Bioorganic & Medicinal Chemistry 19 (2011) 4820–4828

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Thiolactone modulators of quorum sensing revealed through library design and screening

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ARTICLE INFO

Article history:
Received 20 May 2011
Revised 22 June 2011
Accepted 26 June 2011
Available online 1 July 2011

Keywords:
N-Acylated L-homoserine lactones
Gram-negative bacteria
LuxR-type receptors
Pseudomonas aeruginosa
Quorum sensing
Thiolactone AHL analogs

ABSTRACT

Quorum sensing (QS) is a process by which bacteria use small molecules or peptidic signals to assess their local population densities. At sufficiently high density, bacteria can alter gene expression levels to regulate group behaviors involved in a range of important and diverse phenotypes, including virulence factor production, biofilm formation, root nodulation, and bioluminescence. Gram-negative bacteria most commonly use *N*-acylated L-homoserine lactones (AHLs) as their QS signals. The AHL lactone ring is hydrolyzed relatively rapidly at biological pH, and the ring-opened product is QS inactive. We seek to identify AHL analogues with heightened hydrolytic stability, and thereby potentially heightened activity, for use as non-native modulators of bacterial QS. As part of this effort, we probed the utility of thiolactone analogues in the current study as QS agonists and antagonists in Gram-negative bacteria. A focused library of thiolactone analogs was designed and rapidly synthesized in solution. We examined the activity of the library as agonists and antagonists of LuxR-type QS receptors in *Pseudomonas aeruginosa* (LasR), *Vibrio fischeri* (LuxR), and *Agrobacterium tumefaciens* (TraR) using bacterial reporter strains. The thiolactone library contained several highly active compounds, including some of the most active LuxR inhibitors and the most active synthetic TraR agonist reported to date. Analysis of a representative thiolactone analog revealed that its hydrolysis half-life was almost double that of its parent AHL in bacterial growth medium.

1. Introduction

While humans and other higher order organisms can use a combination of sound, scent, movement, and sight to communicate, bacteria use a language of chemical signals to express their messages. This chemical communication process is termed quorum sensing (QS),[1] and allows bacteria to assess their local population densities using a class of signaling molecules called autoinducers.[2–4] In general, autoinducer concentration increases with bacterial cell density. Once a threshold population density is achieved in a given environment,[5] bacteria can alter gene expression levels in order to initiate processes that are largely only possible as a multicellular community. Such processes can play critical roles in both mutualistic symbiosis and the pathogenesis of bacterial infections.[6] Phenotypes under the control of QS include biofilm formation, antibiotic production, bioluminescence, root nodulation, swarming, and virulence factor production. As several of the most prevalent human pathogens use QS to control virulence,[7] there is significant interest in delineating the mechanisms of QS and targeting QS as an anti-infective strategy.[8] Over the past decade, the use of abiotic small molecules[9–12] and macromolecular probes[13,14] to attenuate QS pathways has emerged as a prominent research strategy in the bacterial communication field. Most of these agents work by intercepting or inactivating the native QS signal.

Gram-negative bacteria use *N*-acylated L-homoserine lactones (AHLs) as their primary autoinducers for QS (Fig. 1A).[4,11,15] These low molecular weight, cell permeable signals are produced by AHL synthases (LuxI-type proteins) and are sensed by their cognate cytoplasmic receptors (LuxR-type proteins). The LuxR-type proteins are AHL-regulated transcription factors. Productive LuxR-type receptor binding occurs once a sufficiently high AHL concentration is achieved in the cell. The AHL:LuxR-type protein complex most commonly dimerizes, binds DNA, and activates the transcription of QS controlled genes.[16] Over 100 Gram-negative bacteria use LuxI/LuxR-type systems to control QS, and many species have multiple LuxI/LuxR-type pairs to regulate different and overlapping aspects of their QS regulon.[17] The known, naturally occurring AHL signals all have a conserved L-HL head group and simply vary in their acyl tail.[4] The selectivity of an AHL for its cognate LuxR-type receptor is therefore controlled by acyl chain structure. These tails are commonly derived from fatty acids and can have between 4 and 18 carbons and different levels of oxidation at the 3-position.[18] Representative naturally occurring AHLs are

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doi:10.1016/j.bmc.2011.06.071

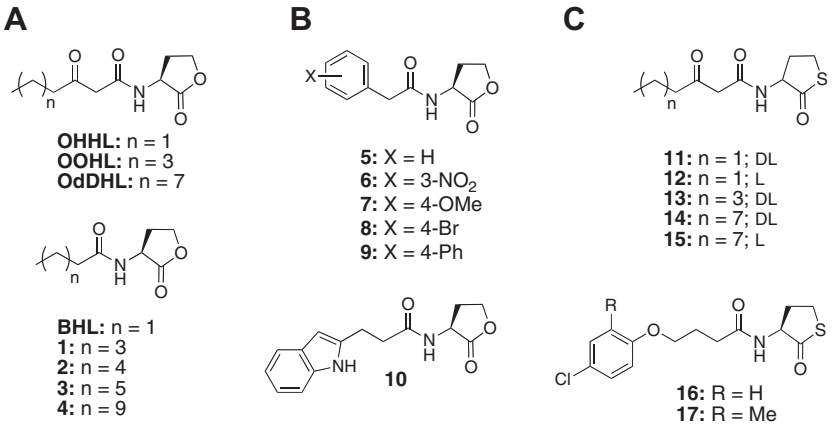

Figure 1. (A) Selected naturally occurring AHLs. OHHL and OOHL are the native AHL signals for *V. fischeri* and *A. tumefaciens*, respectively. OdDHL and BHL are native AHLs used by *P. aeruginosa*. (B) Representative AHLs with non-native acyl groups studied in our laboratory. (C) Previously reported thiolactone AHL analogues.

shown in Figure 1A, including *N*-(3-oxo)-hexanoyl HL (OHHL) from the marine symbiont *Vibrio fischeri*, *N*-(3-oxo)-octanoyl HL (OOHL) from the plant pathogen *Agrobacterium tumefaciens*, and *N*-(3-oxo)-dodecanoyl HL (OdDHL) and *N*-butanoyl HL (BHL) from the opportunistic pathogen *Pseudomonas aeruginosa*.

Our research laboratory has focused on the design and synthesis of non-native AHLs that differ in their acyl chain structures for use as probes to study LuxI/LuxR-type QS.[9,19] Several of these AHLs (**5**–**10**) are shown in Figure 1B. In our initial studies, we sought to determine the structural features of non-native acyl chains that engendered LuxR-type protein selectivity,[20–22] and we uncovered several highly potent and selective modulators of LuxR in *V. fischeri*, LasR in *P. aeruginosa*, and TraR in *A. tumefaciens*. In more recent work, we have evaluated the activity of these compounds in an expanded set of receptors, including QscR in *P. aeruginosa*,[23] CviR in *Chromobacterium violaceum*,[24] and ExpR1/ExpR2 in *Pectobacterium carotovora*.[25] Overall, these past studies have revealed that even subtle changes to a non-native AHL acyl chain can impart dramatic changes in ligand activity, ranging from converting a poor agonist into a strong and broad spectrum agonist, to converting an agonist into a receptor-selective antagonist.[22]

An important goal of our current research is to improve the potency of our lead AHL agonists and antagonists. One approach to address this objective is to increase or tune the hydrolytic stability of the lactone ring, as an intact lactone ring is required for activity.[26,27] In the work reported herein, we examined the effects of replacing the lactone head group in several of our AHLs with a thiolactone (i.e., derived from L-homocysteine) on LuxR-type receptor agonism and antagonism. Previous work has demonstrated that selected thiolactone analogues of native AHLs (Fig. 1C) can behave as agonists or antagonists of LuxR-type proteins. For example, Passador et al. reported that thiolactone **15**, the analogue of LasR's native ligand, OdDHL (Fig. 1A), had comparable agonistic activity to OdDHL in LasR.[28] Thiolactone **15** was also analyzed in LuxR by Schaefer et al., who found that both **15** and OdDHL were unable to agonize LuxR in an *E. coli* reporter, while **12**, the thiolactone analogue of LuxR's native ligand, OHHL (Fig. 1A), was a weak LuxR agonist. A later study by Chhabra et al. directed at the use of AHL analogues as possible immune modulators revealed thiolactone **15** to cause approximately 40 times less of an immune response in mice than the native OdDHL, suggesting that thiolactone-derived QS agonists and antagonists could be useful in clinical settings.[30] Janssens et al. have studied the effects of non-native AHLs on the (orphan) LuxR homolog from *Salmonella enterica*, SdiA, and uncovered several thiolactone-derived agonists (e.g., racemic **11**, **13**, and **14**, Fig. 1C).[31] Thiolactone **11** was markedly potent, activating SdiA at single nanomolar concentrations in their bacterial reporter strain. Most recently, Bassler and co-workers have reported a set of thiolactone AHL mimics (e.g., **16** and **17**) that are highly active antagonists of CviR in *C. violaceum*.[32,33] In view of these past studies, we reasoned that chimeric ligands made by uniting L-homocysteine thiolactone with our lead AHL acyl groups could yield LuxR-type protein modulators with heightened or novel activity profiles.

Here, we report the design and parallel synthesis of a focused library of thiolactone analogs based on native and non-native AHLs studied in our laboratory. The library was examined for agonistic and antagonistic activity in the well-characterized QS receptors LuxR, LasR, and TraR using bacterial reporter strains. For a subset of these compounds, both the racemic and enantiopure (L) thiolactone were evaluated. Several highly potent and receptor selective thiolactone agonists and antagonists were found for these systems, further underscoring the potential utility of thiolactone derivatives as chemical probes to study QS. This study represents the first comparative analysis of thiolactone analogues across different LuxR-type receptors. In addition, the results of this study allowed us to formulate a new hypothesis with regards to how several of the thiolactone agonists elicit their activity in LuxR-type receptors. An analysis of the hydrolysis rates for a representative thiolactone and its AHL analog concludes this study, and suggests that thiolactones can exhibit heightened stability relative to AHLs in standard bacterial growth media.

2. Results and discussion

2.1. Library design and synthesis

We designed a 21-member library of thiolactone AHL analogues, with all of the compounds containing a conserved thiolactone head group and a variable acyl tail (Fig. 2C). A brief description of our design process and compound synthesis is provided here. Thiolactones **12** and **15**, previously shown to be agonists in LuxR and LasR (see above) and analogs of OHHL and OdDHL, respectively, were included in the library to serve as control compounds.[28,29] To complement these two controls, we also included the thiolactone analog (**18**) of TraR's native ligand, OOHL, along with five other thiolactone analogs (**19**–**23**) of native AHLs used by a range of bacteria, including *P. aeruginosa* (BHL, **19**), *P. chlororaphis* (C6-HL, **20**), *Pantoea ananatis* (C6-HL, **20**; C7-HL, **21**; C8-HL, **22**) *Burkholderia cepacia* (C8-HL, **22**), and *Acidithiobacillus ferrooxidans* (C12-HL, **23**).[34] The remaining thiolactone library

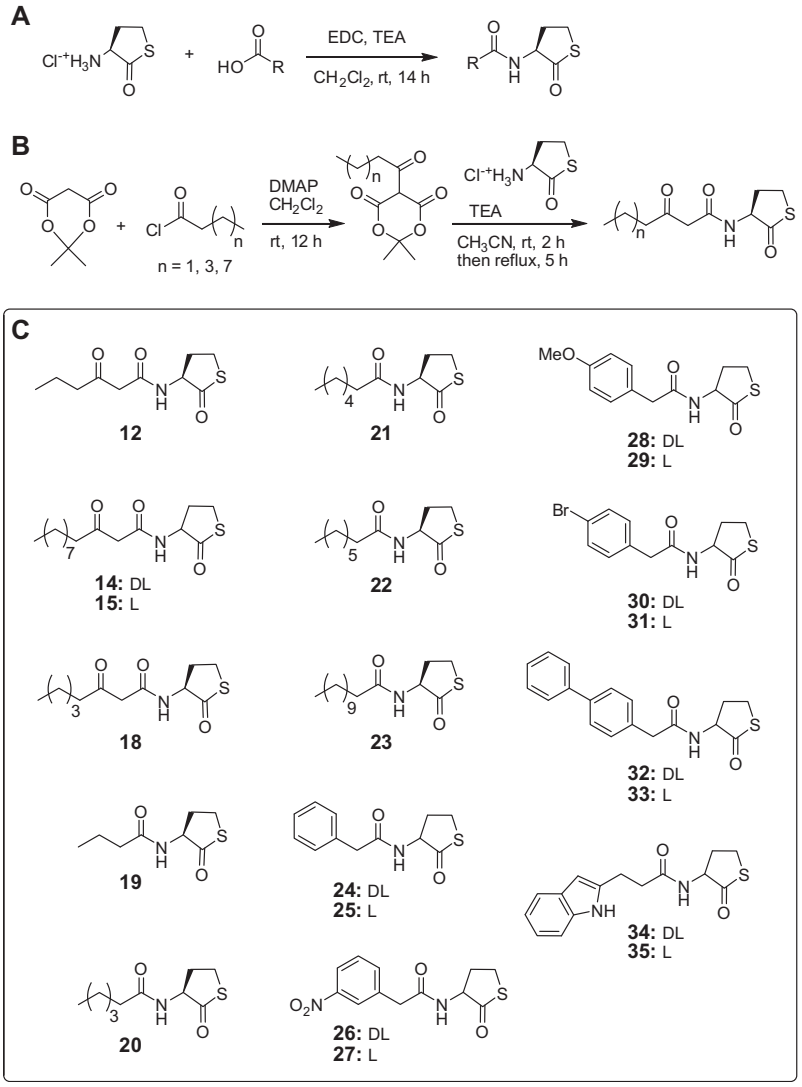

Figure 2. Thiolactone library. (A) Synthesis of non 3-oxo thiolactones. EDC = 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide. TEA = triethylamine. (B) Synthesis of 3-oxo thiolactones. DMAP = dimethyl amino pyridine. (C) Library of thiolactones analyzed in this study.

members were chimeric ligands based on acyl groups that we have previously identified in AHL-based LuxR-type receptor agonists and antagonists (Fig. 1B).[20–22,35] Thiolactones **30**/**31** and **32**/**33** were modeled after AHLs **8** and **9**, which are strong antagonists of both LuxR and TraR. Likewise, thiolactones **28**/**29** were based on AHL **7**, which is a moderate antagonist of LuxR. Phenylacetanoyl HL **5** was previously shown to be largely inactive in many LuxR-type receptors,[21] and we therefore included thiolactone analogs **24**/**25** of AHL **5** to test whether this inactivity profile would be maintained in thiolactones. The 3-nitro phenylacetanoyl thiolactones **26**/**27** were based on AHL **6**, which is an extremely strong LuxR agonist[20] and a moderate LasR antagonist.[21,22] To assess the importance of stereochemistry on ligand activity, thiolactones **14**, **15**, and **24**–**35** were synthesized in both racemic (DL) and enantiopure (L) form. The L-thiolactone enantiomer was chosen based on several previous studies that have shown that the active enantiomer of native AHL signals is the L-form.[21,27] Such an analysis of the stereochemical requirements for thiolactone modulation for LuxR-type proteins is yet to be reported.

The thiolactone derivatives that lacked 3-oxo functionality were synthesized by routine EDC couplings between L-homocysteine thiolactone and various carboxylic acids. (Fig. 2A). The remainder of the library was synthesized by reacting Meldrum's acid with the requisite alkyl acid chloride to afford the Meldrum's acid derivative, which was then coupled to L-homocysteine thiolactone (Fig. 2B). Racemic thiolactones were made in similar manner from DL-homocysteine thiolactone (see Section 4).

2.2. Library assay design

Small molecules are usually screened for LuxR-type agonism or antagonism using a bacterial strain containing a reporter gene for a given LuxR-type protein.[9] These strains typically lack a functional LuxI-type synthase, yet retain the functional LuxR-type receptor. Exogenous native AHL therefore must be added to activate the LuxR system. These strains provide a straightforward way to examine the agonistic and antagonistic activities of non-native ligands (by adding only the compound of interest or the compound in competition with the native AHL ligand (at its EC_{50} value), respectively).

We utilized four bacterial reporter strains in this study to examine the LuxR-type modulatory activities of the thiolactone library in LasR, LuxR, and TraR. Two strains were selected for the LasR screens: *Escherichia coli* DH5 (pJN105L + pSC11)[36] and *P. aeruginosa* PA01 MW1 (pUM15).[37] *E. coli* DH5 (pJN105L + pSC11) is a heterologous reporter strain containing one plasmid for the LasR gene and

a second plasmid containing the promoter region for LasI fused to β-galactosidase (β-gal). LasR activity is read-out using a standard colorimetric assay with *ortho*-nitrophenyl-β-galactosidase (ONPG) as the substrate for β-gal. The PA01 MW1 (pUM15) strain is a LasR reporter in *P. aeruginosa* that lacks a functional LasI and contains a plasmid with a LasR responsive promoter for Yellow Fluorescent Protein (YFP), which facilitates straightforward evaluation of LasR activity using fluorescence. Examining the thiolactone library in both of these strains allowed us to study the effects of these compounds on LasR in an isolated system (*E. coli*) and then in the presence of *P. aeruginosa*'s more complex QS network (including RhlR and QscR, in PAO1). (We note that *E. coli* and *P. aeruginosa* have different compound uptake/efflux profiles, and this feature should be taken into account when comparing small molecule screening data between the two strains (see below)).

V. fischeri ESI 114 (-LuxI)[38] and *A. tumefaciens* WCF (pCF372)[39] were used to examine the activity of the thiolactone library in LuxR and TraR, respectively. The *V. fischeri* mutant strain lacks a functioning LuxI synthase, but retains its native *lux* operon, allowing a quantitative luminescent readout based on LuxR activity. Similarly, *A. tumefaciens* WCF (pCF372)[39] lacks a functioning TraI, yet contains a plasmid with a TraR responsive promoter for the β-gal gene, thereby allowing for direct quantitation of TraR activity.

We used bacteriological assay protocols for small molecule screening that were analogous to those reported in our earlier studies (See Section 4). All synthetic compounds were screened at 10 μM in both agonism and antagonism assays in the bacterial reporter strains. Notably, these compound concentrations parallel those used by our laboratory in past studies,[22,40] allowing for comparisons to be made between the assay data reported here and this past work. No effects on bacterial growth were observed over the time course of the reporter gene assays (4–16 h).

2.3. Antagonism and agonism assays in LasR

The primary antagonism and agonism data for the thiolactone library in the LasR *E. coli* and *P. aeruginosa* reporter strains are shown in Table 1. Several intriguing trends in ligand activity are immediately apparent upon analysis of these data. First, all of the thiolactones exhibited slight to moderate *agonistic* activities in the *antagonism* assay in the *P. aeruginosa* LasR reporter strain. Natural AHL thiolactone analogues **12**, **14**/**15**, **19**, and **20** and phenylacetanoyl thiolactones **24**/**25**, **29**, and **32**–**34** were capable of agonizing LasR by greater than 20% in the antagonism assay, yet only the natural AHL analogs (**12**, **14**/**15**, **19**, and **20**) showed appreciable agonistic activity in the corresponding agonism assays in the same strain. Compounds capable of agonizing LasR in both assays can be clearly defined as LasR agonists,[21] but those that fail to agonize in the agonism assay could operate by a different mechanism (see below). Second, while native AHL thiolactone mimics **19** and **20** exhibited an expected correlation between agonism and antagonism trends in the *P. aeruginosa* LasR reporter strain, this pattern is not mimicked in the *E. coli* LasR reporter strain. Conversely, 3-NO_2 thiolactones **26**/**27** were highly active agonists in the *E. coli* LasR reporter strain, yet minimally active in the *P. aeruginosa* reporter strain. This activity profile is the opposite of its parent AHL **6**, which is one of the stronger LasR inhibitors known.[21] We note, however, that 3-NO_2 AHL **6** is also an extremely potent agonist of LuxR.[20] Third, the remaining potent LasR agonists identified in the *E. coli* reporter strain (**22** and **34**) were also significant LasR *antagonists* in this strain. On the whole, the only LasR agonists in the thiolactone library capable of agonizing LasR in the agonist and antagonist assays in both reporter strains were thiolactone analogs of natural AHL ligands: OdDHL analog **14**/**15**, OHHL analog **12**, and *N*-dodecanoyl thiolactone **23**. These results, while unexpected, corroborate with those previously reported by Passador and co-workers for **15**.[28]

In contrast to the *P. aeruginosa* LasR reporter strain in which no antagonists were found, several highly potent LasR antagonists were found using the *E. coli* reporter strain. The non-3-oxo aliphatic thiolactones **20**–**22** and phenylacetanoyl thiolactones **24**/**25** and **28**-**34** were all found to be ~50–80% inhibitors of LasR this strain. Many of these compounds show complex trends in activity. For example, both the racemic and enantiopure phenylacetanoyl thiolactones

Table 1
LasR primary antagonism and agonism assay data for the thiolactone library in *P. aeruginosa* and *E. coli* reporter strains[a]

Compound	*P. aeruginosa* LasR		*E. coli* LasR	
	Antagonism[b] (%)	Agonism[c] (%)	Antagonism[b] (%)	Agonism[c] (%)
12	−23	76	−119	96
14	−49	127	−61	102
15	−55	88	−40	94
18	−8	3	−30	0
19	−24	22	17	4
20	−23	20	59	1
21	−8	0	78	8
22	−7	0	64	85
23	−11	42	−93	94
24	−24	17	61	3
25	−25	2	65	2
26	−15	3	−3	81
27	−7	5	−13	82
28	−18	4	48	0
29	−23	7	51	1
30	−18	5	68	8
31	−17	8	80	2
32	−23	1	45	0
33	−43	3	56	9
34	−22	3	54	72
35	−7	10	16	4

[a] All synthetic compounds were screened at 10 μM. All assays were preformed in triplicate; error did not exceed ±10%. Positive controls were OdDHL at its EC_{50} value for the strain in antagonism assays and 100 times its EC_{50} value for the strain in agonism assays. Negative controls contained neither thiolactone nor natural AHL, and were subtracted from each sample to account for background. Negative antagonism values indicate that the compound activates at the tested concentration. See text for details of strains.

[b] Antagonism assays were preformed against the EC_{50} value for OdDHL in each strain: *P. aeruginosa* PA01 MW1 (pUM15) = 1 M; *E. coli* DH5 (pJN105L + pSC11) = 10 nM.

[c] Agonism assays were normalized to the positive control (OdDHL) in each strain.

28–**31** are good to excellent inhibitors in the *E. coli* LasR reporter strain, yet are mild *agonists* in the *P. aeruginosa* reporter strain *antagonism* assays. This is particularly intriguing since neither the 4-methoxy nor the 4-Br phenylacetanoyl parent AHLs (**7** and **8**) were very active in LasR; rather, they are both active antagonists in LuxR.[20] Thiolactones **24/25** and **32/33**, which contain either an unsubstituted phenyl or a biphenyl tail, exhibit even more exaggerated differences in activity; they are simultaneously good antagonists in the *E. coli* strain and good agonists in the antagonism assay in the *P. aeruginosa* strain. The parent AHLs (control phenyl AHL **5** and biphenyl AHL **9**) display minimal activity in LasR as well.[21] These data trends for thiolactone analogs versus their parent AHLs clearly suggest that minor perturbations (i.e., O to S) to the AHL head group can have dramatic effects on overall compound activity and receptor selectivity.

We note again here that the *P. aeruginosa* reporter strain retains the two other LuxR-type proteins that govern QS in this organism (QscR and RhlR), while the *E. coli* strain lacks these receptors. The lack of these receptors in *E. coli* could be one reason for the complicated thiolactone activity trends for LasR outlined above when comparing the two strains. Differences in membrane permeability between the two strains could be another contributing factor (see above). Both RhlR and QscR can regulate LasR to some degree, with QscR directly repressing LasR using its identical cognate ligand, OdDHL.[19] In previous work, we have shown that many of our non-native AHLs can simultaneously inhibit LasR and QscR. In this context, thiolactones **24**, **25**, and **28**–**33** could also be inhibiting QscR in addition to LasR. A QscR inhibitor would yield LasR agonistic activity in the *P. aeruginosa* strain, yet would exhibit LasR antagonistic activity in the QscR null *E. coli* strain. This is the trend observed above for these compounds. Additional experiments with QscR are necessary to evaluate this hypothesis.

Since it was difficult to determine a definitive acyl group SAR for the thiolactone LasR agonists and antagonists, we scrutinized the thiolactone stereochemistry to determine what role it played, if any, in dictating compound activity. If stereochemistry played a large role in receptor binding, we hypothesized that the enantiopure L-thiolactones would be approximately twice as active as the racemic thiolactones (screened at the same concentration). Our reasoning proved incorrect, however, as the enantiopure L-thiolactones and their racemates largely exhibited approximately the same activity (Table 1). (We note that no appreciable racemization was observed in our synthesis procedure for the L-thiolactones). This result suggests that however these ligands modulate LasR, the interaction is less specific than that of native AHL ligands. Thiolactones **35** and **34**, however, are a special case. Although they are LasR antagonists in the *E. coli* strain and LasR agonists in the *P. aeruginosa* strain, **34** (the racemate) has higher activity in both cases than **35** (the enantiopure L-form). For these compounds, the D-enantiomer may be the more active form of the compound.

2.4. Possible agonism model in LasR for thiolactones

The interesting trends in LasR agonism for the thiolactones in the presence of OdDHL *within the individual strains* caused us to consider the mechanism of LasR activation in more detail (Fig. 3). Many of the thiolactone library members have similar architectures as OdDHL (most notably the aliphatic derivatives), allowing us to make the reasonable assumption that they can potentially bind in the OdDHL binding pocket in LasR. A simple schematic of LasR's mechanism of action is shown in Figure 3A. OdDHL binds to LasR, which then homodimerizes and binds to the promoter region of one of many genes under LasR control including LasI, RhlI, RhlR, and RsaL, acting as a transcriptional activator.[16] Similarly,

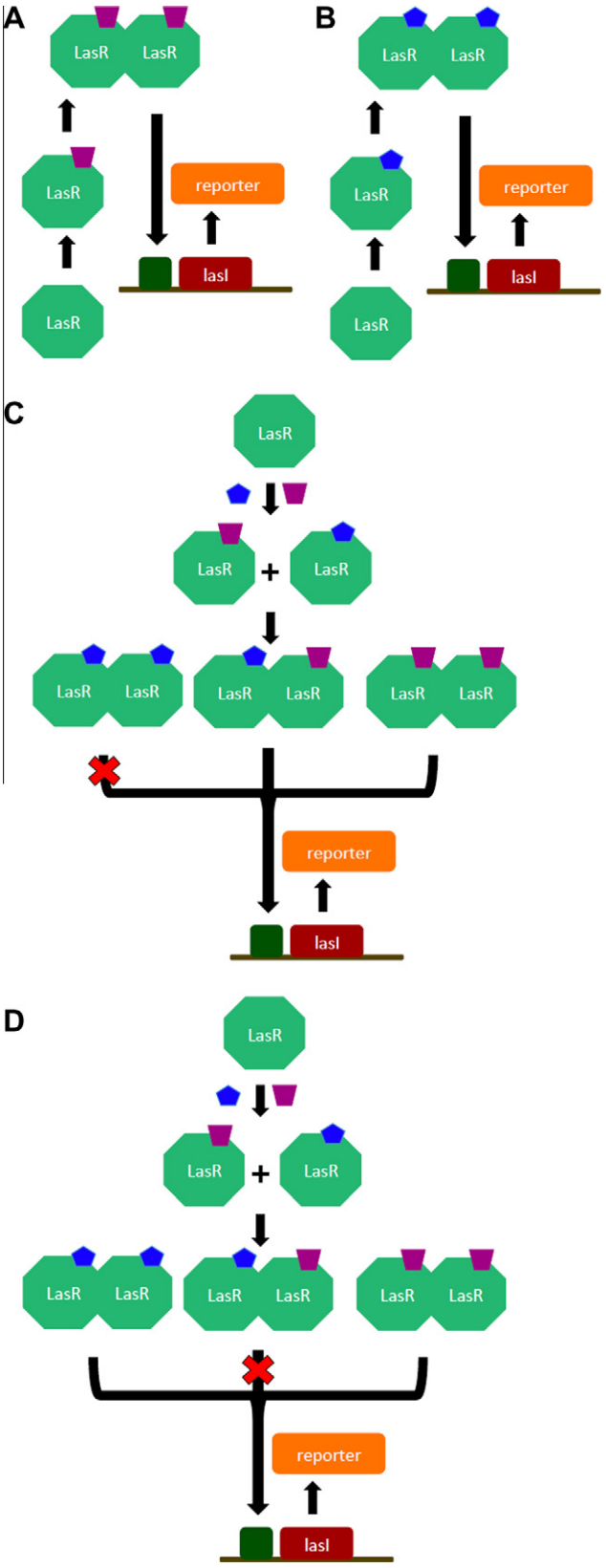

Figure 3. A schematic diagram of possible mechanisms for LasR agonism and antagonism by OdDHL (native AHL) or a non-native AHL analog. OdDHL is shown as the purple trapezoid, and the non-native ligand is shown as the blue pentagon. (A) The accepted mechanism for native ligand (OdDHL) activation of LasR. (B) Synthetic ligand binding to LasR. (C) Proposed binding mode for cooperative agonists. (D) Proposed binding mode for 'bimodal binders.' See text for further detail.

when a non-native LasR agonist replaces OdDHL in reporter gene assays, LasR:non-native ligand homodimers are believed to form (Fig. 3B). In competitive antagonism assays, it is generally assumed that the non-native ligand is able to outcompete OdDHL for the binding pocket, either by creating inactive homodimers or by preventing dimerization from occurring. Based on the work of Bassler and co workers,[32] it is also possible that the inactive homodimers are able to bind DNA, but the homodimer:DNA complex is then unable to successfully interact with RNA polymerase. Two alternate scenarios are possible, however, if one considers LasR heterodimer formation. Such heterodimers would consist of one LasR bound to OdDHL and one LasR bound to a non-native ligand (Fig. 3C and D).

In one scenario (Fig. 3C), non-native ligands may act as what we term cooperative agonists if they are able to form viable heterodimers with LasR, yet are either unable to form homodimers or form inactive homodimers. This could lead to an observed high *agonistic* activity in a competitive antagonism assay against OdDHL Therefore, if the homodimers of the non-native ligand are either inactive or unable to form, negligible agonistic activity would be observed for the non-native ligand alone in an agonism assay. For example, thiolactones **24**/**25**, **29**, and **32**–**34** exhibit this trend in the *P. aeruginosa* strain. Conversely, in a second scenario (Fig. 3D), if the synthetic ligand is an agonist when OdDHL is absent and is an antagonist when OdDHL is present, both the non-native and OdDHL homodimers could likely agonize LasR, while the heterodimers are inactive. An antagonistic effect would therefore be observed when the natural ligand is present (Fig. 3D). We have named compounds that could bind in this latter manner 'bimodal binders' because of their dual antagonistic and agonistic properties. We uncovered thiolactones displaying this activity profile in both LuxR and TraR (see below). Additional biochemical experiments are of course required to validate these models for LasR and related LuxR-type proteins, and are currently underway in our laboratory. Nonetheless, we find these models helpful for understanding the screening data obtained above for the thiolactones in LasR, and below in LuxR and TraR.

2.5. Antagonism and agonism assays in LuxR and TraR

The antagonism and agonism primary screening data for the thiolactone library in LuxR and TraR are shown in Table 2. Several exquisitely active compounds were identified, indicating the ability of acylated thiolactones to strongly modulate LuxR-type receptors beyond LasR. Sixteen thiolactones (including racemic compounds) were found to inhibit LuxR by over 50%. Notably, thiolactones **15**, **18**, **20**, **27**/**28, 30**/**31**, and **32**/**33**, all of which are over 90% inhibitors, represent some of the most potent LuxR antagonists reported to date. The strong antagonistic activity of **15**, the thiolactone mimic of OdDHL, is analogous to OdDHL itself in LuxR.[22] Thiolactone **15** was also incapable of activating LuxR, corroborating the work of Schaefer et al.[29] Surprisingly, 3-NO_2 phenylacetanoyl thiolactones **26**/**27** were only weak agonists of LuxR, even though their parent 3-NO_2 AHL (**6**) is a 'super activator' of LuxR.[20] This change in activity may be a result of the possible bimodal binder activity profile for **26/27** in LuxR (see above), as it is also behaves as a weak LuxR antagonist. However, several thiolactone displayed activities that tracked well with their parent AHLs. Thiolactones **28/29** were found to have analogous LuxR antagonistic activities as their methoxy AHL analog (**7**), which is a moderate inhibitor of LuxR. Similarly, thiolactones **30**–**34**, modeled after our lactone-based LuxR inhibitors, were all strong LuxR antagonists, while **24**/**25** (based on inert AHL **5**) were only weakly inhibitory.

Turning to TraR, we only found one thiolactone, the OOHL mimic **18**, which displayed appreciable agonistic activity in TraR (50%; Table 2). Thiolactone **18** is notable as very few non-native TraR agonists have been reported to date.[21] Several thiolactones, however, appeared to be either capable of what we now propose to be cooperative agonism (see above), or were extremely strong TraR antagonists. Compounds **19**, **20**, **26**, and **27** behaved as cooperative agonists, with **19** and **20** agonizing TraR in the presence of OOHL to over 70% more than OOHL alone. The *N*-hexanoyl thiolactone **20** is the non-3-oxo analog of OHHL, suggesting that close similarities in ligand structure may permit active heterodimer formation. In turn, thiolactones **21** and **22** were very strong TraR

Table 2
LuxR and TraR primary antagonism and agonism assay data for the thiolactone library in *V. fischeri* and *A. tumefaciens* reporter strains[a]

Compound	*V. fischeri* (LuxR)		*A. tumefaciens* (TraR)	
	Antagonism[b] (%)	Agonism[c] (%)	Antagonism[b] (%)	Agonism[c] (%)
12	62	0	93	1
14	91	0	51	1
15	99	0	92	1
18	99	0	−89	50
19	42	0	−70	0
20	93	0	−78	0
21	78	0	78	0
22	80	0	99	0
23	57	0	6	0
24	30	0	13	0
25	34	0	−22	3
26	30	26	30	0
27	19	21	33	1
28	59	0	−24	1
29	70	1	−107	0
30	91	0	25	1
31	97	0	−60	0
32	85	0	26	11
33	98	0	9	0
34	75	0	−26	6
35	68	0	−80	0

[a] See footnote a in Table 1.
[b] Antagonism assays were preformed against the EC_{50} value for the native AHL each strain: *V. fischeri* ESI 114 (-LuxI) = 2 M OHHL; *A. tumefaciens* WCF (pCF372) = 200 nM OOHL.
[c] Agonism assays were normalized to the positive control (native ligand) in each strain.

inhibitors, with **22** surpassing the activity of its parent *N*-octanoyl HL (**3**) and inhibiting TraR by 99%.[22] Compound **21** has a one carbon shorter acyl chain than **22** and is 20% less active, correlating well with acyl chain length activity trends seen for similar AHL analogs in TraR.[22]

Analogous to the LasR primary screening data, thiolactone stereochemistry did not appear critical for LuxR agonism or antagonism. However, it did influence TraR agonism and antagonism trends in some cases. For example, the racemic thiolactone **30** and L-thiolactone **31** showed a reversal in activity; **30** is a 25% TraR antagonist, while **31** is a 60% TraR agonist in the antagonism assay. These data suggest that the D-stereoisomer may have higher TraR inhibitory activity, and the L-stereoisomer then has higher agonistic activity. In another example, L-thiolactone **29** exhibited very strong *agonistic* activity in the TraR antagonism assay (2x the activity of OOHL), while the racemic thiolactone **28** is only a 24% TraR agonist. For both **28** and **29**, agonistic activity is only observed when OOHL is present. These data suggest that **28** and **29** could function as cooperative agonists of TraR (see above), with the L enantiomer imparting the majority of the activity. Indole thiolactones **34** and **35** showed a similar agonistic activity profile as **28** and **29**, although not quite as exaggerated.

Overall, the thiolactone analog of OdDHL (**15**) was highly active in all strains tested—either as a LasR agonist (94% in *E. coli* and 88% in *P. aeruginosa*) or an antagonist in LuxR (92%) and TraR (65%). These trends match those observed for OdDHL in each strain, and suggest that, at least for **15**, sulfur replacement in the lactone ring does not significantly affect its interactions with a LuxR-type receptor.

2.6. Dose response analyses for selected L-thiolactones

Dose response analyses for selected active, L-thiolactones (**12**, **14**, **15**, **20**–**23**, **25**, **27**, **29**, **31**, **33**, and **35**) were conducted to determine their IC_{50} and EC_{50} values in the bacterial reporter strains. These data are listed in Table 3, and revealed several agonists and antagonists with sub-micromolar activities (**15**, **18**, **20**–**22**, **31**, and **33**). The majority of these compounds were LuxR antagonists. The most active compound identified overall was *N*-octanoyl L-thiolactone (**22**), which could antagonize LuxR by 50% at a 10-fold lower concentration than OHHL. Interestingly, thiolactone **22** was also the strongest LasR antagonist identified in the *E. coli* reporter strain, capable of antagonizing LasR by 50% at a 10-fold higher concentration than OdDHL. Following this trend, the second and third most active LuxR antagonists were also the next most active LasR antagonists in the *E. coli* strain (i.e., the *N*-heptanoyl (**21**) and biphenyl (**33**) L-thiolactones).

The dose response data for four thiolactone agonists are worthy of attention (**12**, **15**, **18**, and **23**; Table 3). Interestingly, all four were native AHL analogs. The thiolactone analog of OOHL (**18**) is especially notable, as to our knowledge it is the most active non-native TraR agonist reported to date (albeit 100-fold less active then OOHL). In turn, OdDHL thiolactone analog **15** had EC_{50} values approximately equal to OdDHL in both the *E. coli* and *P. aeruginosa* LasR reporter strains (~10 nM and 1 mM, respectively). OHHL thiolactone analog **12** was also a strong agonist in both LasR reporter strains, but with EC_{50} values approximately 10-fold higher than OdDHL in each strain. Interestingly, unlike the thiolactone analogs of OOHL (**18**) in TraR and OdDHL (**15**) in LasR, OHHL thiolactone analog (**12**) was a mild LuxR *antagonist* instead. Finally, thiolactone **23**, the non-3-oxo thiolactone analog of OdDHL, was found to be ~100-fold less active then OdDHL in the *E. coli* LasR reporter strain**,** yet was only ~10-fold less active than OdDHL in the *P. aeruginosa* reporter strain. This difference in activity is significant because many of the thiolactones examined in the primary assays displayed the opposite trend in the two strains, that is, heightened activity in the *E. coli* versus the *P. aeruginosa* LasR reporter strain.

Further comparison of the thiolactone dose response data to that for our previously reported AHLs (**1**–**9**) revealed that the oxygen→sulfur replacement can both augment and diminish relative compound activities in AHL analogs (Table 3).[20,21] For example, thiolactones **21**, **22** and **33** largely exhibit similar agonistic or antagonistic potencies as their parent AHLs (**2**, **3**, and **9**) in LasR (*E. coli*), LuxR and TraR. The *N*-dodecanoyl thiolactone (**23**), however, displayed reduced agonistic activity relative to its AHL analog (**4**), suggesting that the sulfur replacement can impact ligand activity in some cases. Likewise, the phenylacetanoyl thiolactone **25** was a moderate LasR inhibitor in *E. coli*, while it parent AHL (**5**) was largely inactive in most LuxR-type receptors. Notably, the 3-NO_2 thiolactone **27** and indole thiolactone **35** were single digit micromolar agonists of LasR in *E. coli*, while their corresponding AHL analogs (**6** and **10**) were sub-micromolar LasR *antagonists*.[21] Clearly, additional thiolactones will need to be studied to gain more insight into the subtle SARs behind these activity switches. Structural studies of these compounds complexed to various LuxR-type proteins could be particularly illuminating, as indicated by the recent X-ray crystallographic work of Hughson and co-workers on related thiolactones bound to CviR.[33]

Table 3
IC_{50} and EC_{50} values for the most active L-thiolactone antagonist and agonists[a]

Compound	*E. coli* LasR		*P. aeruginosa* LasR	*V. fischeri* LuxR		*A. tumefaciens* TraR	
	IC_{50}[b] (μM)	EC_{50} (μM)	EC_{50} (μM)	IC_{50}[b] (μM)	EC_{50} (μM)	IC_{50}[b] (μM)	EC_{50} (μM)
12	–[c]	0.13	13			3.2	
15		0.092	3.2	0.45		1.8	
18				0.35			20
20	1.1			0.84			
21	0.79			0.31		10	
22	0.14			0.13		2.8	
23		1.9	21				
25	2.5						
27		4.1			11		
29	7.2						
31	0.40			0.77			
33	2.9			0.35			
35		1.8					

[a] See footnotes for Table 1 and Table 2 for details.
[b] The IC_{50} values were determined in the presence of the native AHL for each strain at its EC_{50} value.
[c] Vacant cells were not determined.

2.7. Thiolactone hydrolysis study

We sought to examine the hydrolytic stability of the thiolactone analogs described above, as generating probe compounds with heightened hydrolytic activity relative to AHLs was one motivation for this study. Like lactones, thiolactones are also hydrolyzable. However, direct comparison of the hydrolysis rates of AHLs versus their thiolactone analogs has, to our knowledge, yet to be reported. We provide such an analysis here.

We chose to analyze **15** as it was the most active thiolactone modulator identified in this study. A biological assay was developed to determine the functional half-life of **15** and its corresponding AHL analog, OdDHL. Our assay did not directly measure lactone hydrolysis, but rather the ability of the remaining non-hydrolyzed ligand to activate LasR. Previous experiments have shown that the hydrolysis half-life for OdDHL is approximately two days, while racemization of the chiral center was found to be less than 5% over the course of a week.[27] These data suggest that most ligand degradation is due to hydrolysis and not epimerization. Previous hydrolysis experiments have been monitored by NMR spectroscopy and performed in deuterated buffers containing 50% DMSO.[27,41] The use of high levels of DMSO reduces the biological relevance of the assay. In order to assess ligand degradation under more biologically relevant conditions, we monitored ligand activity levels using a LasR reporter strain (PA01 MW1 (pUM15); introduced above).[36]

Our hydrolysis assay protocol utilized the *P. aeruginosa* LasR reporter to detect ligand activity after incubation in growth media for specified times using fluorescence (YFP; see Section 4). Fluorescence values corresponded to the degree of ligand degradation prior to contact with the reporter strain, and were normalized to that of a freshly prepared ligand sample (i.e., the positive control). Since we predict that the ligand degradation is due to hydrolysis, we assumed a pseudo first-order rate and plotted the natural log of the ligand activity as a percent of the positive control versus time. The slope of the graph therefore could be used to determine the half-life of the ligand according to the formula $t_{1/2} = \ln(2)/\text{-slope}$ (Fig. 4).

Using this reporter assay procedure, we determined the half-life for OdDHL to be 48.2 h. This value corresponds closely with the hydrolysis half-life for OdDHL previously reported by Spring and co-workers.[27] A similar analysis of thiolactone **15** revealed its half-life to be 82.3 h, almost twice that of OdDHL. Yates and co-workers have shown that once ring-opened, the lactone does not reclose in appreciable quantities until pH values of less than 2.[41] Since sulfur compounds are stronger nucleophiles than their oxygen analogs,[42] we hypothesize that **15** is either able to remain closed for longer than OdDHL or it is able to ring close at higher pHs, shifting the half-life to longer times. We note that the LasR, LuxR, and TraR reporter gene assays above were conducted for no longer than 16 h, so the majority of both OdDHL and **15** is likely in the closed form and ligand stability (or free thiol reactivity) did not have a significant impact on the observed activities. Nevertheless, these hydrolysis data suggest that thiolactone AHL analogs, such as **15**, could have value for biological experiments conducted over longer time periods.

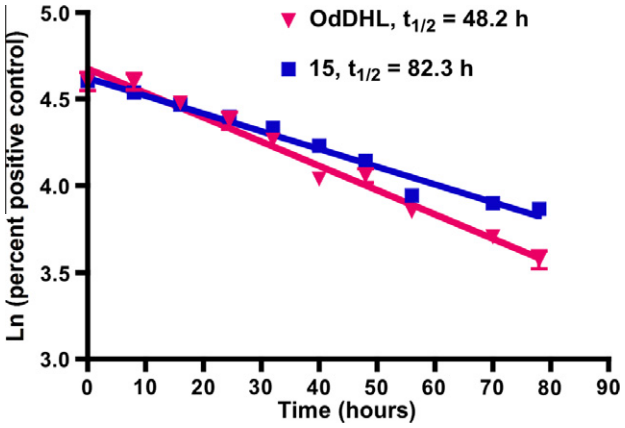

Figure 4. Hydrolysis of OdDHL and its thiolactone analog (**15**) plotted as a function of time versus the natural log of the percent of the positive control. The slope is −0.01399 ± 0.0006 for OdDHL and −0.01025 ± 0.0004 for **15**. The R^2 value for both slopes is 0.96. Error is less than ±2% for each time point.

3. Conclusion

We have designed and synthesized a library of thiolactone analogs of both naturally occurring and non-native AHLs. These thiolactones were evaluated for both antagonistic and agonistic activity in three relevant LuxR-type receptors (LasR, LuxR, and TraR) using cell-based reporter gene assays. The screening data provided a complex set of activity profiles for these compounds (most notably in LasR). However, several new and highly active QS modulators were discovered, many with multi-receptor activity. Compounds **15**, **18**–**22**, and **30**–**33** all had nanomolar IC_{50} values in *V. fischeri* and are some of the most potent LuxR antagonists to be reported. OOHL thiolactone analog **18** is also a strong LuxR antagonist, and simultaneously is the strongest non-native TraR agonist to be reported. In terms of LasR, thiolactones **21**, **22**, and **31** displayed nanomolar IC_{50} values in the *E. coli* LasR reporter. The thiolactone analogs of both OdDHL and its non-3-oxo HL analog (**15** and **23**) are strong LasR agonists in the *E. coli* system, supporting previous studies that showed that acyl chain length is important for receptor selectivity. However, this trend does not always hold true, as we found that the OHHL thiolactone analog with a six-carbon shorter acyl tail (**12**) had similar LasR agonistic activity as **15**.

The complexity of the thiolactone activity profiles observed in LasR motivated us to propose models for what we term cooperative agonism and bimodal binding in LuxR-type receptors. Several of the active thiolactones reported herein could behave via these pathways. While thiolactones that had the highest structural similarity to their parent AHL appeared to retain the highest degree of activity, the relatively minor change from a lactone to thiolactone could either reduce activity or flip activity from antagonism to agonism for many of the other thiolactone library members. Unexpectedly, thiolactone stereochemistry only appeared critical for ligand activity in TraR from *A. tumefaciens*, which is known to have fairly rigid binding pocket.[43,44]

In the course of these studies, a new assay was developed to assess the rates of thiolactone and lactone hydrolysis. Using this assay, we found that the half-life of the thiolactone derivative of OdDHL (**15**) is almost double that of OdDHL itself. As such, the new thiolactone agonists and antagonists reported here may offer a set of compounds with enhanced hydrolytic stabilities relative to AHLs, and could find utility as new chemical probes to study QS in Gram-negative bacteria.

4. Experimental section

4.1. Acylated thiolactone synthesis

Thiolactones lacking 3-oxo functionalities in their acyl groups were synthesized in solution using previously reported EDC coupling protocols and purified using acid-base extraction procedures.[45,46]

Thiolactones containing 3-oxo acyl groups were prepared from acylated Meldrum's acid derivatives. The Meldrum's acid

derivatives were synthesized by dissolving Meldrum's acid (0.5 g, 3.5 mmol) in CH_2Cl_2 (50 mL) under nitrogen at 0 °C. DMAP (0.86 g, 7 mmol) was added and allowed to dissolve completely. Thereafter, the alkyl acid chloride (3.5 mmol) was added to the reaction mixture slowly over 1 h at 0 °C. The reaction mixture was allowed to stir for an additional hour at 0 °C, and then overnight (~14 h) at rt. The reaction mixture was washed 2× with 2 M HCl and 1× with brine, and the organic layer was dried over $MgSO_4$, filtered, and concentrated to yield the crude acylated Meldrum's acid derivative.

The 3-oxo thiolactones were synthesized by stirring the homocysteine thiolactone (25 mg, 0.16 mmol) with the Meldrum's acid derivative (0.16 mmol) in acetonitrile (15 mL) at rt under nitrogen for 2 h, followed by refluxing for 5 h. The acetonitrile was then removed, and the reaction residue was redissolved in ethyl acetate and washed 1× each with saturated sodium bicarbonate, 1 M $NaHSO_4$, and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated to yield the 3-oxo thiolactone products. Further purification by flash silica gel column chromatography (25% EtOAc/hexanes) was necessary for **14** and **18**.

Purities and isolated yields for these compounds were 91–99% and 35–60%, respectively. See Supplementary data for full compound characterization details.

4.2. Bacterial reporter gene assays

Reporter gene assays for LasR (in *E. coli*), LuxR, and TraR were performed according to our previously reported methods.[21] The LasR assay in the *P. aeruginosa* PA01 MW1 (pUM15) strain was modified from a literature procedure.[37] See Supplementary data for full details of bacteriological protocols.

4.3. Thiolactone hydrolysis study

Thiolactone ligand was incubated in Luria-Bertani medium at 37 °C for predetermined times, and then added to a 96-well plate. The *P. aeruginosa* PA01 MW1 (pUM15) reporter strain was cultured overnight, pelleted, washed, and resuspended in a minimal amount of media. These cells were then added to the 96-well plate containing ligand, incubated for 8 h, and the wells were analyzed for optical density and YFP fluorescence. Fluorescence was normalized to cell density per well, and each time point was referenced to the cell-density-normalized readout of ligand prepared at time 0 (i.e., the positive control).

Acknowledgments

Financial support for this work was provided by the NIH (AI063326) ONR (N00014-07-1-0255), Greater Milwaukee Foundation Shaw Scientist Program, Burroughs Welcome Fund, Camille & Henry Dreyfus Foundation, Research Corporation, and Johnson & Johnson. C.E.M. was supported in part through a DOD (Air Force Office of Scientific Research) National Defense Science and Engineering Graduate (NDSEG) Fellowship (32 CFR 168a). We gratefully acknowledge Professors Peter Greenberg (University of Washington), Stephen Winans (Cornell University), and Edward Ruby (UW–Madison) for donations of bacterial strains and advice on their manipulation.

Supplementary data

Supplementary data (compound characterization data, biological assay protocols, and additional screening data) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.071.

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