruginosa* virulence phenotypes is often more difficult than simply disrupting LasR in an *E. coli* "biosensor" strain. Small molecule modulators must contend with a number of obstacles presented by *P. aeruginosa*, including but not limited to enzymatic degradation,[33] low membrane permeability,[34] active efflux,[35] and constitutive production of the native autoinducers.[36] Thus, compounds that fail in these assays may do so for reasons other than low intrinsic activity on LasR. Determining the most promising small molecule scaffolds for further development as LasR modulators—ideally, ones that subvert the aforementioned obstacles present in wild-type *P. aeruginosa*—is of paramount importance to researchers working at the growing interface of chemistry and biology in the QS field. Identifying such compounds was the motivation for the current study.

Herein, we report the first comparative analysis of the most promising synthetic LasR modulators reported to date. This set of compounds comprises natural and non-natural AHLs, AHL analogues, natural products, and structurally unique molecules (Figure 2). We began by comparing compound potency in a single *P. aeruginosa* LasR reporter strain, and thereafter examined these compounds for *direct* LasR modulation in a single *E. coli* LasR reporter. The activity trends uncovered in these standardized reporter studies were also recapitulated in our QS phenotypic assays in wild-type *P. aeruginosa*, most


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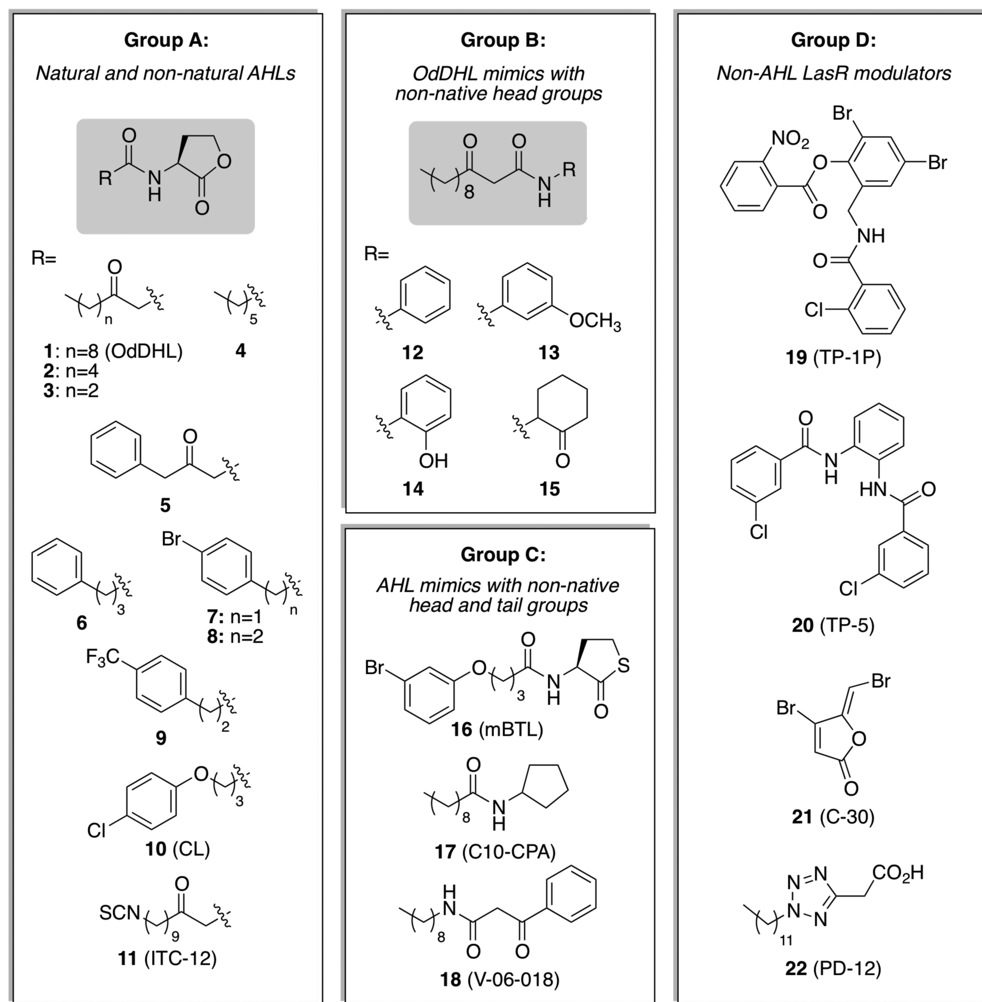


**Figure 2.** Structures of natural (**1**−**3**) and non-natural (**4**−**11**) AHLs, OdDHL mimics retaining the native 3-oxo-C12 tail (**12**−**15**), AHL mimics with non-native head and tail groups (**16**−**18**), and structurally unique compounds (**19**−**22**) chosen for evaluation of LasR modulatory activity and *P. aeruginosa* phenotypic response. Compounds were developed by the following laboratories: **4**, Winans and co-workers; **5**, **6**, Doutheau and co-workers; **7**−**9**, **12**, Blackwell and co-workers; **10**, **16**, Bassler and co-workers; **11**, Meijler and co-workers; **13**, Spring and co-workers; **14**, **15**, Suga and co-workers; **17**, Kato and co-workers; **18**−**20**, **22**, Greenberg and co-workers; **21**, Givskov and co-workers. See Supplementary Note S1 and Table S2 for key citations for each library member.

notably revealing two compounds capable of completely inhibiting the QS-dependent production of a key virulence factor. In the course of our investigations, we also discovered a series of interesting, and unexpected, dose−response phenomena for certain LasR modulators. These observations are significant, as they provide mechanistic insights—with respect to active efflux, receptor overexpression in heterologous strains, and the competitive or noncompetitive interactions of antagonists—that most likely apply not only to LasR, but also to the many other LuxR-type homologues found in bacteria.

## ■ EXPERIMENTAL SECTION

**Chemical Reagents and Instrumentation.** All chemical reagents and solvents were purchased from commercial sources (Acros, Alfa-Aesar, Fisher, Sigma-Aldrich) and used without further purification. See Supporting Information for details of NMR, HPLC, and MS instrumentation.

**LasR Modulator Library Compounds.** Compounds **1**−**4**, **15**, and **21** were purchased from Sigma-Aldrich. Compounds **5** and **6**,[18] **7**−**9**,[21] **10**,[11] **12**,[37] **13**,[25,38] **14**,[31] **16**,[11] **17**,[22] **18**,[11] **20**,[14] **21**,[39] and **22**[25] were synthesized as reported previously and yielded spectra that matched those reported. Compounds **11** and **19** (TP-1P) were generously supplied by the laboratories of Prof. Michael Meijler and Prof. Peter Greenberg, respectively. $^{1}H$ and $^{13}C$ NMR spectra of **19** (TP-1P) matched those reported by Janda and co-workers (see Supporting Information).[40] As the reported potencies of **19** (TP-1P) and its related isomer TP-1R are similar (an activity trend that we also observe; data not shown),[40] we examined only **19** herein. The two AHL analogues reported by Bassler and co-workers, **10** and **16** (evaluated previously as racemates),[11] were synthesized for this study in enantiopure form, using L-homoserine lactone and L-homocysteine thiolactone, respectively. Characterization data (HPLC, MS, and/or NMR) for compounds **1**−**4**, **10**, **11**, **13**, and **15**−**22** (i.e., those not characterized in our prior studies) are provided in the Supporting Information.

**Compound Handling.** Stock solutions of library compounds (100 mM, unless limited by solubility of the compound) were prepared in DMSO and stored at −20 °C in sealed vials. Solvent-resistant polypropylene or polystyrene 96-well microtiter plates (Costar) were used when appropriate for LasR reporter gene assays.

**Biological Reagents and Strain Information.** All standard biological reagents were purchased from Sigma-Aldrich or Gold Biotechnology and used according to enclosed instructions. Buffers and solutions for Miller absorbance assays in *E. coli* (Z buffer, 0.1% aqueous SDS, and phosphate buffer) were prepared as described


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previously.[41] Water (18 MΩ) was purified using a Millipore Analyzer Feed System.

The bacterial strains and plasmids used in this study are listed in Table S1. Bacteria were grown in a standard laboratory incubator at 37 °C with shaking (200 rpm) in Luria−Bertani (LB) medium unless otherwise noted. Absorbance and fluorescence measurements were obtained using a Biotek Synergy 2 microplate reader using Gen5 1.05 data analysis software. All biological assays were performed in triplicate. $EC_{50}$ and $IC_{50}$ values, as well as respective 95% confidence intervals, were calculated using GraphPad Prism software (v. 4.0) using a sigmoidal curve fit (see Supplementary Note S6 for more information regarding curve fitting).

***P. aeruginosa* LasR Reporter Assay Protocol.** Compound activities in the *P. aeruginosa* LasR reporter strains were measured according to our previously reported method,[35] with the following modifications: Overnight cultures were grown for exactly 20 h; for antagonism assays in *P. aeruginosa* PAO-JP2, the 1:100 subculture was pretreated with 150 nM OdDHL; for antagonism assays in *P. aeruginosa* PAO-JG21, the 1:100 subculture was pretreated with 20 nM OdDHL. For full assay protocol, see Supplementary Note S3.

***E. coli* LasR Reporter Assay Protocol.** Compound activities in the *E. coli* JLD271 LasR reporter strain were measured according to previously reported methods (Blackwell and co-workers[21] for LasR reporter strain growth; Wolf and co-workers[42] for *β*-galactosidase activity measurement), with the following modifications: The *E. coli* Δ*sdiA* strain JLD271[43] was used to harbor the LasR expression and reporter plasmids pSC11 and pJN105L, respectively; the 1:10 subculture was grown to an $OD_{600}$ of 0.450 before inducing LasR expression with 4 mg/mL L-arabinose and pretreating with 2 nM OdDHL; the cell permeabilization mixture was optimized to contain 200 *μ*L Z-buffer, 8 *μ*L $CHCl_3$, and 4 *μ*L 0.1% aqueous SDS; the *β*-galactosidase substrate chlorophenol red-*β*-D-galactopyranoside (CPRG) was used, and thus no termination/quenching step was necessary. For full assay protocol, see Supplementary Note S4

***P. aeruginosa* LasR Overexpression/Reporter Strain Construction and Protocol.** The LasR overexpression plasmid pJN105L was introduced into *E. coli* S17−1::*λ*pir by electroporation and then transferred to *P. aeruginosa* PAO-JP2 by conjugation and selection on LB supplemented with gentamicin (10 *μ*g/mL) and tetracycline (12 *μ*g/mL). Reporter assays measuring compound activities on LasR were performed as in the above *P. aeruginosa* assays, but L-arabinose (4 mg/mL) was added to subcultures immediately prior to dispensing subculture into compound-treated plates.

**Elastase B Production Assay in Wild-Type *P. aeruginosa*.** The activity of elastase B in *P. aeruginosa* culture supernatants was measured colorimetrically using an elastin-Congo red substrate.[44] A 10 mL overnight culture of *P. aeruginosa* PAO1 (wild-type) was grown for 16 h as described above. DMSO stock solutions of test compounds (10 mM) were prepared, and 2 *μ*L aliquots were added to the wells of a clear plastic 96-well microtiter plate (Costar 3370). An inoculating subculture was prepared by pelleting an aliquot of the overnight culture at 1500*g* for 10 min, followed by resuspension of the cell pellet into a 100× volume of fresh LB medium (effecting a 1:100 dilution of the overnight). To each well, a 198-*μ*L aliquot of subculture was added (final compound concentrations were 100 *μ*M, with 1% DMSO), and the plates were incubated for 20 h. The final cell density was measured by reading $OD_{600}$. The cultures were pelleted by centrifugation of the assay plate at 2000*g* for 30 min, and 50 *μ*L of supernatant from each well was transferred to a new 96-well plate. A 150-*μ*L aliquot of 0.5% (w/v) elastin-Congo red conjugate (Elastin Products Co.) in Tris buffer (10 mM Tris, 1 mM $CaCl_2$, pH 7.2) was added to each well, and the plate sealed with a polypropylene storage mat (Costar 3080). The plate was incubated at 37 °C with shaking (200 rpm) while attached to a Labquake rotator (8 rpm) to ensure complete mixing. After 12 h, undigested elastin was pelleted by centrifugation at 1500*g* for 2 min, 100 *μ*L of the supernatant was transferred to a new 96-well plate, and the absorbance at 490 nM was measured. Elastase B activity values for all cultures were background-corrected to that of wells containing no bacteria, then growth-normalized by dividing the resulting absorbance value by the final $OD_{600}$ and plotted relative to a DMSO-treated *P. aeruginosa* PAO1 control. Elastase activity of *P. aeruginosa* PAO-JP2 was included in each experiment as a fully QS-inhibited positive control.

## ■ RESULTS AND DISCUSSION

**LasR Modulator Library Curation.** We selected 22 compounds for our comparative analyses based on a combination of the following factors: (i) noteworthy reported potency and/or efficacy as a LasR antagonist or agonist, (ii) ready synthetic tractability, (iii) commercial availability as a reported LasR modulator, and/or (iv) unique structural or QS-modulatory characteristics. The compound library was then divided into four distinct structural classes (Groups A−D; Figure 2), which roughly follow the main research approaches used to develop LasR modulators over the past decade.

**Group A** includes natural and non-natural AHLs, with a focus on OdDHL analogues that have shown effective modulation of LasR and closely related homologues.[45] Because AHLs naturally derive their receptor specificity from variations in acyl tail structure, many laboratories (including our own)[16,21] have attempted to rationally extend these properties to new AHLs with non-native tails.[46]

Other research groups have taken a complementary approach to rationally designing LasR modulators by retaining (presumably) important ligand−receptor contacts in the 3-oxo-C12 acyl tail of OdDHL, while varying the structure of the cyclic headgroup. This approach can bypass the liabilities associated with the hydrolytically unstable homoserine lactone. **Group B** comprises such OdDHL mimics with alternative head groups.

Some laboratories have sought to combine the advantageous properties of Groups A and B by simultaneously altering both halves of the canonical AHL structure. **Group C** contains the most promising OdDHL mimics with non-natural head and tail groups.

Finally, **Group D** is made up of either lead compounds identified through high-throughput screens or natural product derivatives that strongly modulate LasR and/or QS-dependent phenotypes in *P. aeruginosa*. As opposed to the other three Groups, these Group D compounds have structures that significantly differ from native AHLs. Taken as a whole, this library serves as a representative subset of the most notable LasR modulators reported to date. (For a more detailed background for each compound and pertinent citations, see Supplementary Note S1 and Table S2.)

***P. aeruginosa* LasR Reporter Screens Reveal Potent Agonists and Antagonists.** To allow for direct comparisons of potency and efficacy across each class of LasR modulator, we first performed our studies in a single *P. aeruginosa* AHL synthase-null strain—PAO-JP2 (Δ*lasIrhlI*) harboring the LasR reporter plasmid p*lasI*-LVA*gfp*—under standardized growth and media conditions (see Experimental Section).[47] Given that the majority of the compounds in the library have been previously reported as LasR antagonists, we expected most compounds to effectively inhibit LasR activity in PAO-JP2; thus, we submitted all of the compounds to full dose−response analysis for competitive LasR antagonism (in the presence of OdDHL) in this *P. aeruginosa* strain (Table 1; for dose−response curves, see Figure S1). However, to perform a more thorough analysis of compound activity, we also evaluated each compound for LasR agonism in a single-concentration agonism screen (Table S3). Compounds showing significant LasR



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Table 1. $IC_{50}$ Values for LasR Inhibition by Library Members in *P. aeruginosa* PAO-JP2 (p*lasI*-LVA*gfp*)[a]

| compound | $IC_{50}$ ($\mu$M)[b] | 95% CI ($\mu$M) | max. inhibition (%)[c] |
|---|---|---|---|
| **2** (OOHL)[d] | 5.5 | 3.1–9.8 | 55 |
| **3** (OHHL) | 40 | 26–61 | 80 |
| **4**[e] | ≥100 | – | 25 |
| **5** | 73 | 54–99 | 40 |
| **6** | 175 | 108–284 | 75 |
| **7** | 116 | 89–151 | 80 |
| **8**[d] | 12 | 3.9–34 | 60 |
| **9**[d] | 3 | 0.92–9.7 | 35 |
| **10** (CL)[d] | 21 | 11–39 | 55 |
| **11** (ITC-12) | agonist | – | – |
| **12**[e] | 9.7 | 6.3–15 | 70 |
| **13**[e] | >200 | – | 55 |
| **14**[e] | – | – | – |
| **15**[e] | ≥100 | – | 45 |
| **16** (mBTL) | agonist | – | – |
| **17** (C10-CPA)[e] | ≥50 | – | 45 |
| **18** (V-06-018)[e] | 5.2 | 3.7–7.3 | 85 |
| **19** (TP-1) | agonist | – | – |
| **20** (TP-5)[e,f] | 69 | 61–78 | 100 |
| **21** (C-30)[g] | no activity | – | – |
| **22** (PD-12) | 2.5 | 1.2–5.1 | 50 |

[a]Dose–response assays were performed for each compound in the presence of 150 nM OdDHL. [b]Compounds labeled "Agonist" showed LasR-modulatory activity only at levels ≥100% (LasR activation level of OdDHL at 150 nM). [c]Denotes the largest amount of LasR inhibition seen for each compound at any concentration tested. For the full inhibition trace, see Figure S1. [d]Dose–response exhibited nonmonotonic behavior. Concentrations at which LasR activity began to increase were excluded for calculation of $IC_{50}$ values. [e]Compound exhibited limited solubility either in DMSO when preparing stock solutions or in media when performing the dose–response assay. Data obtained at these compound concentrations were excluded from the efficacy and potency analyses. See Note S5 for rationale of data exclusion and Figure S5 for absorbance data at 600 nm. [f]Compound exhibited a dose–response curve with a Hill slope ≠ 1. [g]Compound exhibited cytotoxicity at concentrations ≤1 mM. Data obtained at these compound concentrations were excluded from the efficacy and potency analyses.

activation were then submitted to agonism dose–response analysis (Table 2; for dose–response curves, see Figure S2).

Table 2. $EC_{50}$ Values for LasR Activation by Library Members in *P. aeruginosa* PAO-JP2 (p*lasI*-LVA*gfp*)[a]

| compound | $EC_{50}$ ($\mu$M) | 95% CI ($\mu$M) | max. activation (%)[b] |
|---|---|---|---|
| **1** (OdDHL) | 0.139 | 0.116–0.167 | 100 |
| **2** | >200 | – | 75 |
| **8** | >200 | – | 45 |
| **9** | 140 | 90–210 | 65 |
| **11** | 2.6 | 1.9–3.7 | 80 |
| **14** | 17 | 11–26 | 45 |
| **15** | >200 | – | 15 |
| **16** | 4.2 | 2.5–7.3 | 90 |
| **19** | 0.071 | 0.044–0.11 | 100 |

[a]Determined by testing AHLs over a range of concentrations for ability to mediate LasR expression of *lasI*-LVA*gfp*. [b]Denotes the highest value of LasR activation seen for each compound at any concentration within the dose–response assay. For the full agonism trace, see Figure S2.

All of the Group A compounds elicited LasR activity in these *P. aeruginosa* dose–response studies. The most potent LasR antagonists in the group were the naturally occurring AHL **2** (OOHL) and the trifluoromethyl-substituted phenyl propionoyl HL (PPHL) **9**. Though both exhibited $IC_{50}$ values in the single-digit micromolar range, their maximum LasR inhibition was modest (<60% relative to OdDHL). Interestingly, the isothiocyanate compound **11** (ITC-12) showed a different activity profile than that reported previously.[24] Meijler and coworkers designated **11** a partial LasR agonist with a maximum efficacy of ~40%; additionally, they reported that **11** decreased production of the virulence factors elastase B and pyocyanin by approximately 50% in the wild-type *P. aeruginosa* strain PAO1. Our assays also revealed **11** to be a partial LasR agonist, but the compound's maximum efficacy was 2-fold higher (80%). Corroborating this strong LasR agonistic activity in the reporter assay, our later QS phenotypic assays (see below) showed that **11** can strongly increase elastase B production in both wild-type PAO1 and synthase-null PAO-JP2 strains of *P. aeruginosa*. The disparate activity profiles for **11** between our two laboratories is unclear, but likely may be due to the use of different reporter plasmids and/or initial cell densities in the *P. aeruginosa* reporter assays, and different media conditions in the phenotypic assays.[24]

Intriguingly, the AHLs that displayed the most potent antagonism of LasR (**2**, **8**, **9**, and **10**) in our assays also displayed a characteristic *inversion of activity to agonism* (i.e., nonmonotonic, or "paradoxical," dose–response behavior) at higher concentrations. We term these compounds with concentration-dependent bimodal activity "non-classical partial agonists", as their dose–response behavior differs significantly from "classical" partial agonists (e.g., **11** above), which display monotonic dose–response curves instead (see Figure 3A for an illustration of each dose–response type). This nonmonotonic behavior has been seen previously for AHL-derived antagonists evaluated in *E. coli* reporter strains that heterologously produce LasR,[21,23,48] but we have only recently observed such nonmonotonic dose–response behavior in *P. aeruginosa*.[35] As we observe this nonmonotonic AHL dose–response for LasR in both species, our data suggest that the behavior is not simply an artifact of using a heterologous reporter system. We return to the origins of this bimodal activity below (see Mechanistic Insight 1). Among this set of compounds, it is worth noting that **10** (CL) has also been reported to inhibit the related LuxR-type receptor, CviR, via displacement of its native AHL and stabilization of receptor in an inactive homodimer.[49] Examining if **10** has the similar ability to simultaneously stabilize and deactivate LasR (at least at lower concentrations) would certainly be of interest.

The Group B compounds generally suffered from lower solubilities in LB medium relative to the other Groups, precluding testing at high concentrations (Figure S5; Note S5). Nevertheless, within the soluble regime of these compounds, our aniline derivative **12**[37] was found to be an effective inhibitor of LasR in the PAO-JP2 reporter strain ($IC_{50}$ = 9.7 $\mu$M; maximum inhibition = 70%). The phenol derivative **14** reported by Suga and co-workers[20] displayed no ability to antagonize LasR in PAO-JP2 in the presence of 150 nM OdDHL (the $EC_{50}$ of the native ligand), corroborating previous assays by our laboratory.[21] Surprisingly, when we submitted the same compound to agonism dose–response analysis, we discovered that **14** was in fact a classical partial agonist of LasR, with a maximum efficacy of 50% (Table 2). This


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observation then explained our antagonism data: When high concentrations of **14** outcompete OdDHL present at a concentration also enabling 50% LasR activation, the antagonism dose−response curve shows no net change in LasR activity. These results illustrate how antagonism screens vs a native ligand present at its $EC_{50}$—analyzed in the absence of accompanying agonism assay data—can obscure the full activity profile of a particular compound. Testing for such partial agonism is certainly prudent, as LuxR-type receptor partial agonists have attracted some attention for their ability to tune receptor responses in ways inaccessible by traditional agonists or antagonists alone.[11,50]

The compounds in Groups C and D elicited a wide range of responses from the LasR receptor in PAO-JP2. The acylated thiolactone **16** of Bassler and co-workers,[11] previously reported to partially antagonize (and agonize) LasR in an *E. coli* reporter, displayed no antagonism of LasR under our conditions, and at concentrations ≥5 μM, it began to activate LasR to a greater extent than 150 nM OdDHL alone. The agonism dose−response analysis for **16** confirmed that this AHL analogue is a LasR classical partial agonist in our PAO-JP2 assay, with a maximal LasR activation of 90%. Compound **17** (C10-CPA) modestly inhibited LasR activity (∼50% at 200 μM), though solubility in the assay medium was too low to test at higher concentrations (Figure S5). Compound **18** (V-06-018; uncovered by Greenberg and co-workers in a high throughput screen),[27] when dosed at single-digit micromolar concentrations, displayed the highest LasR inhibition efficacy (>80%) of any library compound dosed at similar concentrations.

Triphenyl compound **19** (TP-1P), also reported by the Greenberg lab,[27] was the only agonist (apart from the native ligand **1**) that maximally activated LasR. It was also the most potent non-native activator of LasR in these *P. aeruginosa* assays, displaying an $EC_{50}$ of 71 nM (∼2-fold lower than OdDHL). Notably, compound **19** is the only non-AHL derivative that has been shown via structural analyses to bind in the LasR ligand-binding site, making analogous contacts as OdDHL.[51] Interestingly, the structurally related TP analogue, **20** (TP-5), is a moderate LasR *inhibitor*. Moreover, it displays a LasR inhibition dose−response that was unique among all compounds tested herein: Complete inhibition of LasR occurred over a remarkably narrow concentration range, and after performing the dose−response assay at higher resolution, we found that the best-fit sigmoidal inhibition curve had a Hill slope of −3. We currently have two hypotheses for the mechanism by which **20** inhibits LasR. The Prinz laboratory has previously postulated that receptor denaturation through allosteric interactions of an antagonist with an unstable protein results in a steep dose−response curve.[52] Given that **20** has been shown to cause LasR instability and aggregation (precluding structural analysis),[51] denaturation through allosteric interactions may explain this behavior. Alternatively, the Shoichet laboratory has attributed such phenomena to the colloidal aggregation or precipitation of small-molecule modulators, followed by deactivation or sequestration of the target protein.[53] Because **20** inhibited LasR at concentrations (50−100 μM) approaching those that showed qualitative precipitation (>125 μM), this phase change mechanism may also contribute to the steep inhibition profile.[54,55]

Turning to the frequently cited natural product-derived QS modulator—halogenated furanone **21**[56]—we found this derivative was toxic to *P. aeruginosa* at concentrations ≥100 μM (Figure S5). At all lower concentrations, **21** elicited no inhibition of LasR activity in PAO-JP2. Though this result conflicts with a recent report by Liz-Marzán and co-workers,[57] we note that **21** showed very little LasR inhibition in their bioassay (<20%) at concentrations as high as 10 μM. Additionally, the concentration of **21** at which the authors saw significant LasR inhibition (100 μM) caused significant growth effects in our assay conditions (Figure S5).

The tetrazole **22** was the most potent inhibitor of LasR activity in our *P. aeruginosa* PAO-JP2 assays, with an $IC_{50}$ of 2.5 μM. This potency value is significantly different from the $IC_{50}$ of 30 nM reported by the Greenberg laboratory;[27] however, similar to this previous report, we found that the greatest magnitude of LasR inhibition at any concentration was about 50%.[58] The incongruity in potency for **22** between our study and Greenberg's work may be due to the use of a different LasR-regulated promoter or due to different growth and media conditions. Such discrepancies (also noted for compounds **11** and **21** above) underscore the necessity of using standardized reporters and assay conditions when comparing the dose−response profiles of different compound classes.

Together, the above-standardized LasR reporter assays in the native *P. aeruginosa* background allow for the first direct comparison of compound activity for the 22 chosen molecules. When taking into account both potency and maximum efficacy of LasR modulation, the two compounds that stand out as the most effective LasR modulators under these conditions are **18** (V-06-018) as an antagonist ($IC_{50}$ = 5.2 μM; maximum inhibition = 85%) and **19** (TP-1) as an agonist ($EC_{50}$ = 71 nM; maximum activation = 100%).

**A Complementary Heterologous *E. coli* LasR Reporter Study Tests Compounds for Direct LasR Modulation.** We next sought to determine if each compound in the LasR modulator library was acting directly on LasR; we thus submitted the library to antagonism and agonism dose−response analysis in an *E. coli* strain (JLD271) harboring LasR that reports on LasR activity via production of β-galactosidase (see Experimental Section).[59] In general, these compounds were more potent LasR modulators in this *E. coli* strain relative to the *P. aeruginosa* PAO-JP2 reporter (Tables 3 and 4). However, the overall shapes of the LasR antagonism dose−response curves for the Group A compounds were conserved between the two strains (see Figures S1 and S3 for full *P. aeruginosa* and *E. coli* curves, respectively). This result supports the common assertion that AHL-type ligands (i.e., ligands like those in Group A) modulate LasR activity directly. Additionally, the maximum percent LasR inhibition trends among highly soluble AHLs in this Group match well between the *P. aeruginosa* and *E. coli* reporters (i.e., **3**, **6**, **7** > **2**, **5**, **8**, **10** > **9**, **11**). Such closely matching trends in activity and dose−response behavior strongly support that the discrepancies in AHL potency between reporter strains are primarily due to mechanisms that affect intracellular availability of the compounds (e.g., active efflux),[35] as opposed to differences in the mechanisms of the LasR receptor−ligand interaction between *E. coli* and *P. aeruginosa* reporters (see Mechanistic Insight 2 below).

The non-AHL-derived compounds in Groups B, C, and D displayed far more varied and unexpected dose−response behaviors in the *E. coli* LasR reporter. OdDHL mimics **12** and **13**, which were LasR *antagonists* in the PAO-JP2 reporter, were found instead to partially agonize LasR in the *E. coli* background (Table 4). Moreover, the maximum LasR responses for partial agonists **14** and **15** were markedly


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Table 3. $IC_{50}$ Values for LasR Inhibition by Library Members in *E. coli* JLD271 (pJN105L, pSC11)[a]

| compound | $IC_{50}$ ($\mu$M)[b] | 95% CI ($\mu$M) | max. inhibition (%)[c] |
|---|---|---|---|
| **2** (OOHL)[d] | 0.078 | 0.032−0.19 | 35 |
| **3** (OHHL)[d] | 10.4 | 5.3−21 | 70 |
| **4**[e] | 2.8 | 1.1−6.8 | 65 |
| **5** | 2.8 | 1.3−6 | 65 |
| **6**[d] | 1.0 | 0.34−3.2 | 70 |
| **7**[d] | 3.5 | 2.6−4.8 | 75 |
| **8**[d] | 0.16 | 0.043−0.57 | 45 |
| **9** | agonist | − | N/A |
| **10** (CL)[d] | 0.49 | 0.1−2.3 | 40 |
| **11** (ITC-12) | agonist | − | N/A |
| **12** | − | − | N/A |
| **13** | 4.7 | 1.9−12 | 40 |
| **14** | agonist | − | N/A |
| **15** | agonist | − | N/A |
| **16** (mBTL) | agonist | − | N/A |
| **17** (C10-CPA) | − | − | N/A |
| **18** (V-06-018)[e] | 2.3 | 0.89−6.1 | 50 |
| **19** (TP-1) | agonist | − | N/A |
| **20** (TP-5)[e,f] | 70 | 56−88 | 85 |
| **21** (C-30)[g] | − | − | N/A |
| **22** (PD-12) | − | − | N/A |

[a]Antagonism dose−response assays were performed for each compound in the presence of 2 nM OdDHL. [b]Compounds labeled as "agonist" showed LasR-modulatory activity only at levels ≥100% (the LasR activation level of OdDHL at 2 nM). [c]Denotes the largest amount of LasR inhibition seen for each compound at any concentration within the dose−response assay. For the full inhibition trace, see Figure S3. [d,e,f,g]See Table 1 footnotes.

Table 4. $EC_{50}$ Values for LasR Activation by Library Members in *E. coli* JLD271 (pJN105L, pSC11)[a]

| compound | $EC_{50}$ ($\mu$M) | 95% CI ($\mu$M) | max. activation (%)[b] |
|---|---|---|---|
| **1** | 0.0018 | 0.0016−0.0021 | 100 |
| **2** | 4.5 | 3−6.7 | 95 |
| **3** | >100 | − | 30 |
| **8** | 8.4 | 4.5−16 | 90 |
| **9** | 0.65 | 0.29−1.4 | 105 |
| **10** | 33 | 23−48 | 60 |
| **11** | 0.017 | 0.014−0.02 | 95 |
| **12** | 0.92 | 0.53−1.6 | 40 |
| **13** | >100 | − | 15 |
| **14** | 0.096 | 0.06−0.15 | 85 |
| **15** | 0.24 | 0.16−0.35 | 90 |
| **16** | 0.013 | 0.0067−0.025 | 90 |
| **17** | − | − | 0 |
| **18** | − | − | 5 |
| **19** | 0.0078 | 0.0047−0.013 | 100 |

[a]Determined by testing AHLs over a range of concentrations for ability to mediate LasR expression of *lasI-lacZ*. [b]Denotes the highest value of LasR activation seen for each compound at any concentration within the dose−response assay. For the full agonism trace, see Figure S4.

increased in the *E. coli* reporter. Such significant alterations of LasR-modulatory ability between native strain reporters and heterologous reporters have been previously observed.[60,61] We further explore this phenomenon in Mechanistic Insight 3 below.

Compounds **17** (C10-CPA), **21** (C-30), and **22** (PD-12) were found to be completely inactive in the *E. coli* LasR reporter (Table 3). Compound **21** caused significant growth effects at concentrations ≥20 $\mu$M, and at lower concentrations, no LasR inhibition was observed, similar to the above experiments performed in *P. aeruginosa*. In turn, while compounds **17** and **22** had elicited weak to strong LasR inhibition in the *P. aeruginosa* reporter, these activities were abolished when LasR was isolated in the heterologous *E. coli* reporter, suggesting these two compounds modulate LasR in *P. aeruginosa* via an indirect mechanism.

In general, the LasR agonism activity trends for the library were largely conserved between the *E. coli* and *P. aeruginosa* reporters (Table 4), although compounds were anywhere from 10- to over 100-fold more potent in the *E. coli* background. Again, we believe this is due to increased intracellular availability in *E. coli* relative to *P. aeruginosa*. Compound **19** remained the most potent LasR agonist in the library, displaying the only single-digit nanomolar $EC_{50}$ value (~8 nM).

Figure 3 summarizes all of the activity trends that we observed for the LasR modulator library using both the *E. coli* and *P. aeruginosa* LasR reporters. Combining data from the two sets of reporters, we were able to systematically classify the compounds as LasR agonists, antagonists, partial agonists, and nonclassical partial agonists. We confirmed that **18** (V-06-018) displays the best combination of efficacy and potency as a LasR antagonist, while the most potent LasR agonist was the triphenyl compound **19**. We were also able to exclude certain compounds from further analysis as LasR ligands as they act via indirect mechanisms. With these results in hand, we next sought to further our understanding of some of the unexpected activity profiles that we encountered in the course of our compound screening.

**Mechanistic Insight 1: "Non-Classical" Partial Agonists Display Nonmonotonic Dose Curves Due to Two Discrete Binding Events—One Competitive and One Noncompetitive.** As highlighted above, we identified seven compounds (**2**, **3**, **6**, and **7−10**) that displayed nonmonotonic dose response curves for LasR antagonism in either the *P. aeruginosa* or *E. coli* reporter assays. Our laboratory has previously noted the occurrence of such paradoxical dose−response curves for non-native AHL modulators of various LuxR-type receptors,[21,23,35,48,62] and we recently hypothesized that the bimodal activity observed during competitive antagonism assays may be due to formation of inactive mixed-ligand heterodimers of the receptor. Thus, at intermediate concentrations of non-native AHL, the formation of inactive heterodimers of receptors bound to native and non-native ligand is read out as antagonism, while at high concentrations of non-native ligand, the non-native ligand fully outcompetes the native ligand, resulting in the formation of active homodimers of the receptor that is read out as (typically weak) agonism.[37] This mechanism has been proposed for other receptor types that can function as dimers when bound to their cognate small molecule ligand, such as nuclear hormone receptors.[63,64] We sought to support or refute this hypothesis through additional experiments on LasR. Accordingly, we performed a converse dose−response study, where we dosed in varying concentrations of **1** (OdDHL) to outcompete a non-native ligand in the reporter strain. Presumably, for the mixed-ligand heterodimer hypothesis to hold, OdDHL would reach a concentration that would favor


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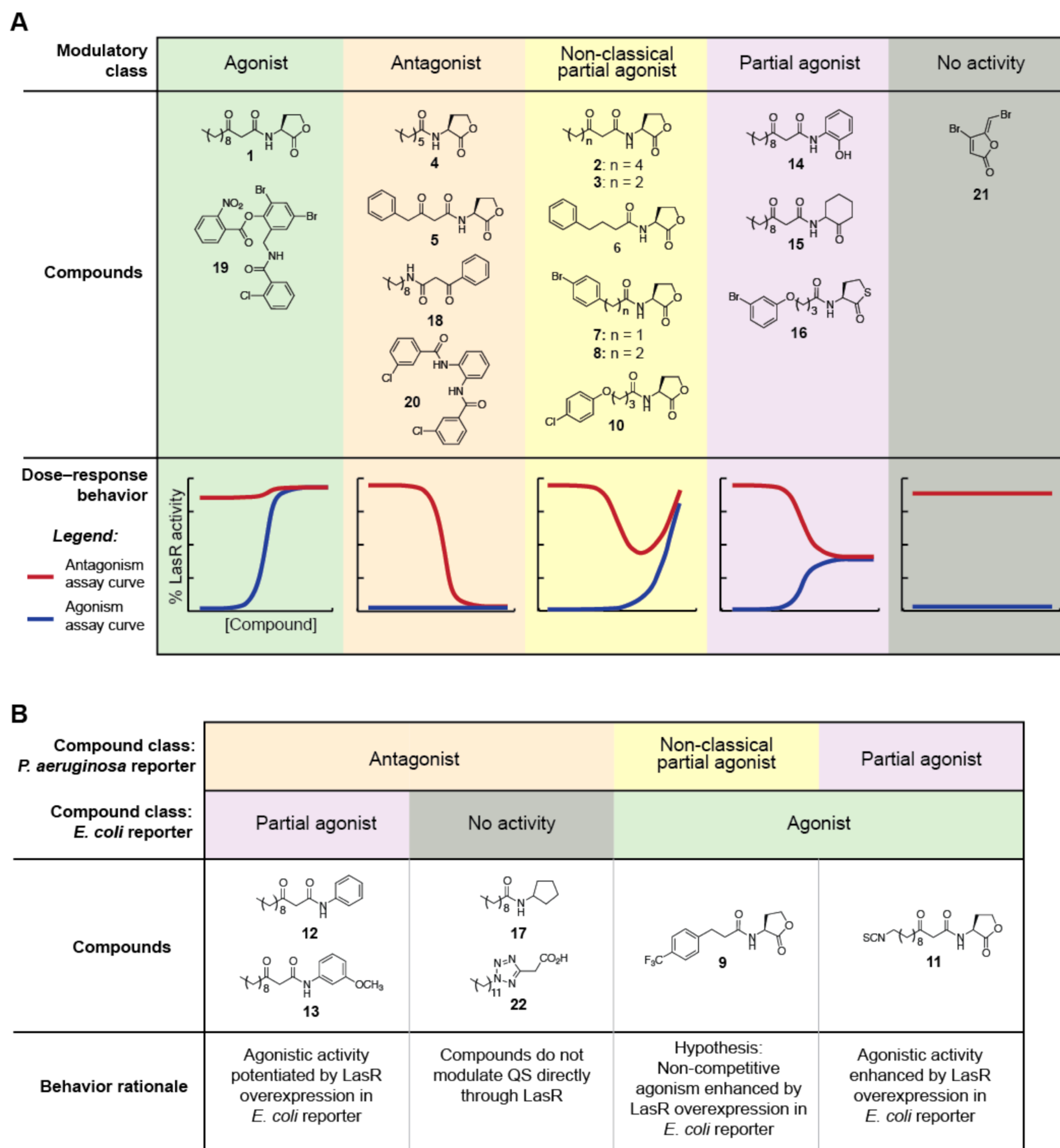


**Figure 3.** Activity trends of LasR modulators, classified by dose−response assay behavior. (A) Compounds with conserved activity across LasR reporters in *P. aeruginosa* and *E. coli*. (B) Compounds showing altered activity profiles between *P. aeruginosa* and *E. coli* reporter strains.

mixed-ligand heterodimer formation and thus elicit a similar nonmonotonic dose−response curve.

We chose to perform this experiment with brominated PPHL **8** due to its potency and strong bimodal activity in both the *E. coli* and *P. aeruginosa* LasR reporters (Figure 4); we used the *E. coli* LasR reporter since both **1** and **8** are more potent in this species. In contrast to the original antagonism dose−response (Figure 4, blue plot), which shows a nonmonotonic curve, the converse dose−response experiment (Figure 4, red plot) showed no bimodal activity that would be expected to accompany the formation of mixed-ligand LasR dimers at intermediate concentrations of OdDHL. Instead, the converse dose−response was entirely monotonic. This result effectively refutes the hypothesis that the bimodal activity is due to formation of inactive mixed-ligand heterodimers at concentration ranges that allow both ligands to bind to the LasR active site.

In view of these results, we needed to alter our hypothesis and next considered whether the bimodal activity of some AHLs may be due to two discrete binding events at two distinct small-molecule binding sites (on LasR or another target). To begin to investigate this possibility, we performed a two-


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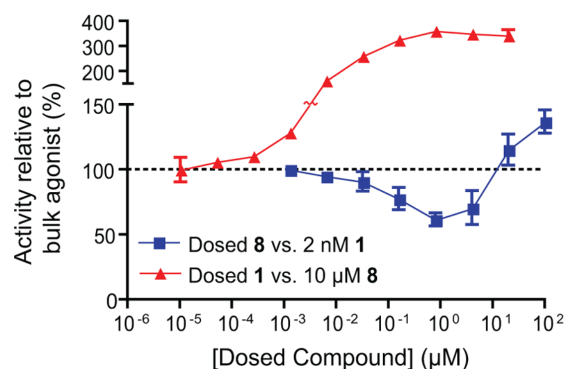


**Figure 4.** Converse dose−response experiments with LasR native ligand **1** (OdDHL) and nonclassical partial agonist **8** in *E. coli* LasR reporter JLD271 (pJN105L, pSC11). Blue squares (original dose−response with bimodal activity): Varying concentrations of **8** in the presence of **1** at its $EC_{50}$ (2 nM). Red triangles (converse dose−response with monotonic activity): Varying concentrations of **1** in the presence of a bulk addition of 10 $\mu$M **8**. Error bars: SEM of $n$ = 3 trials.

dimensional dose−response analysis of the nonclassical partial agonist **8** with native ligand **1** in the *E. coli* reporter (Figure 5).

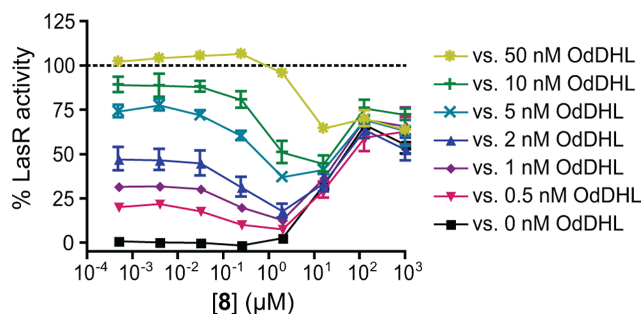


**Figure 5.** Nonclassical partial agonist behavior of compound **8** in a two-dimensional dose−response study with LasR native ligand **1** (OdDHL). Assay was performed using the *E. coli* LasR reporter JLD271 (pJN105L, pSC11). The antagonistic behavior (at concentrations <10 $\mu$M) is competitive with **1** and shifts to higher potency when competed against higher concentrations of **1**. The partial agonist behavior of **8** (at concentrations >10 $\mu$M), on the other hand, is insurmountable with increasing concentrations of **1**. Quantitative $IC_{50}$ values from the antagonistic regime of each curve are shown in Figure S6. Error bars: SEM of $n$ = 3 trials.

Interestingly, we observed that the inhibitory regime of the dose−response curve of **8** (at concentrations <10 $\mu$M) shifts to higher concentrations against increasing doses of OdDHL (**1**),[65] whereas the $EC_{50}$ of the partial agonism regime (at concentrations >10 $\mu$M) exhibited no such shift. Thus, we can conclude that the partial agonism binding event occurring at high concentrations of **8** is noncompetitive with native ligand **1**. We confirmed that this behavior is replicated in the *P. aeruginosa* reporter (Figure S7) and, consequently, is not an artifact of the heterologous *E. coli* background. We additionally performed this two-dimensional dose−response assay with native ligand **1** and a different compound, **2** (OHHL), a naturally occurring AHL with a nonaromatic acyl tail that exhibited the same nonmonotonic activity profile. Despite the structural differences between compounds **2** and **8**, the two compounds displayed the same noncompetitive agonism at high concentrations (see Figure S8). Again, we note that this noncompetitive binding event may be allosteric on LasR or may involve a different distinct protein and/or other target(s); the cell-based reporter gene assay utilized here cannot distinguish between these possibilities. Additional experiments—for example, in vitro studies with purified LasR (or a related, more soluble homologue) and a target DNA sequence—are clearly needed to refine this hypothesis and are ongoing in our laboratory. Nevertheless, we believe this alternative ligand binding interaction may represent an interesting new target for the modulation of LasR (and most likely other LuxR-type receptor) activity, and is worthy of future study.

**Mechanistic Insight 2: A *P. aeruginosa* ΔmexAB-oprM LasR Reporter Shows AHLs Are More Susceptible to Active Efflux than Non-AHLs.** Our laboratory recently reported that the presence of the RND efflux pump MexAB-OprM in *P. aeruginosa* reduces the potency of QS modulators;[35] we concluded that these compounds (primarily AHL-type) were being pumped out of the cell, thereby reducing their intracellular concentration. We also showed via a nonspecific pump inhibitor that, despite the presence of multiple homologous pumps in *P. aeruginosa*, MexAB-OprM was the primary cause of compound potency reduction.[35] Now with access to a wider range of compound scaffolds (relative to our past study)[35] in our LasR modulator library, we sought to identify compounds that resisted efflux-induced losses in potency. Such an activity profile, even if resistance to efflux were only moderate, would mark a compound as a choice scaffold for further development. More broadly, we reasoned that screening the library would reveal structural features that either enhance or reduce compound efflux. To evaluate these properties, we performed LasR agonism and antagonism dose−response activity assays on the library using a *P. aeruginosa* mutant strain that lacked a functional MexAB-OprM pump (PAO-JG21) and harbored the LasR reporter plasmid *plasI*-LVA*gfp*. (Though this wider range of compounds may act as substrates of other homologous pumps in *P. aeruginosa*, the MexAB-OprM pump is the most likely cause of efflux.) We observed that for the majority of the compounds, trends in activity (dose−response curve shape, slope of sigmoidal curve, etc.) were conserved (see Figures S1 and S2), and only the potencies of compounds were shifted. Thus, the fold-change in compound $IC_{50}$ (or $EC_{50}$) from the pump-active reporter to the pump mutant reporter served as the metric by which susceptibility to active efflux was evaluated (Table 5).

This study of efflux susceptibility revealed four clear trends that are directly dependent on compound structural class. First, AHLs with aromatic or long, aliphatic tails (≥8) were more susceptible to active efflux than those with shorter acyl tails (≤6), corroborating previous reports;[3,4,35] for example, compounds **1** (OdDHL) and **2** (OOHL) show 10-fold shifts in potency between pump-active and pump mutant *P. aeruginosa* reporters, while **3** (OHHL, with a six-carbon acyl tail) shows no discernible shift. Second, perhaps unsurprisingly, a covalent (i.e., "irreversible") binding mechanism for LasR modulation reduces susceptibility to active efflux—the isothiocyanate **11**, despite its close structural similarity to OdDHL, exhibits only a 2-fold shift in potency between pump-active and pump mutant agonism dose−response studies, presumably because (at least a percentage of) it is covalently linked to LasR.[24] Third, the presence of a homoserine lactone headgroup greatly increases recognition by MexAB-OprM; compounds with alternative head groups (i.e., **12**, **14**, and **18**) showed significant reduction in susceptibility to active efflux. Fourth, the triphenyl scaffold appears to not be strongly recognized by MexAB-OprM. For


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**Table 5. Comparison of LasR Antagonist or Agonist Potency between Pump-Active (PAO-JP2) and Pump-Mutant (PAO-JG21) *P. aeruginosa* LasR Reporter Strains**[a]

| antagonism | | | |
|---|---|---|---|
| compound[b] | PAO-JP2 $IC_{50}$ ($\mu$M) | PAO-JG21 $IC_{50}$ ($\mu$M) | fold change[c] |
| **2** (OOHL) | 5.5 | 0.57 | 9.6 |
| **3** (OHHL) | 40 | 41 | **1.0** |
| **5** | 73 | 8.9 | 8.2 |
| **6** | 175 | 20 | 8.8 |
| **7** | 116 | 8.2 | 14.1 |
| **8** | 12 | 1.5 | 8.0 |
| **9** | 3 | 0.42 | 7.1 |
| **10** (CL) | 21 | 1.3 | 16.2 |
| **12** | 9.7 | 3.7 | 2.6 |
| **18** (V-06-018) | 5.2 | 6.1 | **0.9** |
| **20** (TP-5) | 69 | 63 | **1.1** |
| **22** (PD-12) | 2.5 | 0.11 | 22.7 |
| agonism | | | |
| compound | PAO-JP2 $IC_{50}$ ($\mu$M) | PAO-JG21 $IC_{50}$ ($\mu$M) | fold change[b] |
| **1** (OdDHL) | 0.14 | 0.019 | 7.4 |
| **2** | >200 | 26 | >7.7 |
| **8** | >200 | 24 | >8.3 |
| **9** | 140 | 8.6 | 16.3 |
| **11** (ITC-12) | 2.6 | 1.3 | 2.0 |
| **14** | 17 | 15 | **1.1** |
| **16** (mBTL) | 4.2 | 0.56 | 7.5 |
| **19** (TP-1) | 0.071 | 0.036 | 2.0 |

[a]Both strains utilize the plasmid p*lasI*-LVA*gfp* to report compound ability to mediate LasR expression of *lasI*-LVA*gfp*. [b]Data for compounds with incalculable fold-changes in potency (due to incomplete dose−response curves) are listed in Table S4. [c]Compounds with statistically insignificant shifts in $EC_{50}$ ($p$ > 0.1) are shown in bold. For statistical analysis, see Table S4.

instance, the LasR agonist **19** exhibited only a 2-fold increase in potency in the absence of MexAB-OprM. Similarly, triphenyl-derived antagonist **20** only exhibited a 1.1-fold shift in potency, within statistical error of the assay. These four structure−activity trends should be strongly considered in the design of next-generation LasR (and likely other LuxR-type receptor) modulators. Namely, short-tail AHLs, AHL analogues with non-native head groups, and triphenyl ligands appear to be a worthwhile chemical space to further explore for potent, efflux-resistant LuxR-type QS modulators. The very recent report of novel, irreversible inhibitors of LasR based on compound **19** by Perez and co-workers provides additional support for the continued study of triphenyl scaffolds.[66]

We further expanded upon our prior study of AHL efflux in *P. aeruginosa* by next comparing compound potencies (see Table S5) in all three LasR reporter strains: pump-active *P. aeruginosa*, pump mutant *P. aeruginosa*, and *E. coli*. We observed that the trend of potency shifts between pump-active and pump mutant *P. aeruginosa* strains did not fully match the trend between the *E. coli* and pump-active *P. aeruginosa* strains; nonetheless, the compounds were almost all more potent in *E. coli* vs the *P. aeruginosa* pump mutant (the only exception being antagonist **20**, which was effectively equipotent in all three strains). These data suggest that, as we anticipated for the broader structural array of compounds studied herein, other factors beyond active efflux are likely contributing to the amplified potency shifts between the *E. coli* and *P. aeruginosa* LasR reporters, such as differential membrane permeability or susceptibility to enzymatic degradation.

**Mechanistic Insight 3: Because of LasR Overexpression, Compound Activity Profiles Can Vary between *E. coli* and *P. aeruginosa* Reporters.** As shown in Figure 3B, a subset of library compounds displayed LasR modulation profiles that significantly changed depending on whether the reporter was in a *P. aeruginosa* or an *E. coli* background. We reasoned that the two compounds displaying a complete loss of efficacy in *E. coli* (**17** and **22**) are likely modulating LasR in *P. aeruginosa* through some upstream interaction (see above). Harder to explain, however, were the compounds that still modulated LasR but had markedly altered activity profiles (e.g., compounds **9** and **11**−**13**; Figure 3B, columns 1, 3, and 4). In 1998, Winans and co-workers hypothesized that heterologous expression of LuxR-type receptors could cause substantial changes in efficacy due to the receptor being overexpressed in such systems relative to the native background; this proposition stemmed from their studies with the LasR-homologue TraR that showed compounds shift from antagonist to agonist upon TraR overexpression in *Agrobacterium tumefaciens*.[67] We sought to test this hypothesis by transforming the same LasR expression plasmid used in our *E. coli* reporter strain (pJN105L) into *P. aeruginosa* PAO-JP2 and performing analogous dose−response analyses while overexpressing LasR via addition of L-arabinose. Control experiments for the test compounds (i.e., **9** and **11**−**13**) in PAO-JP2 (i) in the absence of the LasR expression plasmid and presence of L-arabinose and (ii) in the presence of the LasR expression plasmid and the absence of L-arabinose indicated that neither the plasmid nor the inducer (L-arabinose) alone were influencing LasR activity (Figure S9).

We postulated that if the Winans laboratory hypothesis were correct for the test compounds, their dose−response behaviors in the PAO-JP2 reporter with LasR overexpressed via pJN105L would mimic their behaviors in the *E. coli* LasR reporter. For compounds **11** and **12**, we did indeed see the anticipated activity profile shifts (Figure 6): Compound **11** (a partial agonist in the PAO-JP2 LasR reporter strain) converted to a full agonist, and compound **12** (an antagonist in the PAO-JP2 LasR reporter strain) converted to a partial agonist. The potencies of both compounds in the *P. aeruginosa* LasR overexpression reporter were still less than those in the *E. coli* LasR reporter, likely due to the differences in active efflux and membrane permeability between *E. coli* and *P. aeruginosa* (as described above). We believe that this loss in potency in *P. aeruginosa* is also the reason behind compound **13** showing no partial agonism in the PAO-JP2 LasR overexpression reporter (Figure S10B). The data for compound **9**, however, refuted our hypothesis (Figure S10A); **9** retained its nonmonotonic dose−response when moving from the PAO-JP2 LasR native-expression reporter to the overexpression reporter (in contrast to its observed monotonic dose−response in the *E. coli* reporter; Figure S10A). We consequently speculate that LasR overexpression may not be the only factor causing the altered activity profile of **9** in *E. coli*. Namely, because the non-monotonic dose−response curves are likely produced from *two* (or more) discrete binding events (see above), we believe that, in the *E. coli* LasR reporter, the potency of the *agonistic* binding event for compound **9** may shift far more strongly than that of the *antagonistic* binding event, causing the agonistic event to subsume the antagonistic one.[68]



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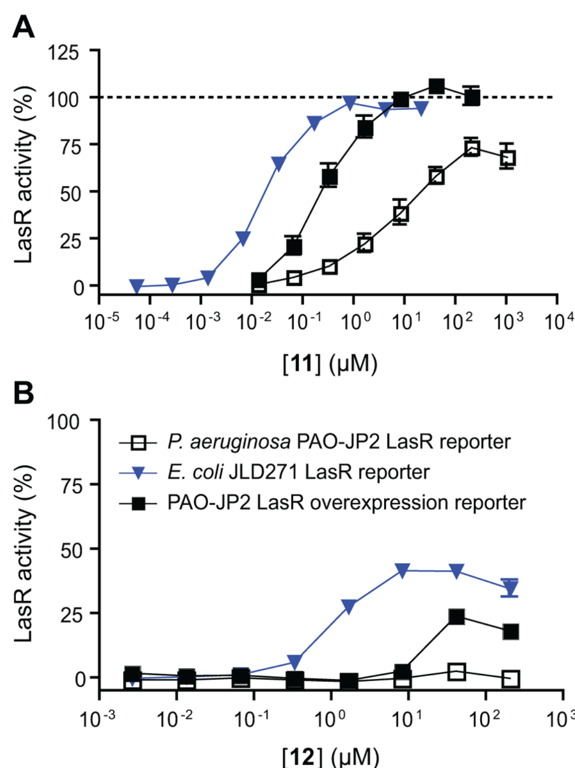


**Figure 6.** LasR overexpression alters dose−response behavior for some compounds. Dose−response assays using compounds **11** (A) and **12** (B) showed that the behavior of the *P. aeruginosa* reporter overexpressing LasR (filled squares) more closely matched that of the *E. coli* LasR reporter (blue triangles) than that of the *P. aeruginosa* native LasR expression reporter (empty squares). Error bars: SEM of $n = 3$ trials.

Although LasR activity profiles were significantly altered in *E. coli* reporters for only a few compounds tested herein, this incongruent behavior in heterologous strains (relative to native backgrounds) is common enough to have been noted by multiple other laboratories studying LuxR-type receptors.[60,61,69] Our results corroborate the claim of the Winans laboratory that heterologous reporters are prone to such anomalies, and ongoing work in our laboratory is focused on developing an *E. coli* LasR reporter and a set of assay conditions that better mimic LasR activity trends in *P. aeruginosa*.

**Elastase Assays Confirm Compound Efficacy on LasR in Wild-Type *P. aeruginosa*.** The comparative activity data for the LasR modulator library above, augmented with new mechanistic insights, allowed us to rigorously choose compounds that we believed would be effective in an assay directly measuring QS-dependent phenotype activity in *P. aeruginosa*. We elected to test the effects of these compounds on the production of the well-studied virulence factor elastase B (LasB). LasB is a metalloprotease that degrades immune components and causes tissue damage within infected hosts.[70] Critically, elastase B production is strongly regulated by the *las* QS circuit.[71] Recent studies have shown that while all phenotypic regulation by LasR is dependent on environmental factors and growth conditions,[72] the influence of the *las* system on elastase B production is much clearer and more direct than that on other prominent virulence phenotypes, for example, biofilm[73,74] or pyocyanin[12] production. We therefore reasoned it would be the most direct test of the compounds' ability to modulate LasR in wild-type *P. aeruginosa*.

To quantify elastase B production, we performed a colorimetric assay in the wild-type *P. aeruginosa* strain PAO1 using an elastin−Congo red substrate (see Experimental Section). We submitted a focused subset of compounds with definitive activity profiles and/or interesting structural features to this assay (Figure 7)—activators **11**, **16** (partial agonists with

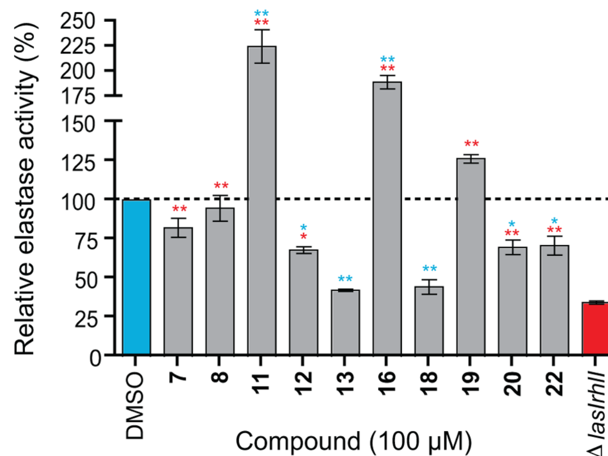


**Figure 7.** Elastase B activity in wild-type *P. aeruginosa* (PAO1) in the absence (DMSO; negative control; blue bar) or presence (gray bars) of 100 $\mu$M LasR modulator, and in Δ*lasIrhlI* mutant PAO-JP2 (full QS-dependent inhibition; positive control; red bar). Error bars: SEM of $n = 3$ trials. Red stars: significance from Δ*lasIrhlI* control; Blue stars: significance from DMSO control. * = $p < 0.05$, ** = $p < 0.01$; For full tabular and statistical data, see Table S6.

high LasR activation) and **19** (full agonist), along with inhibitors **7**, **8** (retention of AHL headgroup), **12**, **13** (retention of OdDHL tail), and **18**, **20**, and **22** (non-AHL scaffolds). As a key control, we used the *P. aeruginosa* Δ*lasIrhlI* mutant PAO-JP2 to mimic a fully QS-inhibited wild-type strain.

Activity trends were well conserved between the elastase B assays in wild-type PAO1 and the LasR reporter assays in PAO-JP2. Interestingly, the AHLs **7** and **8** showed only modest (<25%) elastase inhibition. Both were shown to be particularly susceptible to efflux by MexAB-OprM, and previous reports have shown that the homoserine lactone head is prone to hydrolysis,[75,76] so we believe that in the presence of continually replenished native ligand (in the wild-type strain), the AHLs are unable to effectively inhibit LasR over the 16 h growth span necessary for the assay. Nonlactone OdDHL mimics **12** and **13** were able to inhibit elastase by ≥50%, though we were surprised to see that **13** inhibited elastase more effectively than **12**, despite its lower potency in the PAO-JP2 LasR reporter. Notably, compound **18** (V-06-018), which displayed potent and efficacious LasR inhibition in all reporter assays, showed complete QS-dependent inhibition of elastase (no statistically significant difference from the Δ*lasIrhlI* mutant).

The results of these elastase assays show that our reporter bioassay experiments offer a largely predictive view of LasR-dependent phenotypic modulation under uniform growth conditions. Those compounds that showed resistance to active efflux and consistently potent LasR antagonism were highly effective at overcoming the common hurdles that make small-molecule modulation of *P. aeruginosa* QS phenotypes so challenging.


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## ■ CONCLUSIONS AND OUTLOOK

In summary, we report the assembly and comparative evaluation of a library of compounds that comprises some of the most potent and efficacious LasR modulators known. We submitted this focused library to standardized screening conditions allowing comparison of LasR modulatory ability across a variety of structural classes. Our biological assays allowed us to measure potency, efficacy, susceptibility to active efflux, and whether or not the modulators are directly targeting LasR. This systematic analysis of *P. aeruginosa* LasR modulators has revealed many salient points to consider when designing future compounds as research tools or for antivirulence applications.

First, we have shown that data obtained using the reporter constructs and assay conditions described herein are largely predictive for small-molecule modulation of QS-dependent virulence phenotypes—here, elastase B production—in wild-type *P. aeruginosa*. These reporters and assay protocols could be readily adopted as standard methods for assaying LasR ligands. We also demonstrate that the simultaneous analysis of LasR activity and active efflux susceptibility allows a very clear picture of compound efficacy in *P. aeruginosa* (at least when grown in the common bacterial growth medium LB).

Second, we have identified a possible alternative site/target for LasR modulation. We identified natural and non-natural AHLs that are ostensibly activating LasR through this noncompetitive site/target, and we believe that further research should be focused on characterizing and exploiting this phenomenon. Perhaps most notably in this regard, a non-competitive antagonist would bypass the challenges inherent in treating wild-type pathogens that are constitutively producing their native QS autoinducers.[36]

Third, our studies serve to highlight two compounds for their ability to strongly modulate LasR and influence QS-dependent phenotypes in wild-type *P. aeruginosa*: (i) triphenyl compound **19** (TP-1) as an agonist and (ii) compound **18** (V-06-018) as an antagonist. Compound **19** exhibits multiple desirable traits for a LasR modulator. We have shown that the triphenyl scaffold is less susceptible to active efflux, and **19** consistently ranks as the most potent LasR modulator in our reporter studies. Further, because **19** is known to bind the OdDHL binding site and makes similar molecular contacts to LasR as OdDHL,[51] we believe that analogues of **19** may have a propensity to mode switch between LasR activation and inhibition—similarly to non-native AHL analogues (indeed, this is already exemplified by the disparate activities of **19** and **20**).[21] A potent triphenyl inhibitor of LasR would circumvent the liabilities associated with the hydrolyzable homoserine lactone head and would likely maintain resistance to active efflux. Again, the recent work of Perez and co-workers on new derivatives of **19** is encouraging in this regard.[66] Finally, compound **18** (V-06-018) displayed consistently high efficacy and potency in all of the reporter and phenotypic assays in this study. Though it is similar in structure to the Group B compounds (which have received significant attention from groups that design LasR modulators),[7,46] it appears to be generally more potent. Consequently, efforts to further refine SAR around the features of **18** and enhance its solubility might result in a very powerful *P. aeruginosa* QS inhibitor.

To close, the past 20 years have seen enormous advances in understanding of the intricate social networks utilized by bacteria, and the chemical tools developed by research laboratories to target QS pathways are certainly contributing to this effort.[11,12,49,77] While these compounds can be uniquely valuable in the process of delineating QS circuits, many researchers have called attention to two particular shortcomings in the field: (i) the dearth of *directly comparative* QS modulator SAR data acquired with standardized screening conditions,[46,78] and (ii) the relative lack of small molecules capable of potently modulating QS-controlled phenotypes in wild-type bacterial strains.[79−81] Herein, we report experiments that now address both deficiencies through a comprehensive study of the QS receptor LasR in *P. aeruginosa*. Looking forward, our findings provide important context for the design of next-generation LasR ligands and effective antivirulence strategies in *P. aeruginosa*. Moreover, the mechanistic insights we gained are likely broadly applicable to small molecule ligand interactions with LuxR-type receptors beyond LasR. Accordingly, these structural features and mechanisms should be considered when designing synthetic modulators of any LuxR/LuxI-type QS network in Gram-negative bacteria.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b06728.

Bacterial strain and plasmid descriptions, reporter and elastase assay data, compound characterization data, and supplemental text. (PDF)

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### Notes

The authors declare no competing financial interest.

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