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N-Phenylacetanoyl-L-Homoserine Lactones Can Strongly Antagonize or Superagonize Quorum Sensing in *Vibrio fischeri*

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Abstract

Bacteria monitor their population densities using low-molecular-weight ligands in a process known as quorum sensing. At sufficient cell densities, bacteria can change their mode of growth and behave as multicellular communities that play critical roles in both beneficial symbioses and the pathogenesis of infectious disease. The development of non-native ligands that can block quorum-sensing signals has emerged as a promising new strategy to attenuate these divergent outcomes. Here, we report that *N*-phenylacetanoyl-L-homoserine lactones are capable of either inhibiting or, in some cases, strongly inducing quorum sensing in the bacterial symbiont *Vibrio fischeri*. Moreover, simple structural modifications to these ligands have remarkable effects on activity. These studies have revealed one of the first synthetic superagonists of quorum sensing, *N*-(3-nitro-phenylacetanoyl)-L-homoserine lactone. Together, these ligands represent a powerful new class of chemical probes with the potential to significantly expand the current understanding of quorum sensing and its role in host/bacteria interactions.

Bacteria can assess their local population densities using low-molecular- weight molecules (auto-inducers) and alter gene expression at high cell number to behave as a group. This process, termed quorum sensing, is widely used by bacteria to initiate group behaviors that have direct and often devastating impacts on human health and the environment (1,2). For example, numerous bacterial pathogens use quorum sensing to initiate infection (3,4). In contrast, symbiotic bacteria use these pathways to commence mutually beneficial relationships with their hosts (2,5,6). Because these important processes are controlled by chemical signals, there is intense and growing interest in the development of non-native ligands that can intercept these signals and attenuate or mimic quorum-sensing outcomes (7).

Quorum sensing is best characterized in Gram-negative proteobacteria, which use diffusible *N*-acylated-L-homoserine lactones (AHLs) and their cognate receptors (R proteins) for intercellular communication (Figure 1, panel a) (2, 8). Considerable research efforts have focused on the synthesis of ligands that can disrupt AHL-R protein binding and inhibit quorum sensing (9, 10), yet potent and general R protein antagonists remain scarce. Likewise, compounds exhibiting heightened activities relative to native AHLs (*i.e.*, superagonists of quorum sensing) are also of significant interest, because they could potentially initiate bacterial group behaviors at lower cell numbers than those required in natural environments. For example, superagonists could be used to determine whether beneficial symbioses could be initiated earlier during colonization by a symbiont or whether a pathogen could be forced to

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initiate infection too early and be cleared by a host's immune response. Such experiments, if possible, would help illuminate the relationships between bacterial group behavior and host responses. However, to our knowledge, only two superagonists of R protein activity have been reported to date, and these compounds have yet to be tested *in vivo* (11, 12).

New synthesis and design strategies are needed to expand the current set of quorum-sensing modulators active in Gram-negative bacteria. Unfortunately, the structures of known antagonists and agonists vary widely, and their mechanisms of action are unclear; thus, no obvious rationales have emerged for new ligand design. To address this problem, we have been engaged in the design of focused combinatorial libraries of ligands for the modulation of quorum sensing (13–15). Here, we report the discovery of a family of non-native AHLs capable of either inhibiting or, in some cases, strongly inducing quorum sensing in the marine symbiont *Vibrio fischeri.* In addition, we report the first superagonist of quorum sensing in *V. fischeri*. These ligands provide a new blueprint for the design of both quorum-sensing agonists and antagonists and represent powerful new chemical probes to investigate the mechanisms of bacterial communication.

V. fischeri colonizes the light-producing organs of certain marine fish and squids and uses quorum sensing to initiate bioluminescence at high cell densities (6,8). Quorum sensing is mediated in part by an AHL signal, *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL, **1**), and its cytoplasmic receptor, LuxR (Figure 1, panel a) (16). OHHL is synthesized by the LuxI protein at low basal levels, and high cell densities are required to achieve a sufficient concentration of OHHL for LuxR binding (≥100 nM *in vivo* (17)); thereafter, the OHHL–LuxR complex activates the transcription of luminescence genes and other genes involved in symbiosis and illuminates the fish or squid light organ. Quorum sensing in *V. fischeri* represents the best-characterized quorum-sensing signaling pathway to date (2); however, the role of quorum sensing in host–*V. fischeri* symbiosis is complex and remains poorly understood (6, 8,16). As a first step toward studying the interplay between quorum sensing and bacterial symbioses, we sought to identify non-native signal molecules that could intercept quorum sensing in *V. fischeri*.

Certain AHLs with non-native acyl chains have been reported to inhibit LuxR protein function in reporter gene assays. These antagonists include *N*-heptanoyl-L-homoserine lactone (**2**) reported by Eberhard *et al.* (18) and Schaefer *et al.* (19) and *N*-(4-phenylbutanoyl)-L-homoserine lactone (**3**) and *N*-pentylsulfonyl-L-homoserine lactone (**4**) reported by Castang *et al.* (20) (Figure 1, panel a). Recent studies in our laboratory have shown that *N*-(4-bromo-phenylacetanoyl)-L-homoserine lactone (4-bromo-PHL, **5**) is a potent antagonist of LuxR homologues in several other Gram-negative bacteria (Figure 1, panel a). For example, 4-bromo-PHL, **5**, inhibits R protein function in *Agrobacterium tumefaciens* at a 1:1 ratio against native AHL ligand, as determined by reporter gene assays (13). Because the putative ligand binding sites of the known R proteins have considerable sequence homology (70–80%) (8, 21), we hypothesized that PHLs might also modulate LuxR activity in *V. fischeri* and, if so, they represented a promising ligand class with which to initiate this study.

PHLs can be readily synthesized using a microwave-assisted, solid-phase route previously reported by our laboratory (Figure 1, panel b) (13). Using this method, we synthesized a small focused library of 24 PHLs to systematically examine the effects of different phenylacetanoyl moieties on ligand activity. This route gave PHL products **11a–x** (Table 1) in excellent purities (~95%), good isolated yields (>65%), and sufficient quantities (*i.e.*, 30 mg per compound) for multiple biological experiments.

Non-native ligands are commonly assessed for R protein agonism and antagonism using bacterial reporter strains (7,9,10). These strains lack AHL synthase genes, but retain their native

R genes. Exogenous AHL ligand is therefore required for R protein activation, which can be measured by reporter gene read-outs based on luminescence or fluorescence. The majority of synthetic LuxR modulators have been evaluated using the *luxR* plasmid pSB401 in various *Escherichia coli* strains (19,20). We therefore began our biological evaluation of PHL library **11** using the *E. coli* strain JM109 (pSB401) (22). Competitive inhibition assays were performed in the presence of both OHHL and PHLs **11**, while agonism assays were performed with PHLs alone. The known LuxR protein inhibitors **2–4** and 4-bromo-PHL **5** served as important controls for these studies (Figure 1, panel a). However, unacceptably large error values in the luminescence data (see Supplementary Figure 1) forced us to seek an alternative strain. We found that a Δ-*luxI* derivative of *V. fischeri* ES114 (16), in which the native *lux* operon behaves as the bioluminescent reporter, gave highly reproducible luminescence data in these assays. This nonstandard reporter strain was used for the subsequent primary antagonism and agonism assays in this study.

The antagonism assays in *V. fischeri* revealed several active PHL ligands and a number of striking structure–activity relationships (SARs) (Table 1). First, the control compound 4-bromo-PHL (**5**) showed 79% inhibition at a 1:1 ratio with native ligand **1** (Table 1, entry 5; both ligands at 5 μM). This result supported our hypothesis that PHLs could modulate LuxR function. Indeed, 50% of the PHL library **11** exhibited ≥50% inhibition in this assay. Inhibitory activity was extremely dependent on the substituents and their locations on the phenylacetanoyl group. For example, replacement of the 4-bromo substituent with a hydrogen in PHL **11a** abolished inhibitory activity (Table 1, entry 6). PHLs with bromo (**5, 11a,b**), chloro (**11g–i**), and iodo substituents (**11j–l**) exhibited a ~10% increase in antagonism as the halogen was moved from the 2- to the 3- to the 4-position on the phenyl ring. Antagonistic activity also increased slightly with increasing halogen size, with 4-iodo-PHL (**11j**) exhibiting the highest activity (Table 1, entry 15; 85%) for the halogen series.

In general, sterically large and lipophilic groups in the 4-position enhanced PHL (**11**) antagonism in *V. fischeri* (Δ-*luxI*). This is exemplified by the high activities of 4-phenyl-PHL (**11q**) and 4-trifluoromethyl-PHL (**11s**) (~80% inhibition; Table 1). In contrast, hydrogen-bond-donating substituents in the 4-position engendered the lowest inhibitory activities (*i.e.*, 4-amino-PHL, **11v**, and 4-hydroxy-PHL, **11w**). The nitro-PHL series (**11m–o**), however, showed a more complicated activity trend, with 3-nitro-PHL (**11n**) showing no inhibitory activity relative to the 2- and 4-nitro-PHLs (entries 18–20; see below). We determined IC_{50} values for the most potent PHL inhibitors identified in this assay, along with the most potent controls (**2** and **5**) for comparison. The 4-iodo-PHL (**11j**) and 4-trifluoromethyl-PHL (**11s**) exhibited the lowest IC_{50} values in this study, with PHL **11s** at least two-fold more active than control compound **2** (0.6 μM *vs* 1.4 μM, respectively; Figure 2, panel a).

Similar assays were performed on PHL library **11** to screen for agonistic activity in *V. fischeri* (Δ-*luxI*). Again, we observed striking trends in the activities for PHLs with halogen and nitro groups (at 200 μM compound; Table 1). In contrast to the antagonism data, the 3-substituted compound in each of these PHL families showed the strongest activity relative to the 2- and 4-substituted derivatives, with the 3-bromo-(**11b**), 3-chloro- (**11h**), and 3-nitro-PHLs (**11n**) exhibiting at least 60% luminescence induction relative to native OHHL at the same concentration. Remarkably, simply shifting substituents on the PHL phenyl ring by a single carbon converted these ligands from LuxR antagonists to LuxR agonists. Moreover, 3-nitro-PHL (**11n**) was able to induce 29% higher luminescence than OHHL in this primary assay (Table 1, entry 19). This result was extraordinary and explained the unusual inhibition trends for the nitro-PHL series (**11m–o**; see above).

For a more quantitative comparison of ligand activity, we determined EC_{50} values for our most active PHL agonists (**11b, 11h**, and **11n**) and OHHL in *V. fischeri* (Δ-*luxI*) (Figure 2, panels

b and c). These studies identified 3-nitro-PHL (**11n**) as the most active LuxR agonist, exhibiting a 10-fold lower EC_{50} than OHHL (0.3 μM *vs* 3 μM, respectively). We performed analogous dose–response studies with **11n** and OHHL in wild-type *V. fischeri* ES114 (23) and observed similarly heightened activity for PHL **11n** relative to OHHL (Figure 2, panel d). The superagonistic activity of PHL **11n** in *V. fischeri* relative to OHHL could be easily visualized by luminescence imaging with a CCD camera; Figure 3 shows a representative image of these experiments. We also examined the activity of **11n** and OHHL in a Δ-*luxR* derivative of *V. fischeri* ES114. Neither **11n** nor OHHL induced any detectable luminescence in this strain (Figure 2, panel d), which suggests that **11n**, like the native ligand OHHL, exerts its activity through the LuxR protein (see below). The discovery of compound **11n** is significant, because it is one of the first synthetic superagonists of quorum sensing reported (11, 12) and to our knowledge the first superagonist in *V. fischeri*.

In view of the structural similarity of PHLs **11** to native AHLs and the assay data and subtle SAR described above, we hypothesize that these ligands target the LuxR ligand-binding site and inhibition or activation is based on the specific binding mode and thus resulting affinity of the ligand. Further, we do not believe that these changes in activity simply reflect the different *chemical* properties of the PHLs (**11**) (10). This view is supported by several observations. First, the percentage of PHL **11** lactone hydrolysis (which abolishes activity for native AHLs (24)) is minimal and identical to that of OHHL over the time course of these luminescence assays. Second, the functionalities on PHLs **11** are unreactive under the assay conditions tested (see Methods). Third, higher lipophilicity within the PHL series, and therefore higher potential cell permeability, does not correlate with enhanced antagonistic or agonistic activity (Table 1). This is further exemplified by the D-enantiomers of control antagonists **3** and **5**, which have identical lipophilicities as **3** and **5** yet exhibit markedly reduced activities (~20% inhibition; see Supplementary Figure 6). We have performed molecular modeling studies of several PHLs and OHHL docked into the putative ligand binding site of LuxR (built *in silico* from the one known structure of a complete R protein, TraR (20,21,25,26); see Supplementary Figure 7–Supplementary Figure 13) to further test this hypothesis. The results of these studies suggest that the LuxR binding site can readily accommodate PHLs yet is better able to accommodate 3-substitutued PHLs (agonists) relative to 4-substitutued PHLs (antagonists) and that activation or inhibition of LuxR may depend on the balance of favorable hydrogen bonding and unfavorable steric interactions within the binding pocket. While additional biochemical and structural studies will be required to fully elucidate PHL function in *V. fischeri*, these initial calculations provide support that PHLs **11** target LuxR.

In summary, we have discovered that PHLs **11** elicit remarkable and varied quorum-sensing responses in the bacterial symbiont *V. fischeri*. This family of ligands includes some of the most active antagonists and agonists of Gram-negative bacterial quorum sensing reported to date. One significant outcome of this work is the observation that subtle alteration of substituents and their placement on the phenylacetanoyl moiety dramatically influence ligand activity. These changes do not simply abolish activity but rather convert potent antagonists into agonists. A second major outcome of this investigation is the discovery of the first synthetic superagonist of quorum sensing in *V. fischeri*, PHL **11n**. This ligand displays 10-fold higher activity relative to native autoinducer OHHL and is one of the first known superagonists of quorum sensing in Gram-negative bacteria (11,12). Collectively, PHLs represent a new and valuable set of chemical tools for the study of quorum sensing in *V. fischeri* and could, with further development, provide broad insights into the roles of quorum sensing in bacterial symbioses. Preliminary experiments indicate superagonist **11n** is well tolerated by the main symbiotic partner of *V. fischeri*, the *Euprymna scolopes* squid (6), and is active *in vivo*; these studies will be reported in due course.

METHODS

Ligand Synthesis

PHL library **11**, OHHL (**1**), and control compounds **2**, **3**, and **5** were prepared using reported methods (13) (Figure 1, panel b), except the final cyclization–cleavage step was performed at RT for 24 h. Control **4** was synthesized in an analogous fashion except 4-dimethylaminopyridine and 1-pentanesulfonyl chloride replaced reagents i and **9** (Figure 1, panel b). D-AHLs were accessible using *N*-Fmoc-D-methionine in place of **7**. Compounds were submitted to biological assays following cleavage and an aqueous work-up without further purification. See Supporting Information for compound characterization data.

Compound Handling and Reagents

Stock solutions of synthetic compounds (10 mM) were prepared in DMSO. The structures of PHLs **11** and the control compounds remained unchanged after prolonged storage in DMSO (~4–6 months) and over the course of the biological assays (as determined by GC/MS, NMR, or both). Nitro-PHL derivatives (**11m–o**) were stored in the dark and not observed to degrade over the course of these studies. All biological reagents were purchased from Fisher Scientific. Luria–Bertani (LB) and LB salt media (LBS) were prepared as instructed with pH = 7.5 (LBS contained an additional 1.5% (w/v) NaCl, 0.3% (w/v) glycerol, and 50 nM Tris-HCl (16)).

Bacterial Strains

The *E. coli* strain used for these studies was JM109 (pSB401) (22). The *V. fischeri* strains were ES114 (23), ES114 (Δ-*luxI*), and ES114 (Δ-*luxR*) (16).

Luminescence Assays

For agonism assays, an appropriate amount of AHL stock solution was added into a 96-well plate. An overnight culture of *E. coli* or *V. fischeri* was diluted 1:10 with appropriate media (LB plus 10 μg mL^{-1} tetracycline for *E. coli*; LBS for *V. fischeri*). A 200-μL portion of the diluted culture was added to each well of the plate. Plates were grown for 4–8 h with shaking (200 rpm; 30 °C for *E. coli* and RT for *V. fischeri*). Luminescence was measured using a multilabel plate reader and normalized to cell density. Antagonism screens were performed in an analogous manner against OHHL at its approximate EC_{50} value (10 nM in *E. coli*; 5 μM in *V. fischeri*). Similar methods were used for dose–response assays, except the concentrations of PHL (**11**) or control varied between 2×10^{-2} and 2×10^{5} nM. None of the PHLs (**11**) or control compounds in this study inhibited bacterial growth or displayed insolubility over the range of concentrations tested in the luminescence assays. All assays were performed in triplicate. Graphpad Prism software (version 5.0) was used to calculate IC_{50} and EC_{50} values. See Supporting Information for detailed assay protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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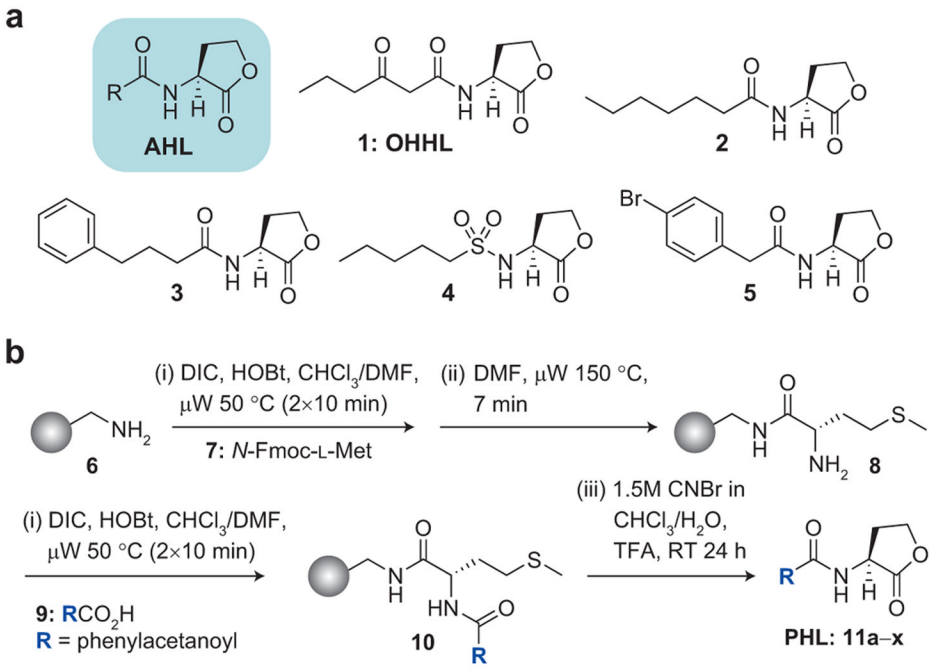


Figure 1. Ligand structures and synthesis

a) Structures of a generic AHL, OHHL (1) (the natural autoinducer ligand of *V. fischeri*), and selected known synthetic inhibitors of LuxR or other R protein function (2–5). b) Solid-phase synthetic route to PHL library 11. DIC = *N*,*N*′-diisopropyl-carbodiimide. HOBt = *N*-hydroxybenzotriazole. μW = temperature-controlled microwave irradiation.

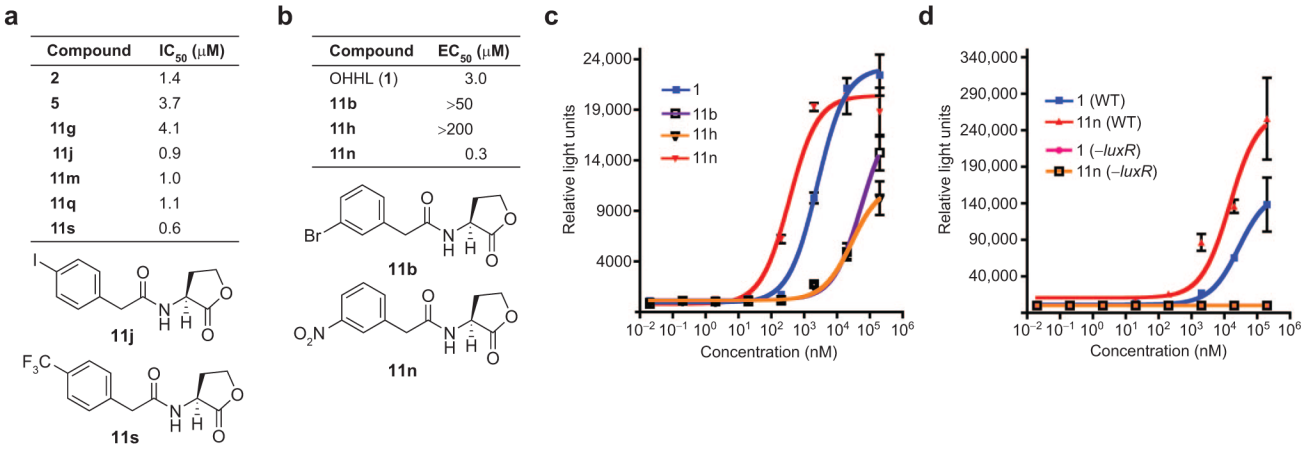


Figure 2. New synthetic modulators of LuxR

a) IC_{50} table of most potent inhibitors and structures of selected compounds. b) EC_{50} table of most potent activators and structures of selected compounds. See Supporting Information for dose–response curves. c) Agonism dose–response curves for OHHL (1) and synthetic ligands 11b, 11h, and 11n in *V. fischeri* ES114 (Δ-*luxI*). d) Agonism dose–response curves for OHHL (1) and 11n in wildtype (WT) *V. fischeri* ES114 and *V. fischeri* ES114 (Δ-*luxR*). (Note, we were unable to achieve a maximum level of luminescence over the concentrations tested and therefore could not determine accurate EC_{50} values in WT *V. fischeri*. Experiments at higher concentrations were precluded by the diminished solubilities of both OHHL (1) and PHL 11n at concentrations >500 μM.) Luminescence measured in relative light units. Error bars, standard deviation of the means of triplicate samples.

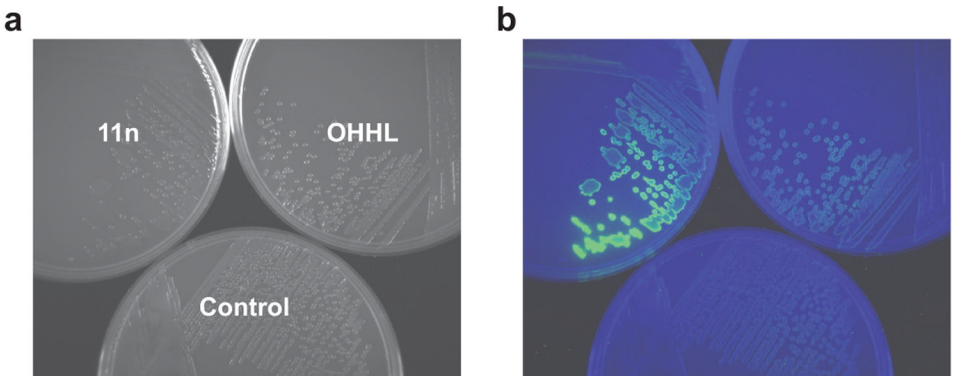
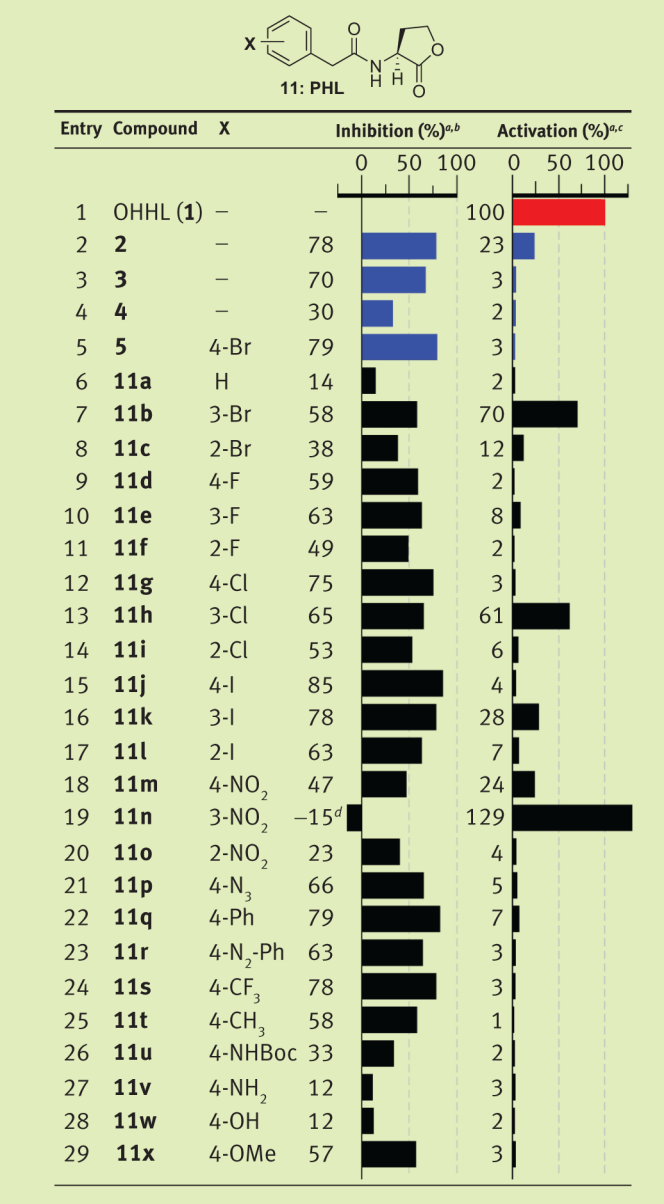


Figure 3. Bioluminescence induction by OHHL and quorum-sensing superagonist 11n in *V. fischeri*

a) *V. fischeri* ES114 (Δ-*luxI*) streaked onto agar plates containing either no compound (control), 0.5 μM OHHL (1), or 0.5 μM superagonist 11n and grown for 8 h and imaged. b) Identical plates from panel a imaged for luminescence using a CCD imager.

TABLE 1

Structures and primary antagonism and agonism screening data for PHL library 11 in *V. fischeri* (Δ-*luxI*)



[a] Strain: *V. fischeri* ES114 (Δ-*luxI*). Luminescence data measured in relative light units and normalized to OHHL (**1**, red). Control compounds shown in blue. All assays performed in triplicate; error did not exceed ±10% of the mean.

[b] Screen performed using 5 μM synthetic ligand against 5 μM OHHL (**1**).

[c] Screen performed using 200 μM ligand.

[d] PHL **11n** displayed 115% activation in this assay.