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Evaluation of a focused library of *N*-aryl L-homoserine lactones reveals a new set of potent quorum sensing modulators

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ABSTRACT

A focused library of *N*-aryl L-homoserine lactones was designed around known lactone leads and evaluated for antagonistic and agonistic activity against quorum-sensing receptors in *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, and *Vibrio fischeri*. Several compounds were identified with significantly heightened activities relative to the lead compounds, and new structure–activity relationships (SARs) were delineated. Notably, 4-substituted *N*-phenoxyacetyl and 3-substituted *N*-phenylpropionyl L-homoserine lactones were identified as potent antagonists of TraR and LuxR, respectively.

Bacteria do not always act alone. Rather, many bacteria can assemble into multicellular communities and initiate processes as a group that they are incapable of as individual cells.[1] These group behaviors are largely under the control of a cell–cell signaling pathway called quorum sensing (QS), and can play a significant role in the establishment of both symbiotic and pathogenic relationships with eukaryotic hosts.[2] For example, virulence factor production and biofilm formation is under the control of QS in many clinically relevant pathogens.[3] QS is mediated by a chemical language of low molecular weight signals, or autoinducers, and their cognate protein receptors. Autoinducer–receptor binding occurs once the bacteria reach a threshold cell density, and this binding event controls the transcription of genes necessary for bacterial group functions. Interception of this binding event represents a strategy to attenuate bacterial group behaviors, and has attracted considerable interest in the drug development and chemical biology fields.[4] Our laboratory recently developed several synthetic autoinducer mimics that are capable of strongly antagonizing and agonizing autoinducer receptors in a range of Gram-negative bacteria.[5] Here, we report a third-generation set of autoinducer mimics that are derived from these initial lead compounds and that display significantly improved activities, most notably in the symbiont *Vibrio fischeri* and pathogen *Agrobacterium tumefaciens*.

Proteobacteria use *N*-acylated L-homoserine lactone signals (AHLs, Fig. 1) and cytoplasmic LuxR-type signal receptors as their primary QS circuit.[6] One of the first approaches to modulate QS in these bacteria was the development of non-native AHLs capable of blocking or intercepting native AHL–LuxR-type receptor binding.[7] These autoinducer mimics retained the native L-homoserine lactone, yet contained non-native acyl tails. The majority of our synthetic QS modulators are of this structural class, and selected AHLs (**1**–**8**) are shown in Figure 1. Compounds **1**–**6** and **8** represent

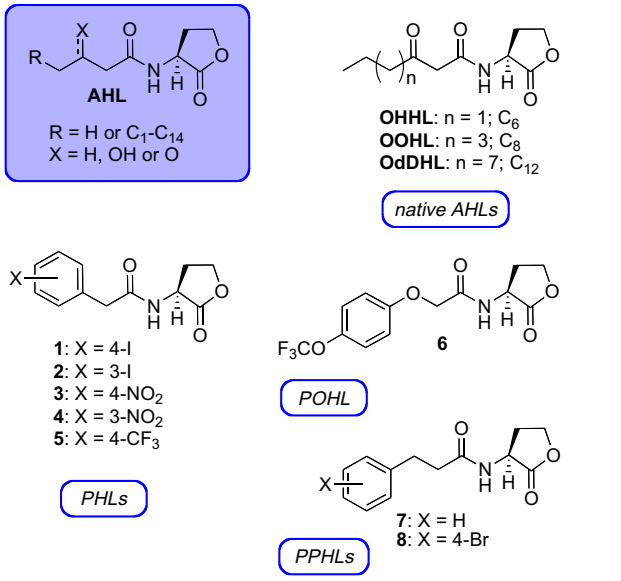

Figure 1. Generic structure for AHLs (top left), structures of selected naturally occurring AHLs (top right), and structures of our previously reported non-native AHLs (PHLs **1**–**5**, POHL **6**, and PPHLs **7** and **8**; bottom). Acyl chain carbon numbers are shown for clarity for native AHLs.

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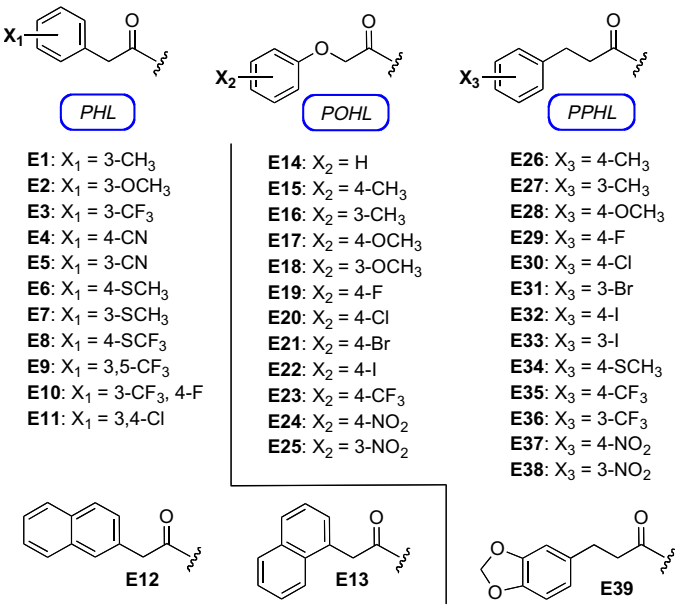

Figure 2. Structures of the acyl groups of the AHLs in Library E (**E1**–**E39**). All 39 AHLs have L-stereochemistry.

some of the most potent agonists and antagonists of LuxR-type receptors that we have reported.[5] These compounds are active in *V. fischeri*, *A. tumefaciens*, and/or the opportunistic pathogen *Pseudomonas aeruginosa*, and can be classified as phenylacetyl HL (PHL, **1**–**5**), phenoxyacetyl HL (POHL, **6**), or phenylpropionyl HL (PPHL, **7**–**8**) derivatives. Our initial studies revealed that subtle structural changes to the PHL phenyl ring, some as simple as moving a substituent by one carbon, had marked effects on ligand activity;[5,8] our examination of the POHL and PPHL structure classes was far more limited, however. In the present study, we sought to further explore the PHL, POHL, and PPHL structure classes as non-native QS modulators, and delineate additional SARs for antagonists and agonists of LuxR-type receptors in *A. tumefaciens*, *P. aeruginosa*, and *V. fischeri* (TraR, LasR, and LuxR, respectively). In addition, we sought to determine structural features that rendered ligands selective for one receptor, or more. We designed a 39-member library (Library E) that incorporated acyl group functionalities that broadly fit into the PHL, POHL, and PPHL structure classes (shown in Fig. 2). A brief discussion of our choice of functional groups is valuable at the outset. In examining the PHLs, we sought to study the effects of the incorporation of an expanded set of 3- and 4-substituents on agonistic and antagonistic activity against LuxR-type receptors. We focused primarily on 3-substituted PHLs, as these ligands are capable of inhibiting LasR in *P. aeruginosa*, and in turn, strongly activating LuxR in *V. fischeri*.[5,8] In both organisms, substituents with some steric bulk and electron-withdrawing capability yielded the most active PHLs (*e.g.*, 3-NO_2 PHL **4**; Fig. 1). We also examined a small set of PHLs with electron-withdrawing substituents in the 4-position, as these compounds are strong inhibitors of TraR in *A. tumefaciens* and LuxR in *V. fischeri* (*e.g.*, PHLs **1**, **3**, and **5**). In this initial study of the POHL and PPHL structure classes, we also selected 3-and 4-substituted derivatives for further examination. The 4-OCF_3 POHL **6** was shown previously to be a moderate to strong inhibitor of LasR and TraR, respectively.[5] In addition, 4-bromo PPHL **8** is a potent inhibitor of TraR, LasR, and LuxR, while the non-substituted PPHL **7** displays minimal activity against all three receptors, suggesting that phenyl ring substitution patterns are also important for activity in this structure class.

Table 1
IC_{50} values for most active antagonists across the three strains[a]

Entry	Compound	*A. tumefaciens* TraR[b] (μM)	*E. coli* LasR[c] (μM)	*V. fischeri* LuxR[d] (μM)
1	***1***	1.25[e]	1.72[e,f]	0.86[e]
2	***2***	–[g]	4.63	–
3	***3***	2.25[e]	–	0.96[e]
4	***4***	–	0.61[e,f]	–
5	***5***	0.81[e]	–	0.61
6	***6***	0.46[e,f]	4.67[e,f]	–
7	***7***	0.92[e]	0.34[e,f]	1.35
8	**E1**	–	–	2.0 (91%)[h]
9	**E2**	–	–	2.5 (85%)[h]
10	**E3**	–	–	1.4
11	**E6**	8.4	–	2.1
12	**E7**	–	–	1.5
13	**E8**	4.7	–	1.3
14	**E10**	–	–	1.0[e,f]
15	**E11**	2.4[e]	–	–
16	**E12**	4.2	–	0.94
17	**E13**	–	–	1.3
18	**E15**	1.8 (84%)[h]	–	–
19	**E16**	1.1[e]	7.8[e]	–
20	**E17**	4.3	–	–
21	**E19**	2.7[e,f]	–	–
22	**E20**	**0.62**[e]	4.7[e]	
23	**E21**	**0.51**[e,f]	**2.1** (67%)[h]	3.0
24	**E22**	**0.44**[e,f]	**2.0** (63%)[h]	1.9
25	**E23**	1.6[e,f]	–	–
26	**E24**	**0.29**[e,f]	–	–
27	**E25**	–	–	2.4
28	**E26**	–	4.3[e,f]	2.2
29	**E27**	–	8.9[e,f]	2.1
30	**E28**	–	6.8[e,f]	–
31	**E29**	–	12[e,f]	2.7
32	**E30**	3.3	**2.2**[e,f]	0.97
33	**E31**	–	3.3[e,f]	**0.53**
34	**E32**	2.1	–	**0.63**
35	**E33**	–	**1.8**[e,f]	**0.39**
36	**E34**	1.1[e]	–	1.7
37	**E35**	**0.21**[e]	–	2.2
38	**E36**	–	–	**0.50**
39	**E37**	1.6[e]	**3.0** (67%)[h]	0.99
40	**E38**	–	3.4[e,f]	**0.88**
41	**E39**	–	3.4	–

[a] See text for details of reporter strains. Values determined by testing compounds over a range of concentrations ($0.01–1 \times 10^6$ nM) against native ligand. All assays performed in triplicate. See Supplementary data for plots of dose–response curves. Bold italicized compounds are controls; key data for Library E is bolded. Control IC_{50} data from Ref. 5a; see also Ref. 12.
[b] Determined against 200 nM OOHL.
[c] Determined against 20 nM OdDHL.
[d] Determined against 3 μM OHHL.
[e] Dose–response curve upturned at higher concentrations; IC_{50} value calculated from partial antagonism dose–response curve. See Ref. 13.
[f] Dose–response curve did not fully level off over the concentrations tested due to upturn.
[g] Not determined.
[h] Dose–response curve levels off, yet not at 100% inhibition. This could indicate eventual upturn. Maximal inhibition level is indicated.

We synthesized Library E according to our previously reported microwave-assisted synthetic route to AHLs.[5a] The 39 library members were isolated in moderate to good yields (55–80%) and high purities (91–99%) on a 10–20 mg/compound scale (see Supplementary data).

Library E was evaluated for antagonistic and agonistic activity in TraR, LasR, and LuxR using cell-based reporter gene assays according to reported procedures.[5,8] We utilized the following three reporter strains that lack native AHL synthases: *A. tumefaciens* WCF47 (pCF372)[9] *Escherichia coli* DH5α (pJN105L pSC11)[10] and *V. fischeri* ES114 (Δ-*luxI*)[11] The *A. tumefaciens* and *E. coli* strains produce the enzyme β-galactosidase upon TraR or LasR activation, respectively, and ligand activity is measured using Miller absorbance assays. LuxR activation or inhibition in the *V. fischeri* strain

Table 2
EC_{50} values for most active agonists across the three strains[a]

Entry	Compound	*A. tumefaciens* TraR (μM)	*E. coli* LasR (μM)	*V. fischeri* LuxR (μM)
1	***OOHL***	0.20	—[b]	—
2	***OdDHL***	—	0.01	—
3	***OHHL***	—	—	3.0
4	***4***	—	—	0.35
5	***6***	—	6.3 (30%)[c]	—
6	**E5**	—	—	**0.30** (75%)[c]
7	**E8**	—	**3.4** (65%)[c]	—
8	**E9**	—	—	**0.37** (70%)[c]
9	**E32**	—	>10[d]	—
10	**E35**	—	>10[d]	—
11	**E39**	—	>10[d]	—

[a] See footnote a for Table 1. Control EC_{50} data from Ref. 5a.
[b] Not determined.
[c] Dose–response curve reached a plateau over the concentrations tested, yet the level of maximal induction was lower than that for the native ligand; EC_{50} value calculated from this dose–response curve. Value in parentheses equals the maximum induction value achievable (at 100 μM ligand) relative to native ligand.
[d] Dose–response curve did not fully plateau over the concentrations tested.

is reported by luciferase production. Antagonism assays were performed in the presence of library compound and native AHL ligand (at its approximate EC_{50} value), while agonism assays were performed with library compound alone (see Supplementary data) The native AHL ligands for *A. tumefaciens (*OOHL), *P. aeruginosa* (OdDHL), and *V. fischeri* (OHHL) and compounds **1**–**8** (Fig. 1) were used as controls for these assays.

The reporter gene assays of Library E revealed that 36 of the 39 library members exhibited strong antagonistic or agonistic activity against one or more LuxR-type receptor. This high percentage of 'hits' (92%) serves to validate our third-generation library design focused on PHLs, POHLs, and PPHLs. Thirty-four compounds were identified that displayed inhibitory activities of >70% against TraR, LasR, and/or LuxR. In turn, six compounds were identified as either LasR or LuxR agonists, with activities of >50% in LuxR and/or >20% in LasR (see Supplementary data for primary assay data.) To obtain more quantitative data about the activity of these hits, we performed dose–response assays in the three reporter strains and determined either IC_{50} or EC_{50} values for the 36 compounds. These values are listed in Tables 1 and 2, respectively. Here, we focus on the most active LuxR-type receptor antagonists and agonists identified in Library E for brevity.

The most active TraR antagonists in Library E were POHLs and PPHLs containing electron-withdrawing groups in the 4-position (Table 1), with POHLs representing the largest subset of actives.[13] 4-CF_3 PHHL **E35** and 4-NO_2 POHL **E24** were the most potent TraR antagonists overall, with IC_{50} values 2- to 4-fold lower than control PPHL **8** and control POHL **6** (~0.25 μM).[12] Notably, **E35** and **E24** were capable of inhibiting TraR by 50% at a ~1:1 ratio with the native ligand OOHL, and represent the most active TraR inhibitors that we have identified to date. The next three most active TraR antagonists in Library E were all 4-halo POHLs (**E20**–**E22**), and displayed increasing antagonistic activity as halogen size increased (Cl → Br → I). We previously found that 4-halo PHLs exhibit the same pattern for TraR antagonistic activity,[5] and interestingly this trend is continued in these one-atom-longer homologs. This activity trend was less apparent in the 4-halo PPHL series, however. In terms of TraR agonists, no compounds with appreciable agonistic activity were found in Library E (Table 2), again suggesting that this receptor has stringent ligand-binding requirements (*i.e.*, specific to OOHL) to adopt an active conformation.[5]

Similar to TraR, the most active LasR antagonists in Library E were POHLs and PPHLs (Table 1). However, PPHLs were the largest set of actives overall, with 3-I PPHL **E33** being the most active LasR antagonist in the library (IC_{50} = 1.8 μM). The four next most active compounds were, in contrast to **E33**, all 4-substituted derivatives: 4-Br and 4-I POHLs (**E21** and **E22**), and 4-Cl and 4-NO_2 PPHLs (**E30** and **E37**). The 4-halo POHLs exhibited the same activity trend in LasR as they did in TraR, with 4-I POHL **E22** being the most active antagonist in the series (IC_{50} = 2.0 μM) and 2-fold more active than control POHL **6**. Notably, POHL **E22** was also identified as a strong TraR antagonist (see above). The 4-halo PPHL series, however, did not exhibit a consistent antagonistic activity trend relative to the POHLs, similar to the data for TraR. Surprisingly, the PHLs in Library E failed to exhibit appreciable antagonistic activity against LasR. Indeed, the overall activities of the LasR antagonists identified in Library E were low relative to the controls, most notably PPHL **8** and PHL **4**. These data, along with those from an earlier study,[5a] suggest that the PHL, POHL, and PPHL structure classes contain more individual, potent LasR antagonists as opposed to families of highly active ligands (as seen for TraR above and LuxR below).

Turning next to LasR agonists, we identified four compounds in Library E that showed weak LasR agonistic activity, one PHL and 3 POHLs (Table 2). The 4-SCF_3 PHL **E8** was the most active agonist overall, capable of activating LasR to 65% the level of native ligand (OdDHL) at 300-fold higher concentration. Interestingly, PHL **E8** is also a weak *antagonist* of both TraR and LuxR (Table 1). To our knowledge, no PHL activator of LasR has been reported to date, so the identification of **E8**, despite its low activity, is noteworthy.

In contrast to the TraR and LasR screening data, the most active LuxR inhibitors identified in Library E were all PPHLs (Table 1). Further, the most active subset of these compounds had electron-withdrawing substituents in the *3-position*, as opposed to the 4-position (*e.g.*, **E31**, **E33**, **E36**, and **E38**). The three most potent PPHLs (3-I **E33**, 3-CF_3 **E36**, and 3-Br **E31**) all had lower IC_{50} values than that of control PHL **5** and control PPHL **8** (~0.47 vs 0.61 and 1.35 μM, respectively), and were capable of inhibiting LuxR by 50% at a ~6-fold lower concentration than native ligand (OHHL). These ligands are among the most potent LuxR antagonists that we have discovered to date. Of note, 3-I PPHL **E33** was also identified as the strongest LasR inhibitor in this study (see above), further highlighting the utility of PPHLs as a general class of LuxR-type receptor modulators. This activity trend for PPHL antagonists is intriguing, as we had previously observed that the one-carbon shorter homologs, that is, PHLs, exhibit higher LuxR inhibitory activity with substituents in the *4-position* as opposed to the 3-position (see above).[7] Indeed, the PHLs in Library E, the majority of which were 3-substituted, were all weaker LuxR antagonists relative to the 3-substituted PPHLs (see Table 1).

The 3-substituted PHLs did provide some exciting results in the LuxR *agonism* assays, however (Table 2). Here, two 3-substituted PHLs were uncovered as highly potent LuxR agonists (3-CN PHL **E5** and 3,5-CF_3 PHL **E9**), with EC_{50} values ~10-fold lower than that of the native ligand (OHHL). Notably, PHL **E9** has an EC_{50} value comparable to our previously reported LuxR activator, 3-NO_2 PHL **4**, yet does not activate to the same threshold level (70% vs ~100%).[8] (Intriguingly, the mono-substituted 3-CF_3 PHL **E3** failed to activate LuxR, and was a moderate antagonist instead; Table 1). Few synthetic LuxR-type receptor activators have been reported; thus, the discovery of LuxR agonists **E5** and **E9** in Library E is significant. In addition, this result serves to further refine our previous SAR data for LuxR activators to include PHLs with specific electron-withdrawing groups in the 3- or the 3- and 5-position.[8]

In summary, analysis of Library E has yielded a new set of synthetic LuxR-type receptor antagonists and agonists. The most active antagonists were largely POHLs or PPHLs with electron-withdrawing groups in the 4- or 3-position, respectively. Several antagonists had markedly improved activities relative to our initial lead compounds, most notably **E24**, **E33** and **E35** in TraR or LuxR. In addition, PHLs **E5** and **E9** were identified as strong activators

of LuxR. These results serve to further underscore the utility of screening focused AHL libraries for the optimization of existing and the identification of new LuxR-type receptor modulators. Ongoing work in our laboratory is focused on evaluating the Library E hits in phenotypic QS assays and will be reported in due course.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.089.

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12. Note, IC_{50} data for controls **1**–**8** were obtained at ~2-fold lower concentration of native ligand relative to Library E. This does not prevent the comparison of relative IC_{50} values in Library E, and indeed provides a more conservative interpretation of the activities of the hits relative to controls **1**–**8**.
13. We note that the dose–response curves for some of the antagonists in this study start to upturn at higher concentrations (~100 μM). Ongoing studies are focused on fully characterizing this phenomenon. However, we believe that these compounds can still be termed antagonists over a specific concentration range, and that this upturn pattern does not preclude our comparison of ligand activity within each receptor data set.