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## ARTICLE

## Unraveling the contributions of hydrogen-bonding interactions to the activity of native and non-native ligands in the quorum-sensing receptor LasR

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Quorum sensing (QS) via the synthesis and detection of *N*-acyl L-homoserine lactone (AHL) signals regulates important pathogenic and mutualistic phenotypes in many bacteria. Over the past two decades, the development of non-native molecules that modulate this cell-cell signaling process has become an active area of research. The majority of these compounds were designed to block binding of the native AHL signal to its cognate LuxR-type receptor, and much effort has focused on LasR in the opportunistic pathogen *Pseudomonas aeruginosa*. Despite a small set of reported LasR structural data, it remains unclear which polar interactions are most important for either (i) activation of the LasR receptor by its native AHL signal, *N*-(3-oxo)-dodecanoyl L-homoserine lactone (OdDHL), or (ii) activation or inhibition of LasR by related AHL analogs. Herein, we report our investigations into the activity of OdDHL and five synthetic analogs in wild-type LasR and in nine LasR mutants with modifications to key polar residues in their ligand binding sites. Our results allowed us to rank, for the first time, the relative importance of each LasR:OdDHL hydrogen bond for LasR activation and provide strong evidence for the five synthetic ligands binding LasR in a very similar orientation as OdDHL. By delineating the specific molecular interactions that are important for LasR modulation by AHLs, these findings should aid in the design of new synthetic modulators of LasR (and homologous LuxR-type receptors) with improved potencies and selectivities.

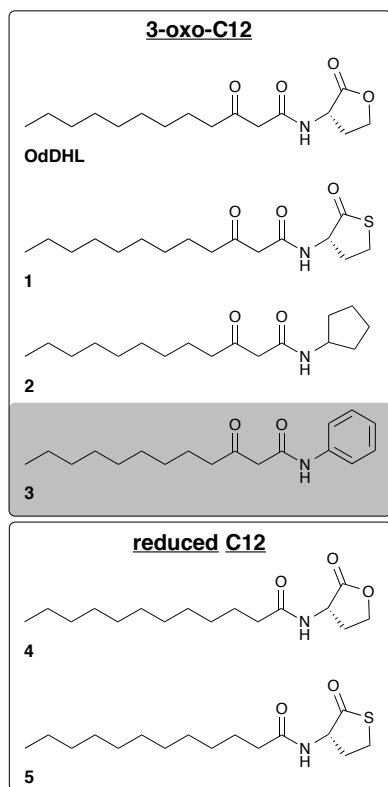
### Introduction

Bacteria, much like higher organisms, must alter their behaviour to have optimal fitness in changing environments. For example, if nutrients are limited, they swim to a new environment,<sup>1</sup> change their metabolic flux,<sup>2</sup> or even enter into spore states.<sup>3</sup> In addition to sensing the presence of nutrients, bacteria perceive which organisms are around them and the density in which they are packed in order to regulate whether or not to secrete toxins (e.g., antibiotics, hemolysins, and reactive small molecules) and to produce shared resources (e.g., siderophores, enzymes, and even light).<sup>4,5</sup> Many bacteria sense their own population densities in a process called quorum sensing (QS). In QS, bacteria biosynthesize a small molecule or short peptide signal that is either secreted or diffuses out of the bacterial cell and then can disperse throughout the environment.<sup>6-8</sup> As the bacteria replicate and their density increases within a confined space, the concentration of signal in this environment likewise increases.<sup>9</sup> Once signal levels reach a threshold concentration, the signals engage in productive interactions with bacterial receptor proteins that ultimately result in changes to gene expression. In the case of Gram-negative bacteria, the signal molecules are primarily *N*-acyl L-

homoserine lactones (AHLs) that are biosynthesized by LuxI-type enzymes. These AHL signals are sensed by intracellular LuxR-type receptors, which upon binding signals, typically form active dimers and function as transcription factors to induce expression of QS-regulated genes.<sup>6,7</sup>

Numerous bacteria that are relevant to human health use QS to regulate pathogenic or mutualistic behaviours. For example, the opportunistic pathogen *Pseudomonas aeruginosa* waits until it amasses a quorum before it expresses many virulence genes that harm the host organism.<sup>4</sup> The notorious human pathogen *Staphylococcus aureus* also uses QS to control a broad range of virulence phenotypes.<sup>5</sup> Alternatively, nitrogen-fixing rhizobia use QS to regulate their conversion into mature root nodules that help feed legumes.<sup>10</sup> Because QS is dependent on signal-receptor binding, blocking this binding event with a synthetic ligand represents a logical approach to artificially control virulent and beneficial phenotypes in bacteria. To this end, our laboratory and others have developed small molecules that competitively bind QS receptors and modulate the myriad phenotypes that they regulate.<sup>11-17</sup> In particular, we have heavily focused on developing inhibitors and activators of the LuxR-type receptor LasR, which is a primary regulator of

virulence in *P. aeruginosa*.<sup>18</sup> Such modulators of LuxR-type receptors have significant utility as chemical probes to study QS with both temporal and spatial control. Notably, because of the selfless communal nature of QS, QS inhibitors are likely to provide a weaker selective pressure for resistance relative to traditional antibiotics that directly inhibit bacterial growth.<sup>19-21</sup> For these reasons and others, the development of small molecule and macromolecular QS inhibitors as anti-virulence agents has attracted considerable attention.<sup>22-26</sup>



**Figure 1.** Native AHL ligand OdDHL and the OdDHL analogs examined in this study. The compounds are divided into groups containing the same tails (3-oxo-C12 or reduced C12) but varying heads (lactone, thiolactone, cyclopentyl, or phenyl). The single LasR inhibitor (3) is shaded grey; the others (OdDHL, 1, 2, 4, and 5) are all LasR activators.

Despite much past research, we still know relatively little about which ligand-receptor interactions are critical for activation or inhibition of LasR and other LuxR-type QS receptors. The first structures of a LuxR-type protein (TraR from *Agrobacterium tumefaciens*) bound to a native AHL were reported over a decade ago,<sup>27, 28</sup> but additional structural data of LuxR-type proteins with either native or non-native ligands remains scarce.<sup>29</sup> The paucity of both structural and biochemical data is primarily due to LuxR-type proteins being difficult to manipulate *in vitro*. These proteins are unstable without a ligand bound,<sup>27, 30-34</sup> the ligands can be difficult to exchange once bound,<sup>31, 32</sup> and often the proteins aggregate and become insoluble when bound to inhibitors.<sup>35</sup> Even when bound to its native AHL signal (*N*-(3-oxo)-dodecanoyl L-homoserine lactone, OdDHL; Figure 1), only structures of the truncated N-terminal ligand-binding domain LasR have been

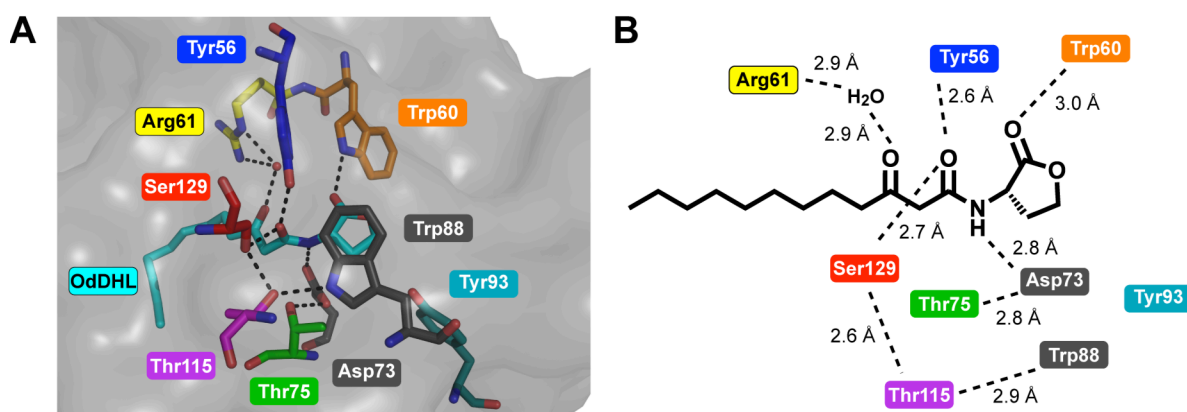
solved to date.<sup>35-37</sup> Further, there have been only two reported studies of LuxR-type proteins bound to non-native ligands: first, the LasR N-terminal domain bound to triphenyl (TP)-type synthetic activators,<sup>35, 38</sup> and second, full-length CviR (from *Chromobacterium violaceum*) bound to three AHLs exhibiting differing degrees of partial agonism (octanoyl L-homoserine lactone, decanoyl L-homoserine lactone, and the “chlorolactone” inhibitor CL).<sup>39</sup>

While these previous structural studies afford some insights into the binding of AHL and non-AHL ligands to LasR and its homologs, they fall short of delineating both the specific ligand-receptor interactions that are most important for activation of LasR and the modes by which non-native OdDHL-like analogs bind to LasR and affect its activity. This information is crucial for our efforts to design improved synthetic LasR modulators, and provided the motivation for the current study. Our investigations reported herein centered on two broad goals: (i) to uncover the relative importance of each hydrogen-bonding residue in the LasR ligand binding pocket for activation by its native ligand OdDHL, and (ii) to develop a better molecular-level understanding of how non-native ligands bind and activate LasR. The latter goal focused on a set of five analogs that closely mimic OdDHL with non-native head groups (1–3), an altered acyl tail (4), or both (5) (shown in Figure 1). In light of the challenges outlined above in manipulating LuxR-type proteins *in vitro*, we used bacterial cell-based  $\beta$ -galactosidase reporter-gene assays on site-directed mutants of LasR in order to investigate specific ligand-receptor interactions. Our results allowed us to rank, for the first time, the significance of each hydrogen-bonding residue in the LasR ligand-binding pocket for receptor activation. Interestingly, we also discovered one mutation that made LasR *more sensitive* to OdDHL. In turn, the results for the non-native ligands strongly supported our hypothesis that straight-chain OdDHL analogs that lack certain hydrogen-bonding moieties, whether they are activators or inhibitors, can still bind the LasR ligand-binding pocket in nearly an identical manner as OdDHL. This finding provides the first empirical evidence of the binding mode of the non-native activators (1, 2, 4, and 5), and affords further support for the predicted binding mode of aniline inhibitor 3.<sup>40</sup>

## Results and discussion

### Importance of hydrogen-bonding residues for LasR activation by OdDHL

We first sought to understand the relative importance of each hydrogen-bonding residue in the LasR ligand-binding pocket for receptor activation. Although X-ray crystal structures reveal the likely interactions present between LasR and a bound ligand (OdDHL or TP-type ligand),<sup>35, 36</sup> they fail to tell us the relative importance of these interactions for LasR activation. To our knowledge, the field still lacks a comprehensive mutational analysis of the hydrogen bonding residues in the ligand-binding pocket of LasR. A catalog of previously reported mutations (both via artificial mutagenesis and via isolation of naturally



**Figure 2.** LasR residues mutated in this study. (A) Image from the LasR X-ray crystal structure (OdDHL shown in cyan)<sup>36</sup> and (B) a corresponding cartoon illustrating suspected hydrogen-bonding interactions (dashed black lines) between eight different LasR residues and OdDHL. Tyr93 is also shown, as it could hydrogen bond with OdDHL if the ligand reorients slightly in the pocket.

occurring mutant bacteria) to polar residues in the ligand-binding pocket of LasR and its related homologs LuxR (*Vibrio fischeri*), TraR (*A. tumefaciens*), and RhlR (*P. aeruginosa*) is presented in Table S1. We selected nine residues in the LasR ligand-binding pocket for site-directed mutagenesis (Figure 2). The side chains of eight of these residues appear to either hydrogen bond directly with OdDHL or hydrogen bond with another LasR residue that hydrogen bonds with OdDHL (i.e., Tyr56, Trp60, Arg61, Asp73, Thr75, Trp88, Thr115, and Ser129), as revealed in the structure reported by Bottomley et al.<sup>36</sup> Closer scrutiny of this LasR structure indicated that the side chain of the ninth residue, Tyr93, could conceivably hydrogen bond with OdDHL if this ligand was slightly reoriented in the pocket, so we also included the Tyr93 residue in our studies. Each residue was mutated to an approximately isosteric residue that lacked a hydrogen-bonding side chain (Asp→Leu, Thr→Val, Trp→Phe, Tyr→Phe, Ser→Ala, Arg→Met, Table 1). Three of these mutants (R61M, W60F, and Y56F) have been previously studied,<sup>40–42</sup> and six are new to the field. We tested the activity of OdDHL in each mutant LasR using a  $\beta$ -galactosidase reporter-gene assay in an *Escherichia coli* background (see Experimental Section). The  $EC_{50}$  values for OdDHL and the maximal activity levels (at OdDHL concentrations well above the  $EC_{50}$  values) were determined for each mutant (listed in Table 1). These values and activity levels were then compared to those for OdDHL in wild-type LasR. In addition, we also gauged each residue's importance for LasR activation from an evolutionary perspective by calculating the percent conservation of each residue among 100 of LasR's most closely related homologs (see Table S4 and Table S5 for additional details).

Surprisingly, one of the LasR mutants (T75V) actually had a greater than 10 $\times$  lower  $EC_{50}$  value for OdDHL relative to wild-type LasR (Table 1). Such a hypersensitive LasR mutant, to our knowledge, has no literature precedence, although other work has revealed hypersensitive mutants of the *Pantoea stewartii* EsaR receptor.<sup>43</sup> We propose that the suspected polar interaction between Thr75 and Asp73 in wild-type LasR (see

Figure 2) decreases the sensitivity of LasR to OdDHL. When this polar interaction was removed by the T75V mutation, the pocket was presumably able to reorient in a manner that promoted improved binding of OdDHL. We also note that a Thr→Lys mutation was previously reported to be responsible for a weakened QS response in a clinically isolated

**Table 1.**  $EC_{50}$  values and maximal activation by OdDHL for wild-type and mutant LasRs, and the evolutionary conservation of each mutated residue

mutation	$EC_{50}$ (nM) <sup>[a]</sup>	maximal activation (% vs. wild-type) <sup>[b]</sup>	identity of residue in 100 LasR homologs <sup>[c]</sup>
wild-type	10	–	–
T75V	0.5	106%	26% (53% Val, 15% Ile)
D73L	inactive	0%	100%
W88F	inactive	0%	100%
Y93F	10	93%	4%
Y56F	10	102%	73%
S129A	–	–	66% (20% Thr, 5% Cys)
W60F	75	73%	98%
R61M	600	56%	14%
T115V	3500	109%	46% (28% Ser, 7% Cys)

<sup>[a]</sup>  $EC_{50}$  value is the concentration of OdDHL at which the activity is half the maximum activity for that mutant. Values are geometric means of biological triplicates (s.e.m. of log-transformed data  $\leq \pm 0.14$ , which corresponds to antilog errors of  $\times/\div 1.4$ ,  $n = 3$ ).  $EC_{50}$  values that are > 3.6-fold different from each other (corresponding to log-transformed  $EC_{50}$  values that differ by > 0.56) are statistically significant ( $p < 0.05$ ). Representative dose curves are shown in Figure S1. We note that mutants R61M, W60F, and Y56F were previously studied by others,<sup>40, 42</sup> and OdDHL  $EC_{50}$  values were previously reported (albeit without any discussion) for all mutants except Y93F,<sup>41</sup> but maximal activation data, compilation of sequence identity data at the mutated residues, and all discussion of these data is new to this manuscript. <sup>[b]</sup> Maximal activation levels of the mutants relative to wild-type LasR were determined using OdDHL concentrations much higher than the  $EC_{50}$  value in that strain. 10  $\mu$ M was sufficiently high to reach maximal activation for all strains except T115V, which had a substantially higher  $EC_{50}$  value. 100  $\mu$ M was used for T115V instead. Values are means of biological triplicates (s.e.m.  $\leq \pm 23\%$ ,  $n = 3$ ). <sup>[c]</sup> LasR was aligned with 100 closely related homologs, and the conservation of each residue of interest is reported (see Table S4 and Table S5 for entire alignment data).

*P. aeruginosa* strain.<sup>44</sup> This finding agrees with our model since lysine would be capable of an even stronger polar interaction than the wild-type threonine with Asp73. Interestingly, an alignment with 100 of LasR's most closely related homologs showed that hydrophilic residues at this position are relatively rare (see Table 1 and Table S5). Most homologs possess an isoleucine or valine at this position instead of a threonine, which like our T75V mutant, would not allow interactions with Asp73. It is intriguing that LasR has a lower-than-maximal sensitivity for its native ligand, given that over evolutionary history one would assume it has had the opportunity to sample valine at the T75 position. These data suggest that *P. aeruginosa* might have a fitness benefit by being less sensitive to OdDHL. If true, this is a surprising discovery, as it would require the synthesis of more OdDHL signal and would therefore be less efficient. Perhaps a counteracting advantage of lessened sensitivity would be the decreased risk of accidental activation by stochastic fluctuations in signal concentration or by similar signals produced by neighbouring bacterial species. Additional experiments are currently underway with this mutant, as well as with less sensitive mutants, to characterize the fitness implications of higher and lower sensitivities to the OdDHL signal.

The other LasR mutants behaved as expected, showing varying degrees of reduced sensitivity to OdDHL. Two mutants were completely inactive (D73L and W88F), suggesting that these residues play a critical role in LasR activation by OdDHL. This finding corroborates published data for other mutations to these residues in LasR and to their homologous residues in LuxR, TraR, and RhlR.<sup>45-50</sup> We note that these two residues are universally conserved among 100 of LasR's closely related homologs (Table 1). This finding provides a satisfying correlation between the evolutionary conservation of the residues and their importance for activity. We cannot say with certainty that the D73L and W88F LasR mutants are inactive due to their inability to bind OdDHL—the residues could be necessary for protein folding regardless of the ligand. Nevertheless, since Asp73 and Trp88 are in the ligand-binding pocket, it is reasonable to suspect that their importance is related to ligand binding. Furthermore, Asp73 appears to hydrogen bond with a ligand amide in every crystal structure of a ligand-bound LasR homolog—even with the TP-1, TP-3, and TP-4 ligands that are structurally distinct from AHLs<sup>35</sup>—underscoring the likely importance of interactions between Asp73 and LasR activating ligands.

Among the other LasR mutants evaluated, two displayed essentially no change (Y93F and Y56F) in activation by OdDHL relative to wild-type LasR, and two showed moderate decreases in activity (S129A and W60F) (Table 1). The results for the Y56F and W60F mutants were comparable to a previous report;<sup>42</sup> however, the Y56F, S129A, and W60F mutations were less detrimental than similar mutations reported in TraR, LuxR, and RhlR, which almost or completely obliterated the activities of these related receptors (Table S1).<sup>46, 49-51</sup> The Y93F mutation has not been previously examined in any LuxR-type protein. Gratifyingly, the relative activity trend for these four LasR

mutants is largely consistent with their relative degree of conservation: Tyr93 is unconserved (4%) and Tyr56 is only moderately conserved (73%), while Ser129 is either Ser, Thr, or Cys in 91% of the homologs and Trp60 is nearly universally conserved (98%). Therefore, mutant activity and residue conservation match well for the T75V, D73L, W88F, Y93F, Y56F, S129A, and W60F mutations in LasR.

Mutation of Arg61 in LasR, which binds the OdDHL 3-keto carbonyl, was found to be detrimental for LasR activation (60-fold increase in EC<sub>50</sub> relative to wild-type, Table 1), consistent with previous studies on LasR and LuxR.<sup>41</sup> However, despite its importance, Arg61 is not conserved among LasR homologs. This apparent inconsistency can be explained in two reasonable ways: first, many of the 100 LasR homologs examined herein have a native AHL ligand that lacks a 3-keto group and they therefore do not have an evolutionary benefit to engage in such a hydrogen bond (e.g., *C. violaceum* CviR,<sup>52</sup> *Ralstonia solanacearum* SolR,<sup>53</sup> and *Rhodospseudomonas palustris* RpaR<sup>54</sup>), and second, some homologs are able to bind the 3-keto via alternative residues (e.g., Ala38 and Thr129 in *A. tumefaciens* TraR<sup>27, 28</sup> and Ser56 in *P. aeruginosa* QscR<sup>55</sup>). Turning to the final LasR mutant (T115V), we found that Thr115 is very important for LasR activation by OdDHL—T115V exhibits a greater than 100-fold increase in EC<sub>50</sub> relative to wild-type (Table 1), corroborating prior reports in both LasR and TraR.<sup>49, 56, 57</sup> Although it does not directly hydrogen bond to OdDHL in the reported X-ray crystal structures of LasR,<sup>35, 36</sup> it appears to serve as a “linchpin” of a hydrogen-bonding network between Trp88 and Ser129 (Figure 2). Surprisingly, Thr115 is not universally conserved—19% of the nearest LasR homologs do not contain a side-chain capable of hydrogen bonding in that position. Therefore, similar to Arg61, hydrogen bonds supplied by Thr115 to properly orient the OdDHL hydrogen-bonding network are likely supplied by other residues in LasR homologs.

Systematically screening these nine LasR mutants in the same reporter system allowed us to rank the relative importance of the five LasR hydrogen bonds to OdDHL as follows: amide NH > 3-keto C=O > lactone C=O > amide C=O. Of note, this trend does not match the predicted hydrogen-bonding strengths of the residues (e.g., amide carbonyls are substantially more basic than ester and ketone carbonyls<sup>58</sup>); therefore, the importance of the hydrogen bonding residues is likely dependent on factors other than bond strength. Nonetheless, this order largely matches the degree of conservation of the residues that bind these moieties in LasR homologs. We believe that this information could be leveraged for the design of new non-native agonists with potentially increased potencies; for example, designing ligands to interact with Asp73 and Arg61 will likely be more important than designing ligands to interact with Tyr56. In addition to determining this ranking, we also gained insights into the relative importance of four residues that do not directly hydrogen bond with OdDHL. One abolished activity (W88F), one dramatically decreased activity (T115V), one had no effect (Y93F), and remarkably, one was over 10× more sensitive than wild type to OdDHL (T75V). As discussed

above, LasR may have evolved to have a lower sensitivity to its native ligand, and we are currently using this mutant to explore the fitness implications of QS signal sensitivity.

### Importance of hydrogen-bonding residues for LasR activation by thiolactone ligand 1

After examining the impact of the mutations in LasR on OdDHL activation, we next tested the ability of ligands with varying head groups and acyl tails (1–5, see Figure 1) to activate and inhibit the seven active LasR mutants identified above. We hypothesized that these ligands, which resemble OdDHL, bind the orthosteric ligand-binding pocket in the same orientation as OdDHL. Our results supported this hypothesis. The thiolactone analogue of OdDHL (ligand 1) exhibits comparable agonistic activity as OdDHL in wild-type LasR,<sup>59, 60</sup> and it behaved in an equivalent manner to OdDHL in activating the LasR mutants (Figure 3), except it had a significantly lower EC<sub>50</sub> value for the R61M mutant (i.e., an ~8× decrease relative to OdDHL; Table 2). These data suggest that ligand 1 binds in the pocket nearly identically to OdDHL. We propose that the differences in activity between OdDHL and 1 (Table 2) in wild-type (slight), Y56F (slight), S129A (slight), and R61M (moderate) are due to slightly different interactions with Trp60. A previous docking study of ours on a related thiolactone AHL analogue and LasR supports this hypothesis.<sup>40</sup> This computational study demonstrated that due to its larger ring size, the thiolactone head group could interact with Trp60 with a subtly different positioning, and we reason that such an effect could also be operative for 1 with LasR. Overall, the data for agonist 1 suggest that it binds very similarly to OdDHL, and provide impetus for the further study of AHL analogues with thiolactone head groups as improved LasR modulators.<sup>59</sup>

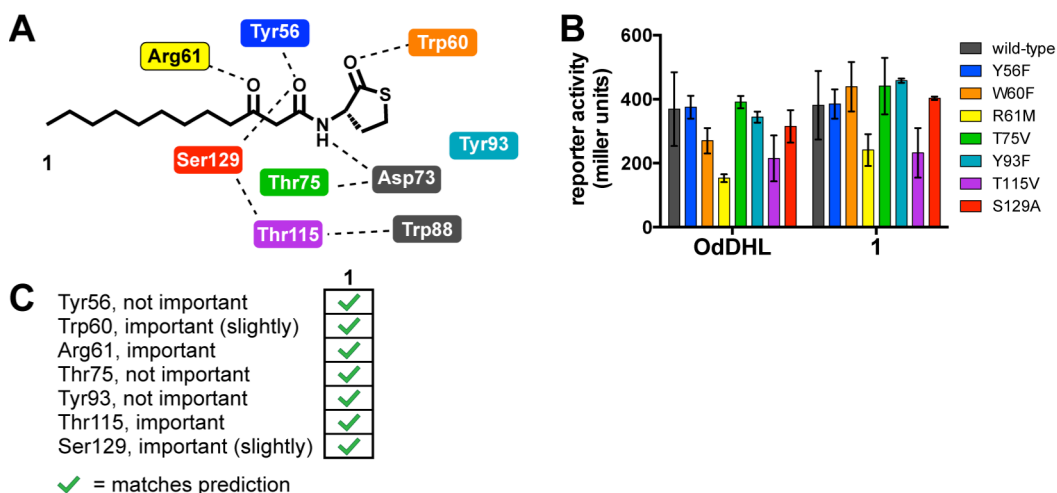
**Table 2.** EC<sub>50</sub> values for wild-type and mutant LasR activation by OdDHL and ligand 1

mutation	OdDHL EC <sub>50</sub> (nM) <sup>[a]</sup>	Ligand 1 EC <sub>50</sub> (nM)
wild-type	10	5
T75V	0.5	0.5
Y93F	10	10
Y56F	10	5
S129A	50	30
W60F	75	70
R61M	600	80
T115V	3500	3500

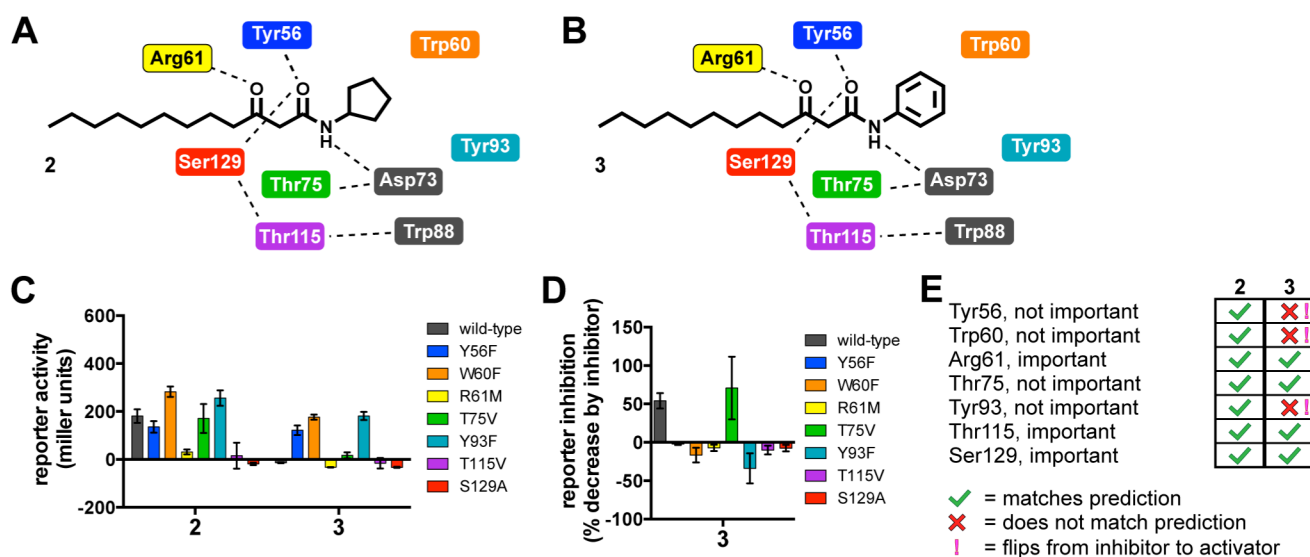
<sup>[a]</sup> EC<sub>50</sub> value is the concentration of OdDHL at which the activity is half the maximum activity for that mutant. Values are geometric means of biological triplicates (s.e.m. of log-transformed data  $\leq \pm 0.14$ , which corresponds to antilog errors of  $\times/\div 1.4$ ,  $n = 3$ ). EC<sub>50</sub> values that are  $> 3.6$ -fold different from each other (corresponding to log-transformed EC<sub>50</sub> values that differ by  $> 0.56$ ) are statistically significant ( $p < 0.05$ ). Representative dose curves are shown in Figure S2.

### Importance of hydrogen-bonding residues for LasR activation and inhibition by non-lactone ligands 2 and 3

We predicted that LasR activation by cyclopentyl ligand 2 and LasR inhibition by aniline ligand 3 would depend on Arg61, Ser129, and Thr115 because (i) these residues were important for OdDHL activity (Table 1), and (ii) no interactions between these residues and the ligands should be affected by changing the ligand head group from a lactone (as in OdDHL) to a cyclopentyl or a phenyl (as in 2 and 3, respectively) (Figure 1 and Figure 4A,B). In addition, ligand activity was initially expected to not depend on Trp60 (because ligands 2 and 3 lack the ability to hydrogen bond with Trp60, as seen in Figure 4A,B) or Tyr56, Thr75, and Tyr93 (as they were not significantly important for OdDHL activity, as shown in Table 1). Activation data for ligand 2 strongly agreed with these hypotheses, as only the R61M, S129A, and T115V mutations



**Figure 3.** Activation of mutant LasRs by thiolactone ligand 1. (A) Predicted interactions made by ligand 1 in the binding pocket of LasR (identical to OdDHL). (B) Activation of each mutant by OdDHL and its thiolactone analog 1, each at 10  $\mu$ M. Error bars indicate s.e.m. from a biological triplicate ( $n = 3$ ) (C) Predicted and actual importance of interactions for activation by 1. A green check indicates the prediction was correct. See also Table 2.



**Figure 4.** Activation and inhibition of mutant LasRs by non-lactone ligands **2** and **3** in the binding pocket of LasR. These ligands lack polar interactions with Trp60. (C) Activation of each mutant by **2** and **3**, each at 10  $\mu$ M for all mutants. (D) Percent inhibition of each mutant by **3** (100% inhibition is complete shutdown of  $\beta$ -galactosidase production). Ligand **3** was added at 10  $\mu$ M, and OdDHL was present at the  $EC_{50}$  value for the given mutant. Negative inhibition values indicate activation greater than that afforded by only OdDHL at its  $EC_{50}$  concentration. Error bars for all plots indicate s.e.m. from a biological triplicate ( $n = 3$ ). Data for ligand **3** has been partially reported previously.<sup>39</sup> (E) Predicted and actual importance of interactions for activation by **2** and inhibition by **3**. A green check indicates the prediction was correct. A red X indicates a substantial deviation from the expectation, and a magenta exclamation point indicates a flip from inhibition to activation activity.

were significantly detrimental for LasR activation by **2** (Figure 4C,E). This finding suggests that **2** binds LasR similarly to OdDHL and simply lacks a polar interaction with Trp60.

As we previously reported,<sup>40</sup> aniline ligand **3** was unexpectedly observed to exhibit two-faced “Janus” behaviour—i.e., it transitions from a good inhibitor into a moderate agonist—in certain LasR mutants (Y56F and W60F, Figure 4C,D). Apart from this Janus behaviour (which itself is consistent with binding LasR similarly to the native OdDHL<sup>40</sup>), every other residue that hydrogen bonds with OdDHL was important for the LasR inhibitory activity of **3** (Figure 4D,E). The only mutation that did not have a consistent negative impact on LasR inhibition was T75V, which was expected because this mutation only improved the sensitivity of LasR to OdDHL (Table 1). Therefore, inhibitor **3** likely binds LasR in a very similar orientation to OdDHL, but as we suggested in our earlier study,<sup>40</sup> the phenyl head group probably makes different interactions with the Trp60 residue to lead to an inactive conformation. Interestingly, we also uncovered a third Janus mutation for ligand **3** in this study. The Y93F mutation, which appeared to have no impact on OdDHL activity in LasR (Table 1), flipped the activity of **3** into an agonist in this mutant (Figure 4C,E). In retrospect, this observation makes sense because Tyr93 would be near the phenyl head group of **3** if it binds similarly to OdDHL, and this Tyr→Phe mutation could provide slightly more space for ligand **3** to bind without displacing Trp60 or other residues from their active conformations. Essentially, this can be considered a “bump-hole” type phenomenon,<sup>61, 62</sup> with the mutation Y93F providing a “hole” in which to accommodate the non-native head group

“bump” of ligand **3**. We previously posited that a related bump-hole interaction could delay the Janus behaviour for **3** in the W60F mutant.<sup>40</sup> Collectively, these data strongly support the hypothesis that ligands **2** and **3** bind the LasR ligand binding pocket in orientations analogous to that of OdDHL; ligand **2** binding leads to an active LasR conformation, but ligand **3** binding leads to a subtly different, yet inactive LasR conformation. We note that subtly different interactions with Tyr56, Trp60, and/or Tyr93 could be causes of the divergent activities of other synthetic LasR modulators with minor structural differences, such as the TP ligands.<sup>35, 38</sup>

#### Importance of hydrogen-bonding residues for LasR activation by ligands **4** and **5**

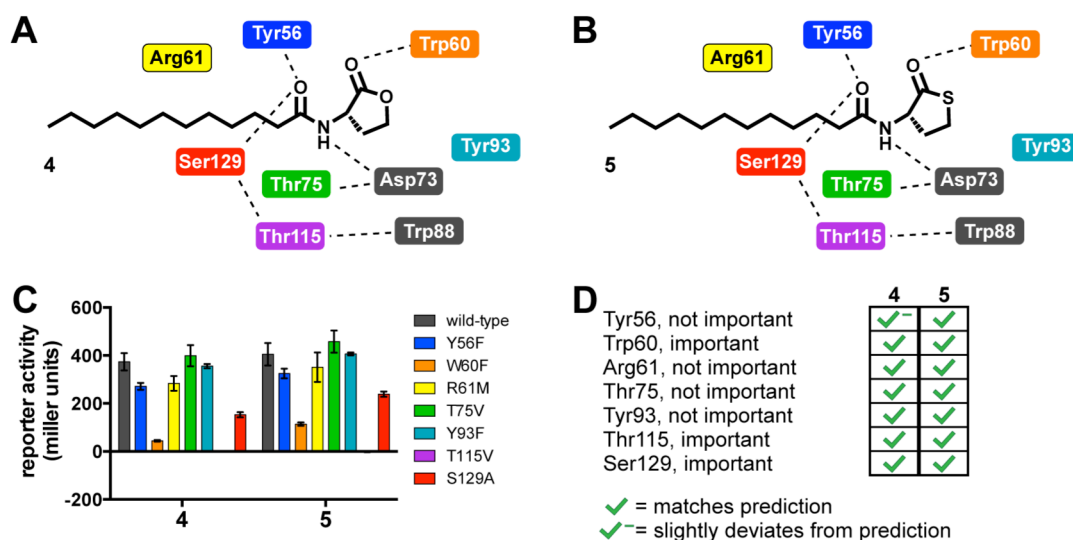
In view of our results thus far, LasR activation by ligands **4** and **5** (the lactone and thiolactone analogues of OdDHL lacking the 3-keto moiety, respectively; Figure 1) were expected to depend primarily on interactions with Trp60, Thr115, and Ser129, if the ligands bind similarly to OdDHL. This hypothesis follows from a similar rationale as above: (i) these three residues were important for OdDHL activity (Table 1), and (ii) no interactions between these residues and the ligands should be affected by loss of the 3-keto group on the ligand tail (Figure 1 and Figures 5A,B). In turn, the R61M mutation was expected to have a minimal impact on LasR activation because ligands **4** and **5** should already be incapable of forming a hydrogen bond with Arg61. The Y56F, T75V, and Y93F mutations were also predicted to have minimal effects on the activities of **4** and **5**, as these mutations were not detrimental to OdDHL activity (Table 1). Since ligand **4** was known to have an  $EC_{50}$  value in wild-

type LasR significantly less than  $10\ \mu\text{M}$  ( $40\ \text{nM}^{63}$ ), we screened ligands **4** and **5** at  $100\ \text{nM}$  instead of at  $10\ \mu\text{M}$  in the LasR mutants to increase the chances of seeing differential activity. As expected, the T115V mutation destroyed LasR activation by **4** and **5**, and the W60F and S129A mutations significantly decreased LasR activation by these two analogues (Figure 5C). None of the other mutations had a substantial impact on activity, except Y56F, which had a small but significant impact on LasR activation by ligand **4**. Most telling was the minimal impact of the R61M mutation on the activities of **4** and **5**. Even though this mutation dramatically affects the activities of **1–3**, it had perhaps only a slight effect on ligand **4** and no significant effect on **5**. This result strongly supports these two ligands binding similarly to OdDHL, because in that orientation, they would be incapable of hydrogen bonding with Arg61 and thus would be unaffected by an R61M mutation. We note that structural data for LasR with ligands **1–5** would help to conclusively answer this question and many of the other interesting questions arising from this study. Nevertheless, the close correlation of the results with our expectations for all five synthetic ligands in the LasR mutants provides the first empirical evidence for these compounds binding to LasR in the same orientation as OdDHL.

## Conclusions

We have performed detailed investigations into the activity of OdDHL and five synthetic analogues on both wild-type LasR and on nine LasR mutants with modifications to their ligand-binding sites. While structural data have been reported for LasR complexed to its native ligand (OdDHL) and to selected non-native TP agonists,<sup>35, 36</sup> prior to the work reported herein, the relative importance of each receptor-ligand polar interaction and the binding modes of AHL-derived LasR modulators were largely unknown. Analysis of the activity profiles for each

LasR mutant with OdDHL revealed the following hierarchy of importance for its hydrogen bonds to LasR: amide NH > 3-keto C=O > lactone C=O > amide C=O. This ranking serves to clarify the interactions that should be maintained in the design of next-generation synthetic LasR modulators. One LasR mutation, T75V, actually afforded a mutant that is *more sensitive* to OdDHL than wild-type LasR. Interestingly, Thr75 is often a valine or isoleucine in LasR homologs, leading us to propose that *P. aeruginosa* actually gains some fitness advantage by having a less-than-maximal sensitivity to OdDHL. Analysis of LasR mutant activity with ligands **1–5** strongly supported the hypothesis that all five of these ligands bind the orthosteric ligand-binding pocket in the same orientation as OdDHL, but simply lack certain hydrogen-bonding interactions. Ligand **3** was the unique LasR inhibitor investigated in this study, and as reported previously, the W60F and Y56F mutations flip it to an activator.<sup>40</sup> We also identified a third, new “Janus” mutation for **3** (Y93F), the location of which is hypothesized to be near the binding location of ligand **3**'s phenyl head group. This discovery further supports our model that aniline ligand **3** binds similarly to OdDHL, but makes subtly different interactions that lead to an inactive LasR conformation.<sup>40</sup> In total, this study serves to deepen our understanding of LasR:OdDHL interactions and augments current LasR:OdDHL structural data by providing an expansive set of new mutant activity data with a series of closely related ligands. Moreover, it provides very strong evidence (in the absence of high-resolution structures) that several non-native ligands bind the LasR ligand-binding pocket in a mode similar to OdDHL—findings that will be helpful in guiding efforts to design new non-native LasR modulators.



**Figure 5.** Activation of mutant LasRs by ligands **4** and **5**. (A,B) Predicted interactions made by ligands **4** and **5** in the binding pocket of LasR. These ligands lack polar interactions with Arg61. (C) Activation of each mutant by **4** and **5**, each at  $100\ \text{nM}$  for all mutants. Error bars for all plots indicate s.e.m. from a biological triplicate ( $n = 3$ ). (D) Predicted and actual importance of interactions for activation by **4** and **5**. A green check indicates the prediction was correct, and a green check-minus indicates the prediction was slightly incorrect.



## Experimental section

### Compound handling and reagents

OdDHL and ligands 1–5 were synthesized as previously reported.<sup>59, 64, 65</sup> Stock solutions of compounds (10 mM) were prepared in DMSO and stored at room temperature in sealed vials. Solvent-resistant polypropylene (Corning Costar cat. no. 3790) and clear polystyrene (Corning Costar cat. no. 3997) 96-well microtiter plates were used as appropriate. All biological reagents were purchased from Fisher and used according to enclosed instructions.

### Instrumentation

Absorbance and fluorescence measurements were obtained using a Biotek Synergy monochromator plate reader running Gen5 v1.05 software. A 600 nm filter was used for reading bacterial cell density. Filters of 420 nm and 550 nm were used for Miller-type absorbance assays.

### Bacterial strains, media, and growth conditions

The bacterial strain used in this study was *E. coli* DH5 $\alpha$  [F $\phi$ 80*dlacZ* $\Delta$ *M15*  $\Delta$ (*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r $\kappa^-$  m $\kappa^+$ ) *phoA supE44*  $\lambda^-$  *thi-1 gyrA96 relA1*]. *E. coli* was cultured at 37 °C in Luria-Bertani (LB) medium and on LB plates with 1.5% agar. For selection and maintenance of plasmids, gentamicin and ampicillin were used at 15  $\mu$ g/ml and 100  $\mu$ g/ml respectively.

### Construction of mutant LasR reporter strains

Mutant LasR strains were based on the *E. coli* LasR  $\beta$ -galactosidase reporter strain reported by Lee et al.<sup>34</sup> Site-directed mutagenesis was carried out on the LasR-expressing plasmid pJN105L<sup>34</sup> by overlap extension polymerase chain reaction (PCR) (see Table S3 for PCR primers).<sup>66</sup> The mutagenized *lasR* genes were digested with *EcoRI* and *XbaI* and cloned into *EcoRI/XbaI*-cut pJN105L. The mutant pJN105L variants were sequenced to verify mutagenesis and transformed via electroporation into the *E. coli* DH5  $\alpha$ /pSC11<sup>67</sup> reporter strain and selected on LB + gentamicin + ampicillin plates. Mutants Y56F, W60F, R61M, D73L, T75V, W88F, T115V, and S129A were previously reported.<sup>40</sup> Mutant Y93F is new to this study (see Table S2 for complete list of strains and plasmids).

### Reporter gene assays

$\beta$ -galactosidase reporter assays were conducted as previously reported for the wild-type LasR strain.<sup>59, 64</sup> All LasR mutant strains were evaluated for activation by OdDHL at both 10  $\mu$ M and through dose-response analysis. Non-native ligands were tested for LasR activation at 10  $\mu$ M in each strain. Similarly, ligands were tested for LasR inhibition at 10  $\mu$ M against OdDHL at its EC<sub>50</sub> value for the mutant LasR strain.

### Determining conservation of homologous residues between LasR homologs

A Concise Microbial Protein BLAST search was performed using blastp on all proteins from complete genomes in the protein cluster database (ProtClustDB) using the LasR sequence aag04819.1 (<https://www.ncbi.nlm.nih.gov/genomes/prokhits.cgi>). The top 100 hits were imported into Geneious Pro 5.5.8 and aligned with a ClustalW alignment using the BLOSUM cost matrix with gap open cost of 10 and gap extend cost of 0.1. See full alignments in Table S4. The frequency of each amino acid at the mutated residues was cataloged within Geneious (comprehensive data in Table S5, summary presented in Figure 2).

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### Notes and references

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