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New and Unexpected Insights into the Modulation of LuxR-type Quorum Sensing by Cyclic Dipeptides

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Abstract

Quorum sensing (QS) is under the control of *N*-acylated L-homoserine lactones (AHLs) and their cognate receptors (LuxR-type proteins) in Gram-negative bacteria, and plays a major role in mediating host-bacteria interactions by these species. Certain cyclic dipeptides (2,5-diketopiperazines, DKPs) have been isolated from bacteria and reported to activate or inhibit LuxR-type proteins in AHL biosensor strains, albeit at significantly higher concentrations than native lactones. These reports have prompted the proposal that DKPs represent a new class of QS signals, and potentially even interspecies or interkingdom signals; their mechanisms of action and physiological relevance, however, remain unknown. Here, we describe a library of synthetic DKPs that was designed to (1) determine the structural features necessary for LuxR-type protein activation and inhibition, and (2) probe their mechanisms of action. These DKPs, along with several previously reported natural DKPs, were screened in bacterial reporter gene assays. In contrast to previous reports, the native DKPs failed to exhibit either antagonistic or agonistic activities in these assays. However, non-natural halogenated *cyclo*(L-Pro–L-Phe) derivatives were capable of inhibiting luminescence in *V. fischeri*. Interestingly, additional experiments revealed that these DKPs do not compete with the natural lactone signal, OHHL, to inhibit luminescence. Together, these data suggest that DKPs are not QS signals in the bacteria examined in this study. Although these compounds can influence QS-regulated outcomes, we contend that they do not do so through direct interaction with LuxR-type proteins. This work serves to refine the lexicon of naturally occurring QS signals used by Gram-negative bacteria.

INTRODUCTION

Quorum sensing (QS) has emerged as a prevalent cell-cell signaling pathway in chemical ecology. This population density sensing mechanism is used broadly by bacteria and is governed by a chemical "language" of small, diffusible signal molecules (or autoinducers) and their associated protein receptors (1–3). QS in Gram-negative bacteria is under the control of diffusible *N*-acylated L-homoserine lactone signals (AHLs) and the LuxR-type family of cytoplasmic receptors, and was first characterized in the luminescent marine symbiont *Vibrio fischeri* (3–6). The AHL ligands are most frequently generated by LuxI-type synthases, and their local concentration correlates with cell density (along with other environmental factors (7)). Above a threshold concentration, the AHLs bind to their cognate LuxR-type receptors, and these complexes most frequently activate the transcription of target genes required for bacterial group behavior. As many of these processes play crucial roles in both pathogenesis and symbiosis, there is significant interest in the development of non-native ligands that can

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Supporting Information Available: Full characterization data for DKPs **5**–**10** and **13**–**16**, details of all bacterial reporter strains tested, assay procedures, and assay data. This material is available free of charge *via* the Internet.

block or mimic native autoinducer signals and attenuate QS (8,9). Such molecules would represent new tools to study the molecular mechanisms of QS and their roles in host-bacteria interactions (10,11).

The structures of native AHLs from selected Gram-negative species are shown in Figure 1 (panel a): *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL, **1**) that binds LuxR in *V. fischeri* and represents the canonical quorum sensing circuit, *N*-(3-oxo-octanoyl)-L-homoserine lactone (OOHL, **2**) that binds TraR in the plant pathogen *Agrobacterium tumefaciens*, and *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL, **3**) that binds both LasR and QscR in the opportunistic pathogen *Pseudomonas aeruginosa*. Interception of AHL:LuxR-type protein binding by synthetic ligands represents one strategy to control QS in Gram-negative bacteria, and considerable research efforts over the past 20 years have been directed in this area (12). The majority of this past work has focused on the design and synthesis of non-native AHL derivatives (5,13,14), and our laboratory has recently made contributions to this area for LuxR, TraR, LasR, and QscR based QS (*e.g.*, the LuxR-type protein inhibitor 4-iodo phenylacetyl HL (4-I-PHL, **4**); Figure 1, panel a) (15–20).

Looking beyond the AHL scaffold would certainly expand the range of possible ligands for use as LuxR-type protein modulators, and several research groups have reported strategies towards this end, including the screening of commercial small molecules libraries (21) and natural product isolates (22,23), and computational pharmacophore modeling (24,25). Recently, a set of cyclic dipeptides (2,5-diketopiperazines, or DKPs) were isolated from a range of Gram-negative bacteria and reported to modulate LuxR, TraR, or LasR activity in sensitive AHL "biosensor" strains previously considered specific for AHLs (DKPs **5**–**12**;Figure 1, panel b) (26–28). These DKPs have been isolated either individually or as mixtures from culture supernatants of *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. alcaligenes*, *Proteus mirabilis*, *Enterrobacter agglomerans*, *Vibrio vulnificus*, and *Citrobacter freundii*. As these compounds appear to be common to a broad number of bacteria, they have been suggested to represent both a new class of naturally occurring QS signals, and potential interspecies signals (4,6,[8],[10],29–31). Further, as DKPs are common in fungi and elicit a range of effects in higher animals and plants (32), these compounds have also been proposed to play roles in interkingdom signaling. Questions remain with regards to their actual physiological function in bacteria, however, as DKPs elicit their activities against LuxR-proteins at significantly higher concentrations than AHLs (up to 10^6 times higher). To date, a critical examination of the DKP structure class as QS modulators is yet to be reported, and would provide insights into their role as putative cell-cell and cell-host signals (33).

Here, we report the design and synthesis of a library of non-native DKPs, an evaluation of these compounds as antagonists and agonists of LuxR, TraR, and LasR, and a comparison of their activities to the naturally occurring DKPs **5**–**10**. Surprisingly, the previously reported DKPs failed to exhibit either antagonistic or agonistic activities in the reporter strains examined in this study. However, several non-native DKPs were identified that are capable of inhibiting (but not activating) luminescence in *V. fischeri*. Interestingly, these compounds exhibited structural features reminiscent of both the naturally occurring DKPs **8** and **9** and several of our recently reported, non-native AHL antagonists of LuxR, TraR, and LasR (15–18). Additional experiments indicated that luminescence inhibition by these non-native DKPs *does not* occur through LuxR in *V. fischeri*. Collectively, these data suggest that DKPs can influence QS regulated outcomes, yet may not do so through direct interactions with LuxR-type proteins. These findings have important implications for the alleged role of DKPs in bacterial QS (34).

RESULTS AND DISCUSSION

DKP Library Design

We designed a focused library of 23 DKPs around the structures of native DKPs **5**–**10** (Figure 1, panel b), as these six DKPs had the most closely related structures of the eight (*i.e.*, each contained L-Pro). We maintained the Pro unit in each library member, and then systematically altered the other amino acid side chain and stereochemistry at each chiral center to generate sub-libraries of *cyclo*(Pro-Xxx) **13**–**16** (shown in Figure 2). Sub-library **13** represented the simplest analogs, containing only one chiral center (L-Pro). DKPs **14** were derived from L-amino acids and designed to display various aliphatic and aromatic functionality (both natural and unnatural) on their non-Pro side chains. We included halogenated- and nitro-Phe derivatives because we have found that AHLs containing halogenated- and nitro-phenylacetyl groups (PHLs) exhibited both strong antagonistic and agonistic activity against TraR, LasR, and LuxR (*e.g.*, 4-I-PHL **4**) (15–18,20). Structures **15** were stereoisomers of control DKPs **6**–**10**, containing L-Pro and D-amino acids. Lastly, DKPs **16** were enantiomers of sub-library **15**, containing D-Pro and L-amino acids.

DKP Synthesis and Purification

We developed an efficient solution-phase synthetic route to DKPs **5**–**10** and sub-libraries **13**–**16** (Scheme 1). In brief, L- or D-Pro-OMe was coupled to *N*-Boc-protected amino acids using standard carbodiimide-mediated conditions. After cleavage of the *N*-Boc group under acidic conditions, intramolecular cyclization proceeded smoothly at room temperature upon treatment with piperidine to generate DKPs in sufficient yields for biological evaluation (38% average overall yield; 60–750 mg scale). All of the DKPs were purified to homogeneity by silica gel column chromatography (>97% purity, 95:5 d.r.; Supplementary Table 1).

Note, we did not utilize routine reverse-phase HPLC methods for DKP purification, as our HPLC instruments are utilized for the analysis of AHLs in related research (15–20), and we sought to minimize the risk of contamination by these highly active compounds. Such contamination, in part due to the similar physicochemical properties of DKPs and AHLs, has impacted research in this area previously. For example, Degrassi *et al*. fractionated *P. putida* WCS358 supernatant extracts using HPLC, tested each fraction against a number of AHL biosensor strains, and isolated DKPs **6**, **8**, **9**, and **12** as the major products in each active fraction (Figure 1, panel b) (27). However, upon re-synthesis of these four DKPs, they found that the synthetic DKPs failed to activate the biosensor strain used for their initial detection, although they did activate other reporter strains. The researchers attributed the initial activities to AHLs that co-purified with their DKPs. For these reasons, we took precautions throughout this study to ensure the purity of our synthetic DKPs.

Primary Screening Methods

We examined the abilities of DKP controls **5**–**10** and sub-libraries **13**–**16** to modulate TraR, LasR, and LuxR activity in the same biosensor strains used in previous DKP reports (26–28) to allow for direct comparisons. We note that these biosensor strains can produce varying levels of LuxR-type protein (most frequently, substantially higher than native levels), and this can directly affect their sensitivity for exogenous ligands (*i.e.*, higher protein levels correlate with higher sensitivity (35)). We sought to obtain data using biosensor strains with both high *and* native protein levels for each protein in this study to increase the stringency of the assays. Therefore, we also investigated DKPs **5**–**10** and **13**–**16** in bacterial reporter strains containing native LuxR-type protein levels (34). All of these strains lack AHL synthases, but contain LuxR-type proteins and cognate promoter sequences that control reporter gene expression. As a result, LuxR-type protein activity, and consequently exogenous ligand activity, can be measured using standard reporter gene read-outs. These assays can be performed in solution

in multititer plates where activity is assessed using a plate reader, or the compounds can be overlaid with bacteria in warm agar and, following incubation, colorimetric reagents allow for visualization of activity.

All of the primary antagonism and agonism assays performed in liquid culture were tested at 500 μM DKP concentrations. Competitive antagonism assays were performed with DKP in the presence of native AHL ligand at its EC_{50}, while agonism assays were performed with DKP alone. Negative controls reported the activity of media and DMSO only.

Screening of DKP Libraries against TraR

We began our investigation of DKPs **5**–**10** and **13**–**16** by overlaying them with a TraR overproducing strain (*A. tumefaciens* NTL4 (pZLR4), a second-generation strain of NT1 (pDCI41E33) (36)) analogous to that described by Holden *et al*. and Degrassi *et al*. (26,27, 37). Although we did not observe activation from *cyclo*(L-Leu-L-Pro) (**6**) as previously reported by both groups, its isomer *cyclo*(L-Ile-L-Pro) (**14a**) was active in this overlay assay, along with DKPs **13a** and **15b** (see Supplementary Figure 2). In contrast to both previous reports, DKP **9** did not activate TraR in our hands. We next sought to test the activity of the DKPs in a β-galactosidase reporter strain that produces TraR at native levels (*A. tumefaciens* WCF47 (pCF372) (38)). In this liquid culture assay, β-galactosidase, and therefore TraR, activity was measured using routine Miller absorbance assays (15,17,18). Neither the control DKPs **5**–**10** nor the DKP sub-libraries **13**–**16** were observed to activate TraR in this strain beyond the level of the negative control (Supplementary Figure 3). In turn, we did not observe inhibitory activity for any of the DKPs (**5**–**10** and **13**–**16**) against OOHL (**2**, at 100 nM) in competitive antagonism assays in this *A. tumefaciens* strain.

Screening of DKP Libraries against LasR

We next screened our DKP controls **5**–**10** and libraries **13**–**16** for agonistic activity against LasR in the heterologous *E. coli* biosensor strain previously used to examine DKP activity (pSB1075) (39). Controls **5**–**10** were inactive in this LasR overproducing strain. This result for control *cyclo*(L-Met-L-Pro) (**7**) contrasted with that of Holden *et al*., who reported that **7** could activate LasR (albeit at concentrations above 1 mM (26)). We did, however, observe partial activation (20%) of this strain with DKPs **14a**, **14c**, and **14h** at 500 μM (Supplementary Figure 4). We sought to compare these data with reporter strain *E. coli* (NH5α (pJN105L pSC11)) (40). This strain contains LasR under the control of an inducible promoter and reports LasR activity *via* β-galactosidase production; LasR protein levels were induced to approximately native levels for *P. aeruginosa* with arabinose (17). Similar to the TraR assay data above, neither the control DKPs **5**–**10** nor the DKP sub-libraries **13**–**16** were capable of activating or inhibiting LasR (*vs.* 7.5 nM OdDHL, **3**) in this native protein level reporter strain (see Supplementary Figure 5).

Screening of DKP Libraries against LuxR

We evaluated the activities of the DKPs against LuxR using the *E. coli* JM109 (pSB401) biosensor (34,39). This heterologous strain contains *luxR* and the *luxI* promoter from *V. fischeri* MJ-1, and the lux operon (*luxCDABE*) from *Photorhabdus luminescens*; LuxR is overproduced and protein activity is reported as luminescence (16). Control DKPs **5**–**10** failed to activate this strain, but **5** and **9** were able to inhibit luminescence by 20% (*vs*. 20 nM OHHL, Supplementary Figure 6). These data conflict with previous reports of controls **7**–**9** activating LuxR and all six controls **5**–**10** inhibiting LuxR in this same strain (26–28). However, several non-native DKPs from sub-libraries **13**–**16** were able to weakly inhibit (but not significantly activate) luminescence in this strain, most notably **14e**, **14f**, and **15f** (~25% inhibition, Supplementary Figure 6).

We next examined control DKPs **5**–**10** and sub-libraries **13**–**16** for LuxR agonistic and antagonistic activities (*vs.* 3 μM OHHL, **1**) in a Δ*luxI* derivative of *V. fischeri* ES114 (41,42). In this strain, LuxR is produced at native levels, and the native *V. fischeri* lux operon behaves as the luminescent reporter (43). Although we identified no LuxR agonists in these screens (Supplementary Figure 7), several non-native DKPs were antagonists at a 100:1 ratio relative to OHHL (**1**). Compounds **14a**, **14e**, **14i**, and **16c** gave moderate inhibitory activities (33–40%, Figures 2 and 3). More notably, *cyclo*(L-Pro-L-4-Cl-Phe) **14b** (the "natural" diastereomer of **16c**) and *cyclo*(L-Pro-L-4-I-Phe) **14c** were capable of inhibiting luminescence by 76% and 95% at 500 μM, respectively. These active DKPs (**14b** and **14c**) exhibit (L, L) stereochemistry and structural features reminiscent of the naturally occurring control DKPs **8** and **9**, yet contain non-native side chains. Interestingly, DKP **14c** shares the 4-iodo phenyl motif with our previously reported, AHL-derived LuxR inhibitor, 4-I-PHL (**4**; Figure 1, panel b).

Dose Response Analyses of Active DKPs

We performed dose response analyses on DKPs **14b** and **14c** against 5 μM OHHL (**1**) in *V. fischeri* ES114 (Δ–*luxI*), and obtained IC_{50} values of 208 μM and 116 μM for luminescence inhibition, respectively (Figure 4, panels a and b). These IC_{50} values are 2–3 orders of magnitude higher than those previously reported for AHL-derived LuxR inhibitors (such as **4**) (16,17).

To obtain further insight into the mechanism of luminescence inhibition by non-native DKPs, we performed competitive dose response analyses of the native ligand OHHL (**1**) in the presence of increasing amounts of the most active DKP, **14c**, in *V. fischeri* ES114 (Δ–*luxI*). Such Schild analyses can reveal whether a compound behaves *via* a competitive or non-competitive antagonism mechanism, either directly interfering with the binding of an agonist or not, respectively (44). In earlier work, Holden *et al*. had presumed that DKPs bind at, or near, the OHHL (**1**) binding site on LuxR because their activators displayed weak competitive inhibition against OHHL (26). However, competitive dose response experiments with **14c** showed that this compound reduces the maximal luminescence induction level of OHHL (**1**) *without* affecting the EC_{50} value of OHHL (**1**) (Figure 4, panel c), suggesting that **14c** does not directly compete with OHHL (**1**) for LuxR binding. In addition, this experiment indicates that DKP **14c** does not affect production of LuxR in *V. fischeri*, as this outcome would also alter the EC_{50} for OHHL (**1**) at different concentrations of **14c**. Later experiments revealed that the luminescence inhibitory activity of DKP **14c** is also reversible in *V. fischeri* ES114 (Δ–*luxI*), as luminescence could be induced by adding fresh OHHL (**1**) after washing cells that had been treated with DKP **14c** (data not shown).

In order to further probe the mechanism of action of DKP **14c**, we performed dose response analyses in several additional mutant strains of *V. fischeri*. These strains lacked either proteins that have been implicated in DKP activity in other *Vibrio* spp. (OmpU and ToxR) (28,31,45, 46), or proteins that have been recently reported to regulate luminescence in *V. fischeri* (CheV, FlrC, and YehT) (47,48). We found, however, that the luminescence inhibitory activity of **14c** was not affected by the loss of these proteins, indicating that this DKP does not directly interact with them (see ref. 45 and 48 for additional details).

Modulation of Luciferase Activity by DKPs

We next turned our focus toward the luciferase enzyme itself. If DKP **14c** interfered with luciferase production in *V. fischeri*, or if it directly inhibited the enzyme, we would expect to observe a reduction in luminescence. To test these possibilities, we measured the levels of active luciferase in cells cultured with and without **14c**. We used our previously reported synthetic LuxR inhibitor 4-I-PHL (**4**) as a control in these experiments (16). We pre-cultured *V. fischeri* ES114 (Δ–*luxI*) in the presence of 3 μM OHHL, DMSO alone, 3 μM OHHL (**1**)

with 1 mM **14c**, or 3 μM OHHL (**1**) with 10 μM 4-I-PHL (**4**) to the same optical density to normalize the number of cells in the culture (~0.4; **14c** and 4-I-PHL were added to the pre-culture at 10x their IC_{50} values). We then pelleted the cells and tested luminescence levels according to the procedure described by Lei and Becvar (49). Briefly, this procedure utilizes an excess of the reactants (decanal and flavin mononucleotide ($FMNH_2$)) to ensure that luciferase is the rate-limiting component in the reaction, and the initial maximum intensity of light emission is proportional to luciferase activity (see Supporting Information for details). The results from this assay are shown in Figure 5. As expected, we observed that OHHL (**1**) is required for luciferase production during the pre-culture (luciferase output (LO) = 72.4 ± 7.6). Addition of 4-I-PHL (**4**) fully inhibited the luminescence of the pre-cultured cells and also decreased the amount of active luciferase produced during the pre-culture (LO = 11.0 ±1.0). When added directly to the enzymatic assay reaction, 4-I-PHL (**4**) had no effect on the levels of luciferase activity (LO = 73.0±7.0). Similarly, the amount of active luciferase was reduced by >80% when the cells were pre-cultured with DKP **14c** (LO = 13.2±4.4). However, in contrast to 4-I-PHL (**4**), **14c** affected the activity of luciferase in the enzymatic assay when added directly to the enzymatic assay buffer (LO = 24.4±1.4), although not to the same degree as in pre-cultured cells. We were unable to test DKP **14c** at higher concentrations in this assay, as the compound is not soluble in the absence of DMSO and greater than one percent DMSO in the assay buffer (v/v) greatly affected luciferase activity (LO ~ 49 ± 6). Investigations are ongoing to further characterize the effects of **14c** on luciferase in *V. fischeri*. Nevertheless, these data suggest that DKP **14c** affects the levels of active luciferase in *V. fischeri*, potentially by inhibiting the enzymatic activity of luciferase itself.

Conclusions and Outlook

Few molecules other than AHLs are known to modulate QS in Gram-negative bacteria, and DKPs have attracted considerable interest as a potential new class of QS signals, and even interspecies or interkingdom signals (50). There have been no reports investigating either their mechanism or the structural features of these molecules that are required for activity. We designed and synthesized focused libraries of non-native DKPs (**13**–**16**) based on the structures of naturally occurring DKPs **5**–**10**, and examined their abilities to agonize and antagonize well-characterized LuxR-type proteins using both sensitive biosensor strains and reporter strains with native protein levels. The results of these assays suggest, in contrast to previous reports, that DKPs do not interact with LuxR-type proteins to affect QS. DKPs **5**–**10** and all of the DKPs in libraries **13**–**16** derived from natural α-amino acids failed to activate or inhibit the native protein level QS reporter strains utilized in this study based on TraR, LasR, and LuxR. The only DKPs capable of significantly modulating LuxR QS outputs (*i.e.*, luminescence) were derived from non-natural amino acids, DKPs **14b** and **14c**. Further experiments showed that these DKPs do not compete with the natural ligand OHHL (**1**) for LuxR. However, DKP **14c** is able to reduce the level of active luciferase in *V. fischeri* when pre-cultured in the presence of OHHL (**1**). As natural DKPs **5**–**12** were discovered largely using AHL biosensors in which the LuxR-type proteins are overproduced, our results also highlight the need to use caution when interpreting such biosensor data for the identification of small molecule LuxR-type protein modulators.

Therefore, the question remains: Do DKPs function in nature as true QS signal molecules? Considerable further experimentation is required to provide a conclusive answer to this question, but the data outlined herein challenge the hypothesis that they do so through interaction with LuxR-type proteins in Gram-negative bacteria. It is important to note that the DKPs in this study were only active in *V. fischeri*, and that naturally occurring DKPs, while present in other *Vibrio* species, have yet to be reported in isolates from this bacterium. Nonetheless, the present work has provided evidence that DKPs do not act as LuxR-mediated

QS signals, and further underscores the value of synthetic chemistry to probe the language of bacterial QS.

METHODS

Small Molecule Synthesis

All chemical reagents were purchased from commercial sources (Sigma Aldrich, EMD Biosciences, Advanced ChemTech) and used without further purification. Solvents were purchased from commercial sources (Fisher Scientific, Mallinckrodt Baker) and used as obtained, with the exception of dichloromethane (CH_2Cl_2), which was distilled over calcium hydride prior to use. AHL controls **1**–**3** and 4-I- PHL (**4**) were synthesized according to our previously reported procedure (15–18).

DKP controls **5**–**10** and sub-libraries **13**–**16** were synthesized as follows: *N*-Boc-α-amino acids were coupled to H-Pro-OMe with 1 equiv. EDC and 1 equiv. Et_3N in CH_2Cl_2 at 4 °C overnight (51). The resulting dipeptides were dissolved in a minimal amount of CH_2Cl_2 and cooled to 0 °C. A solution of AcCl (~5 equiv.) in MeOH (1:2.4 v/v) was added drop-wise to affect amine deprotection, and the reaction was stirred for 3 h. The solvent was removed *in vacuo*, and the deprotected dipeptides were dissolved in a minimal amount of DMF. Approximately 2 equiv. of piperidine were added to these solutions to facilitate cyclization, and the reactions were stirred at room temperature for 1 h. The solvent was removed, and the resulting solids were purified by flash silica gel chromatography (1–4% (v/v) gradient of MeOH in CH_2Cl_2) to give DKPs in 15–86% yields and >97% purities with 95:5 d.r.

Bacterial Strains

References to the strains can be found throughout the text. Additional information about these strains and culture conditions is given in Supplementary Table 2.

Reporter Gene Assay Formats

Stock solutions of synthetic compounds were prepared in DMSO and stored in sealed vials. For agonism assays, an appropriate amount of DKP stock solution was added into wells of a 96-well multititer plate to give a 500 μM final concentration. For competitive antagonism assays, an appropriate amount of AHL stock solution was also added to each well to give a final AHL concentration equal to the EC_{50} value in that strain. The concentration of DMSO was normalized in all wells (<2% (v/v)). Absorbance assays for *A. tumefaciens* WCF47 (pCF372) and *E. coli* DH5α (pJN105L pSC11), and luminescence assays for *E. coli* JM109 (pSB401) and *V. fischeri* strains were performed according to our previously reported methods (15–18,20). All assays were performed in triplicate. GraphPad Prism software (version 4.0c) was used to calculate IC_{50} and EC_{50} values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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45. Park *et al*. recently reported that DKP **8** increases the expression of the major outer membrane protein (or porin), OmpU, and this expression was dependent on the transmembrane transcriptional activator, ToxR, in several *Vibrio* spp (27,30). OmpU has recently been shown to play a role in cell membrane integrity and symbiotic colonization pathways in *V. fischeri*, but is not directly regulated by ToxR *(43)*. We screened DKPs **5–10** and **13–16** at 500 μM in Δ*ompU* and Δ*toxR* derivatives of *V. fischeri* (ESR1 OM3 and ESR1 KR–*tox1 (43)*) for luminescence inhibition against OHHL (**1**) at 0.35 μM and 5 μM (EC_{50} in these strains), respectively. However, the inhibitory activities of these compounds were not significantly affected by the absence of either OmpU or ToxR in *V. fischeri*, with the most active DKPs (**14b** and **14c**) exhibiting IC_{50} values in these two mutant strains analogous to those in the Δ-*luxI* strain (see Supplementary Figures 8–11). These data indicated that neither OmpU nor ToxR is required for DKPs to inhibit luminescence in *V. fischeri*.
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48. Visick and coworkers have recently uncovered three new luminescence regulators in *V. fischeri*: CheV, FlrC, and YehT (44). To determine if DKPs affected these regulators, we tested our DKPs in *cheV*, *flrC*, and *yehT* mutant strains of *V. fischeri*. All knock-outs were tested as previously outlined for other *V. fischeri* strains. DKP **14c** fully inhibited each of these mutant strains with varying, but similar, IC_{50} values (Supplementary Figures 12–13). These results suggest that **14c** is not causing luminescence inhibition in *V. fischeri* through interaction with these luminescence regulators.
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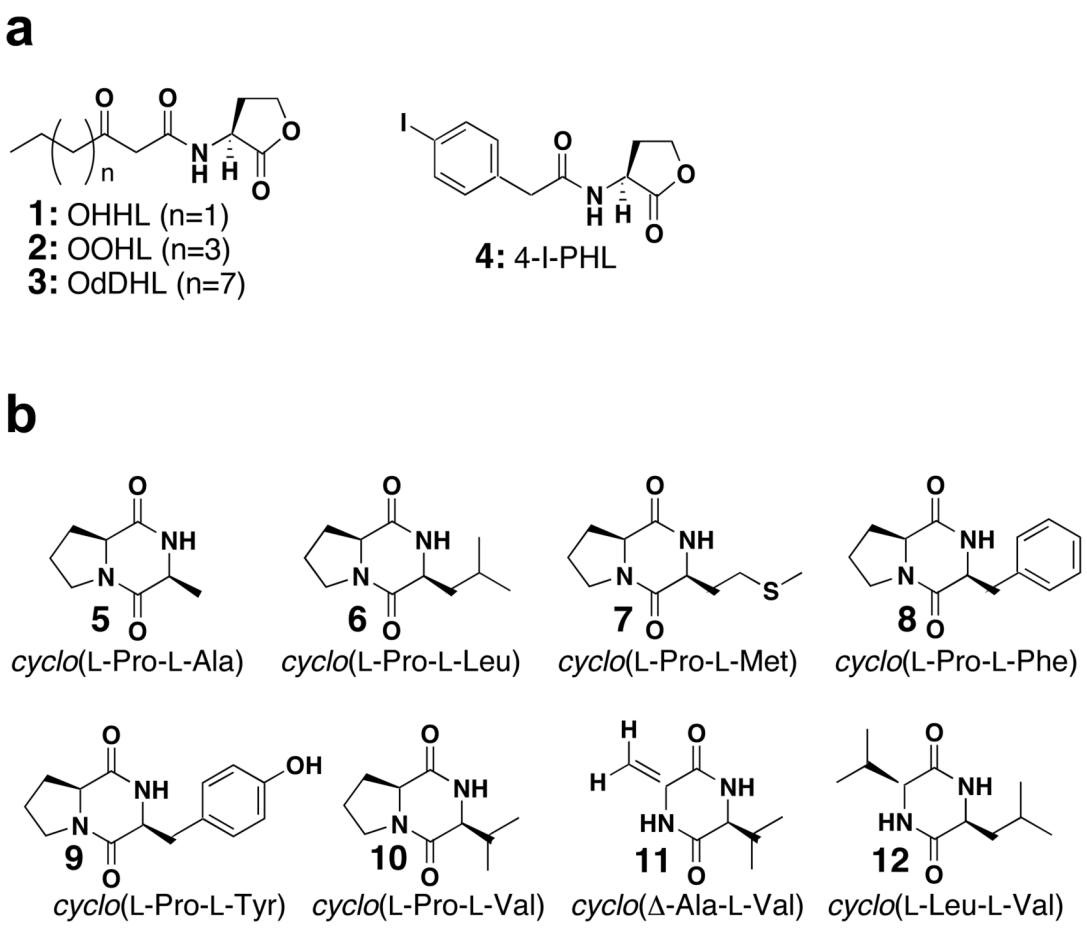


Figure 1.
QS modulators and natural products. a) Selected naturally occurring AHLs **1**–**3** and LuxR inhibitor 4-I-PHL (**4**). b) DKPs **5**–**12** reported to modulate LuxR-type protein activity.

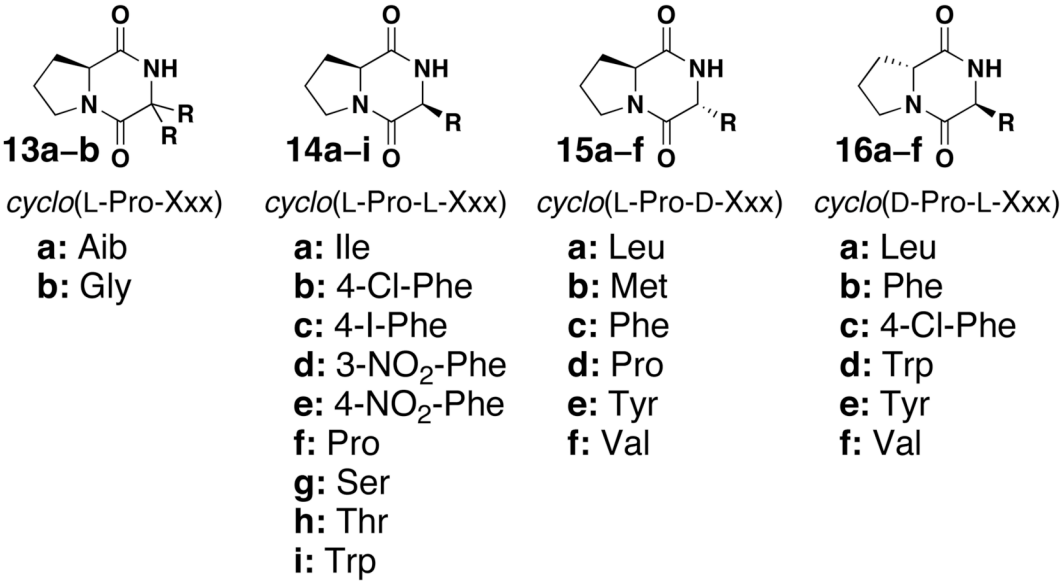


Figure 2.
Structures of the four focused DKP sub-libraries (**13**–**16**) synthesized and evaluated in this study. Aib = aminoisobutyric acid.

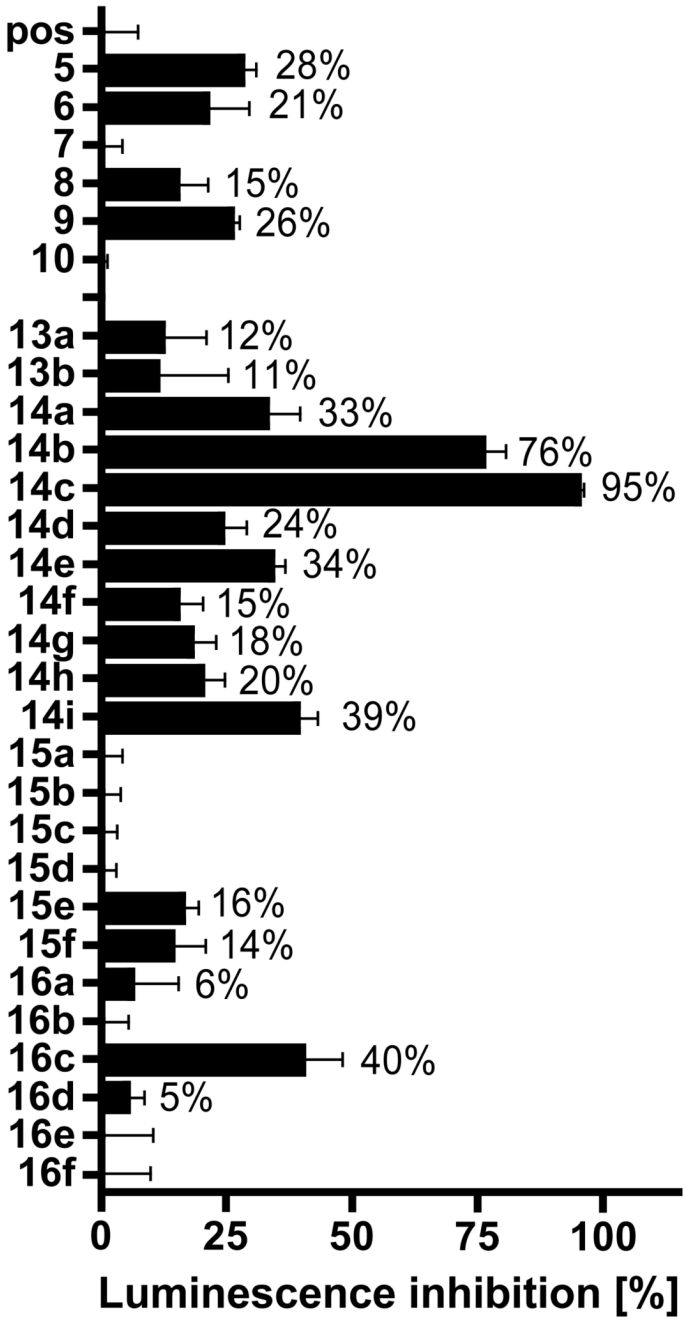


Figure 3.
Primary LuxR antagonism data for controls **5**–**10** and DKPs libraries **13**–**16** tested at 500 μM against 5 μM OHHL (**1**) in *V. fischeri* ES114 (Δ–*luxI*); positive control (pos) contains 5 μM OHHL (**1**).

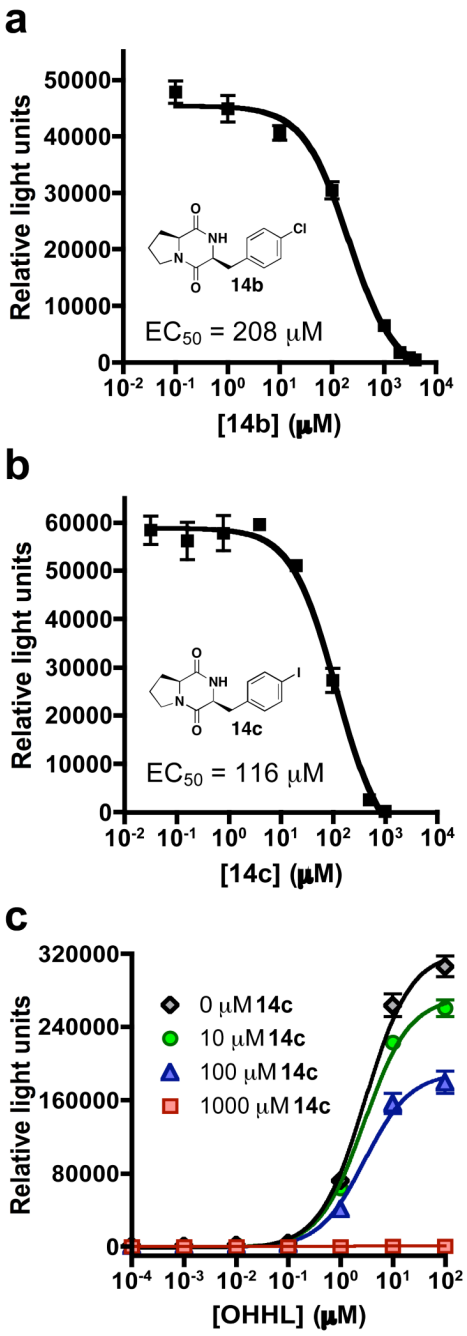


Figure 4.

Dose dependent inhibition of luminescence by DKPs. a) Dose response curve for DKP **14b** against 5 μM OHHL in *V. fischeri* ES114 (Δ–*luxI*). b) Dose response curve for DKP **14c** against 5 μM OHHL in *V. fischeri* ES114 (Δ–*luxI*). c) Dose response curves of OHHL (**1**) in the presence of increasing concentrations of DKP **14c** in *V. fischeri* ES114 (Δ–*luxI*). Relative light units report luminescence.

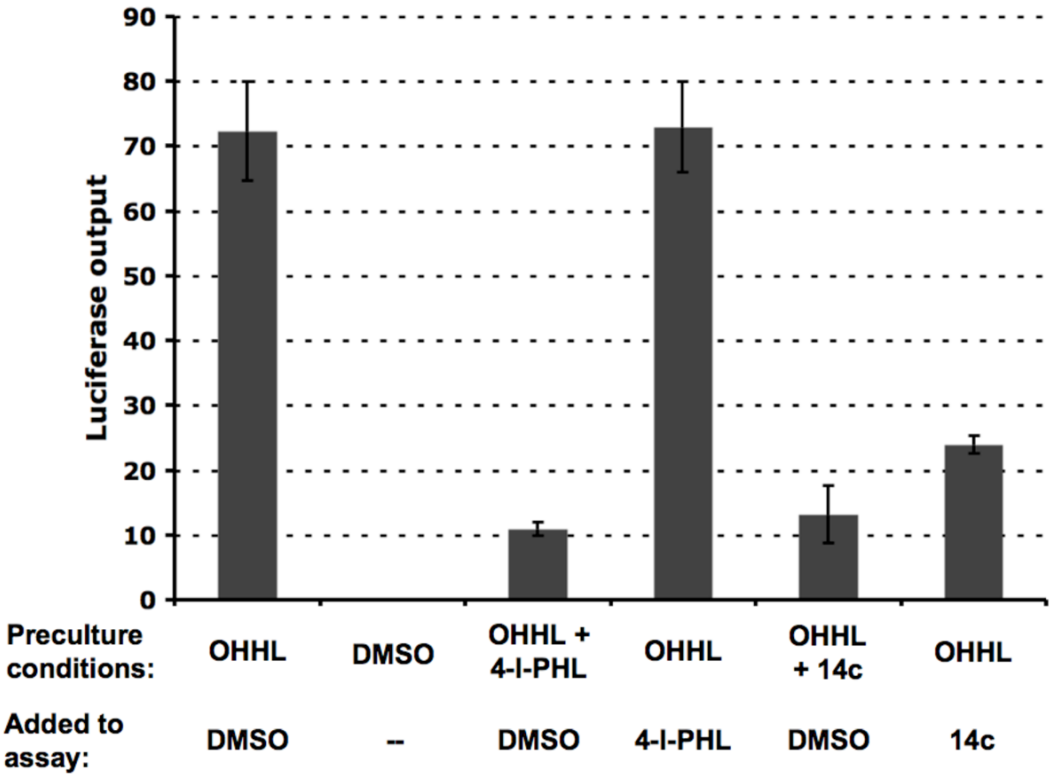
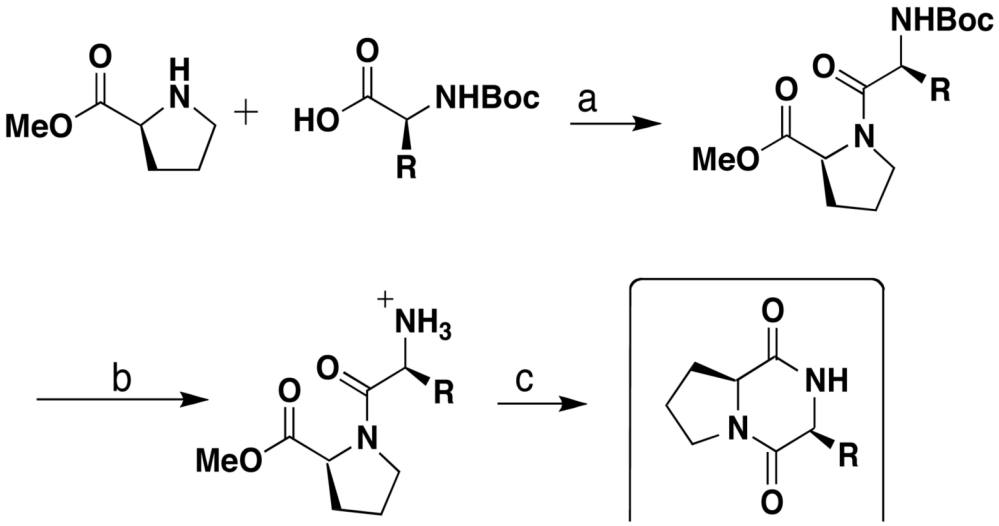


Figure 5.

Luciferase output of *V. fischeri* ES114 (Δ–*luxI*) cell pellets pre-cultured with or without 4-I-PHL (**4**) or DKP **14c** in the presence of OHHL (**1**). Luciferase output was measured as the initial light intensity of the reaction as described by Lei and Becvar (49). The concentration of OHHL (**1**) in the pre-culture was equal to its EC_{50} (3 μM) and the luminescence of the cells before pelleting was ~19,000 RLU. The luminescence of the DMSO pre-culture before pelleting was ~6,000 RLU. The OHHL (**1**) + 4-I-PHL (**4**) culture was grown in the presence of 3 μM OHHL (**1**) and 10x the IC_{50} of 4-I-PHL (10 μM); the luminescence of the cells before pelleting was ~6,000 RLU. The OHHL (**1**) + **14c** culture was grown in the presence of 3 μM OHHL (**1**) and 10x the IC_{50} of **14c** (1 mM); the luminescence of the cells before pelleting was ~6,000 RLU. Compounds that were added during the luciferase assay were added as DMSO stocks (10 μL) to the assay buffer (5 μM 4-I-PHL (**4**) or 500 μM **14c**; 5x IC_{50}). The volumes of DMSO used in these assays did not affect the luciferase output of the positive control.



Scheme 1.

General synthetic route for the generation of DKP libraries **13**–**16** and controls **5**–**10**. Reagents and conditions: a) EDC•HCl, Et_3N, CH_2Cl_2, 4 °C, 16 h; b) AcCl, MeOH, 0 °C, 180 min; c) piperidine, DMF, 25 °C 60 min. EDC = 1-ethyl-3-(3- dimethylaminopropyl)carbodiimide; DMF = dimethylformamide.