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N-Acyl Homoserine Lactone Analog Modulators of the *Pseudomonas aeruginosa* RhlI Quorum Sensing Signal Synthase

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Abstract

Virulence in the Gram-negative pathogen *Pseudomonas* aeruginosa relies in part on the efficient functioning of two LuxI/R dependent quorum sensing (QS) cascades, namely, the LasI/R and RhlI/R systems that generate and respond to *N*-(3-oxo)-dodecanoyl-L-homoserine lactone and *N*-butyryl-L-homoserine lactone, respectively. The two acyl homoserine lactone (AHL) synthases, LasI and RhlI, use 3-oxododecanoyl-ACP and butyryl-ACP, respectively, as the acyl-substrates to generate the corresponding autoinducer signals for the bacterium. Although AHL synthases represent excellent targets for developing QS modulators in *P. aeruginosa*, and in other related bacteria, the identification of potent and signal synthase specific inhibitors has represented a significant technical challenge. In the current study, we sought to test the utility of AHL analogs as potential modulators of an AHL synthase and selected RhlI in *P. aeruginosa* as an initial target. We systematically varied the chemical functionalities of the AHL headgroup, acyl chain tail, and head-to-tail linkage to construct a small library of signal analogs and evaluated them for RhlI modulatory activity. Although the native *N*-butyryl-L-homoserine lactone did not inhibit RhlI, we

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Supporting Information

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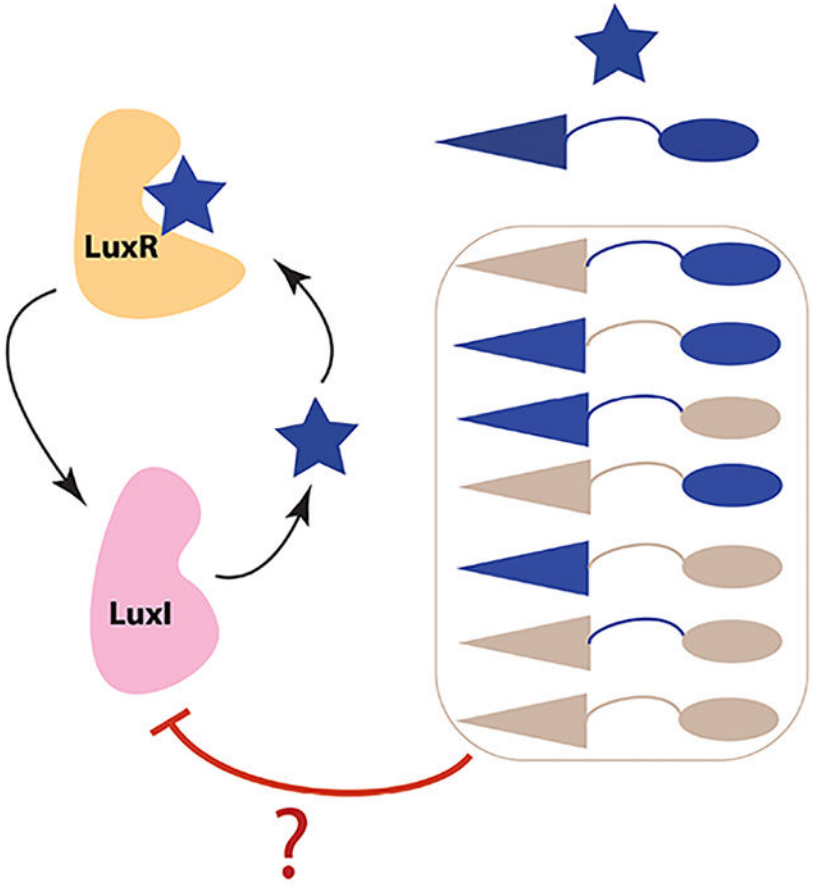
discovered that several of our long-chain, unsubstituted acyl-D-homoserine lactones and acyl-D-homocysteine thiolactones inhibited while a few of the 3-oxoacyl-chain counterparts activated the enzyme. Additional mechanistic investigations with acyl-substrate analogs and docking experiments with AHL analogs revealed two distinct inhibitor and activator binding pockets in the enzyme. This study provides the first evidence of the yet untapped potential of AHL analogs as signal synthase modulators of QS pathways.

Graphical Abstract



Quorum sensing (QS) is a cell counting mechanism used by certain bacteria to assess their local population densities. At low cell densities, these bacteria usually operate as individual cells. At higher cell densities, however, their behavior turns to a teamwork mode in support of collective processes such as production and sharing of public goods within the immediate microbial community, toxin secretion, and the development of resistance to an imminent antibiotic attack.[1,2] This transition from a solitary to social behavior occurs when the bacterial population attains a “quorum” level. Bacteria rely on sensing specific chemical signals, or autoinducers, in their local environment to determine if a quorate population necessary to trigger cooperative, social traits is achieved. A perturbation in this cell counting (i.e., QS) process would disrupt bacteria’s social networking skills, making them vulnerable

against external threats such as a looming antibiotic attack.[3,4] QS inhibitors work toward achieving this end goal.

Gram-negative bacteria typically use LuxI-type AHL synthase and LuxR-type AHL receptors for QS.[5] The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* utilizes QS to form biofilms in a range of clinically relevant environments, including the lungs of cystic fibrosis patients and many common implanted devices.[6–9] The virulence of this pathogen relies, at least in part, on the efficient functioning and control of two LuxI/R QS signaling cascades, namely, the Las and Rhl QS systems. Although both the LasI and RhlI AHL synthases utilize S-adenosyl-L-methionine (SAM) as the common acyl acceptor, they differ in the selective recognition of 3-oxododecanoyl-ACP (ACP: Acyl Carrier Protein; LasI) and butyryl-ACP (RhlI) to synthesize the corresponding *N*-acyl-L-homoserine lactone autoinducers for the bacterium (Figure 1). *P. aeruginosa* also has a third *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4(1H)-quinolone) made by a set of five genes pqsABCDE.[10] The three *P. aeruginosa* QS circuits (las, rhl, and pqs) contribute to the overall virulence, albeit via different mechanisms and virulence factors.

In general, AHL-based QS pathways such as those found in *P. aeruginosa* could be interrupted by one or more of the following methods: (a) sequestration and destruction of AHL signals using catalytic antibodies, (b) quorum quenching enzymes, (c) small molecules that target the LuxR-type receptor, and (d) LuxI-type AHL synthase inhibitors.[10–15] Although numerous chemical and biologic-based QS interrogators have been identified using the first three approaches, inhibitors of LuxI-type AHL synthases are far fewer in number.[16] Small molecules that inactivate AHL synthases would limit signal synthesis, interfere with the bacteria's ability to census count in the vicinal environment, and could provide useful leads to develop QS inhibitors. Several studies indeed report AHL synthases as viable targets for inhibiting QS in pathogenic bacteria.[14,17,18] Molecules that mimic the AHL products are particularly well positioned to act as AHL synthase inhibitors due to their following unique advantages: (1) AHL analog inhibitors are likely QS specific. (2) AHLs have favorable diffusion characteristics. (3) AHLs could, in principle, inhibit both LuxI- and LuxR-type proteins, thereby increasing the potency and QS specificity of these compounds, and (4) AHL analogs that either inhibit LuxI- or LuxR-type proteins would provide valuable chemical tools for mechanistic interrogation of QS pathways. In addition, depending on their structure, compounds resembling AHLs could potentially evade efflux pump recognition resulting in enhanced potency of these analogs *in vivo*.[19] Since many AHL analogs have been previously identified as both QS agonists and antagonists, we were interested in determining if some of these analogs could also inhibit the AHL synthase.[10,15,20] To test this question, we decided to conduct an exploratory study with the enzymatically well-characterized AHL synthase from *P. aeruginosa*, RhlI.

To the best of our knowledge, we have not found any report supporting the concept of AHLs or their analogs as AHL synthase inhibitors. In fact, Parsek *et al.* describe holo-ACP and MTA as weak inhibitors of RhlI, but no inhibition was observed for *N*-butyryl L-homoserine lactone even at 1 mM concentration.[21] While this result seemed discouraging at first, the paucity of AHL synthase inhibitors discovered to date, and the unique advantages offered by AHL analogs to elicit QS specific inhibition motivated us to further explore this line of

inhibitors for the RhlI AHL synthase. In the current study, we made systematic modifications to the AHL headgroup, acyl-chain tail, and linkage connection between the headgroup to the tail to determine "AHL" molecular features that could be utilized in the development of modulators for the RhlI enzyme. This approach revealed the *first set of AHL-based activators and inhibitors* for the RhlI AHL synthase. We believe this strategy could be easily adopted to identify potent and specific modulators of other AHL synthases. In addition, our study underscores the utility of AHL analogs in intercepting both LuxI- and LuxR-type proteins, for mechanistic interrogation of QS signaling pathways and in potentially controlling virulence in pathogenic bacteria.

RESULTS AND DISCUSSION

Compound Selection and Enzymatic Assay.

Our first set of AHL analogs was selected from our recent study developing RhlR modulators to evaluate the importance of the following structural features on RhlI enzymatic rate: (a) modifications in the headgroup (compounds **2**–**4**, **8**–**12**), (b) the "L" vs "D" stereocenter in the headgroup (compounds: **5**, **12**), and (c) tail-to-headgroup linkage (compounds **6**, **7**, **10**–**12**).[15,22] We used the redox dye DCPIP to determine the amounts of holo-ACP thiol released upon acylation of SAM amine by the C4-ACP substrate to estimate initial rates of RhlI-catalyzed C4-homoserine lactone (C4-HSL) synthesis. Initial rates were then measured in the presence of increasing concentrations of AHL analogs to determine the IC_{50} (concentration of AHL analog to attain 50% maximum inhibitory effect) or EC_{50} (half-maximal effective concentration) of these analogs in RhlI-catalyzed C4-HSL synthesis (Table 1, Figures 1, 2, S1; see also assay and dose–response curve methods in the SI).

Initial Assay Results.

The assay data revealed several motifs capable of modulating RhlI. For instance, a change in the chirality (**5**) and linkage (**12**) in the AHL decreased the rate of RhlI-catalyzed C4-HSL synthesis (Table 1, Figures 2 and S1). Although analog **8**, the thiolactone analog of C4-HSL, failed to inhibit RhlI, prior work from the literature revealed significant antagonistic and agonistic effects of acyl-/aryl-thiolactones on QS receptor proteins.[20,23] Therefore, we decided to continue exploring thiolactone analogs in an effort to identify compounds that could potentially modulate both RhlI (AHL synthase) and RhlR (AHL receptor) proteins.[24]

These initial observations prompted us to further expand the AHL analog libraries with alterations to the headgroup stereocenter ("L" vs "D"), headgroup structure (lactone vs thiolactone vs nonlactone), and tail-to-headgroup linkage (amide vs sulfonamide). We therefore synthesized and screened the following classes of AHL analogs in the RhlI enzymatic assay to investigate the impact of varying acyl-/aryl-chain lengths on RhlI activity: (a) homoserine lactones, (b) acyl-sulfonamide-homoserine lactones, (c) homocysteine thiolactones, and (d) nonlactone compounds (Figure 3).

Investigations of Homoserine Lactone Analog RhlI Modulators (l-HSL and d-HSL).

In many studies of LuxR-type receptors, modifications to the acyl-chain had significant antagonistic and agonistic effects on the receptor. To determine whether the same was true

for RhlI, the native headgroup, L-HSL, was acylated with various acyl, 3-oxoacyl, and 3-hydroxyacyl-chains (compounds **1**, **13**–**27**; Figures 2 and 3). Regardless of the modification to the hydrocarbon chain, none of the 16 analogs in the L-HSL series inhibited RhlI (Figure S2). Interestingly, two out of seven acyl-D-HSLs inhibited the enzyme (Figure S3): compound **5** (C4-D-HSL), IC_{50}: 688 ± 91 μM, and compound **31** (4-phenylbutanoyl-D-HSL), IC_{50}: 20 ± 10 μM (Table 1). The inhibition observed for compound **31** was a surprise because the phenylbutanoyl chain was expected to be too large to fit in the acyl-chain binding pocket of RhlI (assuming this compound targets that site). The activity of 3-oxoacyl-D-HSL derivatives, however, showed an interesting trend. While compound **35** (3-oxoC8-D-HSL), a medium chain-length analog, inhibited RhlI (IC_{50}: 282 ± 34 μM), the shorter chain derivative, compound **34** (3-oxoC6-D-HSL), activated RhlI (EC_{50}: 224 ± 55 μM; Figure S3).

Evaluation of Sulfonamide-Linked Acyl-Homoserine Lactones.

Unlike the amide-linked acyl-HSLs, both L and D short-chain sulfonamide derivatives inhibited RhlI (Figures 3 and S4). Compound **12**, a sulfonamide-D-HSL analog, was a more potent inhibitor than compound **37**, a sulfonamide-L-HSL analog (Table 1). However, the long-chain acyl-sulfonamide analogs failed to inhibit RhlI.

Homocysteine Thiolactone Analog-Based Modulators of RhlI (l-HCTL and d-HCTL).

Although compound 8 (C4-L-HCTL) did not inhibit RhlI, expansion of the hydrocarbon tail library for the L-thiolactones to 12 carbons yielded two weak inhibitors: C12-L-HCTL (**46**) and 3-oxoC12-L-HCTL (**55**) with IC_{50} values of 387 ± 89 μM and 621 ± 50 μM, respectively (Table 1; Figures 4 and S5). Except for the 12-carbon chain analogs, none of the short-chain derivatives affected RhlI activity. Mirroring the homoserine lactone series, the D-thiolactone analogs also produced much more potent inhibitors than L-thiolactones (Figures 3 and S6). For instance, the IC_{50} for C12-D-homocysteine thiolactone, compound 60 (11 ± 1 μM), was about 34-fold lower than the IC_{50} for the L-thiolactone counterpart, compound **46** (387 ± 89 μM). A similar trend was observed in the 3-oxoacyl series as well (e.g., compare the IC_{50} of compound **55** with compound **64**; Table 1). Like the 3-oxoacyl-D-HSL series, which was activating with a shorter chain and inhibiting with a longer chain, the 3-oxoC6-, 3-oxoC8-, and 3-oxoC10-D-HCTL (compounds **61**–**63**) activated while the 3-oxoC12-D-HCTL (compound **64**) inhibited RhlI. As the chain length increased from 3-oxoC6-D-HCTL (compound **61**) to 3-oxoC8-D-HCTL (compound **62**) and 3-oxoC10-D-HCTL (compound **63**), the activation potency increased proportionately as revealed by decreasing EC_{50} values of 1506 ± 110 μM (**61**), 855 ± 74 μM (**62**), and 57 ± 23 μM (**63**). Since molecules that activate enzymatic activity are relatively uncommon, we were pleasantly surprised to identify a few of them from a small library. Enzymatic activation could potentially occur due to one or more of the following mechanisms: an activator (a) could increase k_{cat} or decrease K_m (or both) of the substrate, (b) could bind at an allosteric site (thereby not impeding substrate binding to the enzyme) promoting an activated, catalytically competent enzyme conformation primed for catalysis, and/or (c) could rapidly bind and dissociate (high k_{on} and k_{off}) from the enzyme before the acyl-substrate binds leaving the enzyme in an “activated” state. Investigations are currently ongoing to address potential mechanisms of activation. Finally, none of the nonlactone compounds inhibited RhlI, suggesting that the fine balance

of polarity and hydrophobicity present in the lactone or thiolactone is required to observe modulatory activity (Figures 3 and S7).

The double reciprocal plot of initial rate versus variable C4-ACP concentrations at various fixed concentrations of C12-D-HCTL (**60**) or 3-oxoC12-D-HCTL (**64**) show a set of parallel lines, indicative of uncompetitive-mode of inhibition, which suggests that AHL analog inhibitors compete for binding to the AHL binding site ([E.MTA] conformation; Figures 1 and S8). Further analysis using Akaike's method (AIC) also confirms uncompetitive inhibition modes for both inhibitors (Figures S8 and S9).

Specific vs Nonspecific Inhibition.

RhlI is a short-chain AHL synthase where we reasoned, at least initially, that the acyl-chain binding pocket should restrict the binding of longer acyl-chains. To test if the inhibitory effects observed for long-chain lactone and thiolactone derivatives arose due to nonspecific binding of the headgroup or acyl tail to the RhlI, the enzymatic initial rate was measured in the presence of increasing concentrations of the headgroup (L-HSL, D-HSL, L-HCTL, and D-HCTL; compounds **71**–**74** in Figure 3) or the fatty acid chain tail (butyric, hexanoic, octanoic, decanoic, and dodecanoic acid; compounds **75**–**79** in Figure 3) or combinations of both (D-thiolactone + dodecanoic acid, compounds **74** + **79**, mimicking compound 60; Figure S7). None of these compounds or combinations had any effect on RhlI activity, suggesting that the observed inhibition/activation effects are not due to nonspecific binding of AHL analogs to the enzyme. Furthermore, the lack of inhibition for "**74** + **79**" also suggests that the two moieties must be covalently linked to each other to observe the modulatory effects described above. Our data also suggest that micelles cannot account for the perceived inhibitory effects of long-chain AHL analogs because the range of inhibitor concentrations used to determine IC_{50} for compound **60** and other related inhibitors are below the critical micelle concentrations reported in the literature (see Figure S6 legend for additional information).[25]

Last, we removed the maltose binding tag from RhlI and determined the (a) K_m's of butyryl-ACP and octanoyl-ACP and (b) IC_{50}'s of butyl-ACP and octyl-ACP with tag-free RhlI. We notice that both the K_m's of acyl-ACPs and IC_{50}'s of alkyl-ACPs with untagged RhlI were almost identical to the MBP-tagged enzyme (Tables 2 and 3, Figures S10–S12). These results provide strong evidence that the modulatory effects observed for longer-chain AHL analogs could not be discounted as an artifact of the maltose binding fusion conjugate protein attached to RhlI.

A Binding Site for the Longer Acyl-Chains?

The X-ray structures of AHL synthases reported to date reveal that these enzymes fold into a mixed $\alpha-\beta-\alpha$ sandwich with a V-shaped hydrophobic cleft to accommodate the nonpolar acyl-chain of the acyl-substrate.[26–28] The amino acids lining the cleft appear to confer some *specificity to the acyl-chain binding* in this pocket.[29–32] For instance, in the 3-oxohexanoyl-ACP utilizing AHL synthase EsaI, the bottom of the acyl-chain pocket is occupied by hydrophobic amino acid residues with larger side chains, restricting the acyl-chain length to six carbons.[5,27] In contrast, the acyl-chain pocket in 3-oxoC12-ACP preferring LasI is deep

to accommodate a long C12 chain.[28] We reasoned that the acyl-chain pocket in the C4-ACP utilizing RhlI AHL synthase would be narrower than the EsaI synthase to limit binding of longer-chains at this site. If this assumption is true, the long-chain AHL analogs should bind to a site (henceforth termed the "inhibition pocket") that is distinct from the acyl-chain pocket. To investigate if this inhibition pocket overlapped with the "bonafide" acyl-chain pocket in the enzyme, we resorted to mechanistic investigations on ACPs bound to shorter and longer acyl-chains.

Acyl-ACP substrates include three important moieties: (a) an acyl-chain, (b) a phosphopantetheine linker that connects acyl-chain cargo to the ACP, and (c) a predominantly acidic three- or four-helix bundle carrier protein.[33] The relatively large contact surface between carrier protein and phosphopantetheine of the acyl-ACP and a complementary polar patch on AHL synthases such as RhlI should limit the placement of the longer chains in or around the "acyl-chain pocket" in the enzyme. Since the acyl-chain pocket of RhlI is (we assume) not optimized to hold longer chains, we expected that [E.(C6–C12)-ACP.SAM] should be unstable relative to the [E.C4-ACP.SAM] ternary complex. Under this assumption, the K_m of long-chain acyl-ACPs should be greater than C4-ACP reacting with RhlI.

To our surprise, the K_m of longer-chain acyl-ACPs progressively decreased from 7 μM in C4-ACP to about 0.2 μM in C12-ACP (Figure S13 and Table 2). The decrease in K_m for longer-chain acyl-ACPs is also consistent with the lower IC_{50}'s observed for the longer-chain AHL analogs (compounds **55**, **60**, and **64**; Figure S14). Since Michaelis constants do not reveal the true substrate binding constants to the enzyme, we determined the relative affinities of short, medium, and long chains by determining the IC_{50} and K_i's of alkyl-ACPs, the inert analogs of acyl-ACP substrates, with the RhlI (Table 3, Figure 3).[34] Both the IC_{50} and K_i values of alkyl-ACPs decreased as alkyl-chain length increased, which mirrors the trends in K_m for acyl-ACPs (compounds **80**–**84**; Tables 2 and 3 and Figures 3, S14, and S15) and IC_{50} values for AHL analogs. Preincubation of RhlI with butyl-ACP or octyl-ACP did not significantly alter the % inhibition or the IC_{50} values, suggesting that alkyl-ACP inhibition is neither slow-binding nor time-dependent (Figure S16). Since both alkyl-ACP and alkyl-CoA contain the common phosphopantetheinyl moiety, we were interested to test if RhlI also bound to longer-chain alkyl-CoAs tighter like the longer-chain alkyl-ACPs (Figure 3). Indeed, we note a similar trend for alkyl-CoAs with butyl-Coenzyme A (butyl-CoA) not inhibiting RhlI up to 1 mM, while a steep increase in inhibition was observed for hexyl-CoA and octyl-CoA (see Table 3 and Figure S17). The above results suggest that the acyl-chains in AHLs, acyl-ACPs, alkyl-ACPs, and alkyl-CoA are likely binding to a common pocket in RhlI.

To further define this common pocket in RhlI, we characterized the inhibition mode for all alkyl-ACPs used in this study by conducting substrate-velocity measurements under different alkyl-ACP concentrations. All alkyl-ACPs displayed noncompetitive inhibition with RhlI, possibly binding to at least two enzyme conformers, most likely the [E.SAM] and [E.MTA] forms, separated by an irreversible holo-ACP product release step (Figures 1, S14).[35] Thus, the inhibition experiments suggest that both AHLs and alkyl-ACPs could compete for binding to the [E.MTA] complex (see above discussion on inhibition mode for 60 and 64;

Figures 1, S8, and S9). The activity trends as a function of acyl-chain lengths in AHL, acyl-ACP, alkyl-ACP, and alkyl-CoA also reaffirm that the long acyl-chains in AHL analogs likely bind to a common pocket that must, at least partially, overlap with the putative acyl-chain pocket of this enzyme. Interestingly, despite the lower K_i's, we notice the % maximum inhibitions are also lower for longer-chain AHLs, alkyl-ACPs, and alkyl-CoAs, reflecting higher k_{off} for these compounds relative to the native C4-chain binding to RhlI (Figures S1–S7, S14, S17). Taken together, these data suggest that the inhibition site is not well-optimized to hold the long aliphatic chain.

Structural Analyses.

The X-ray structures of EsaI (3-oxoC6-ACP), TofI (C8-ACP), LasI (3-oxoC12-ACP), and BjaI (isovaleryl-CoA) reported to date confirm that all of these LuxI-type synthases adopt a GNAT-fold with β4 and β5 strands forming a V-shaped cleft to hold the acyl-chain of the acyl-substrate.[5,27,28,36] Although the structure of RhlI has not been reported yet, we used the Phyre2 program to build a homology model for this protein. The RhlI homology model was found to conform to the GNAT-fold adopted by the other AHL synthases. In the RhlI enzyme model, the bottom of the acyl-chain pocket was filled with larger residues such as leucine and valine that would effectively seal off the bottom-half of this pocket, restricting only small acyl-chains to bind at this site. Using AutoDock Vina, we computationally docked compounds **31** (4-phenylbutanoyl-D-HSL; IC_{50}: 20 ± 10 μM), **34** (3-oxoC6-D-HSL; EC_{50}: 224 ± 55 μM), **35** (3-oxoC8-D-HSL; IC_{50}: 282 ± 34 μM), **46** (C12-L-HCTL; IC_{50}: 387 ± 89 μM), **55** (3-oxoC12-L-HCTL, IC_{50}: 621 ± 50 μM), **60** (C12-D-HCTL; IC_{50}: 11 ± 1 μM), **63** (3-oxo-C10-D-HCTL; EC_{50}: 57 ± 23 μM), and **64** (3-oxoC12-D-HCTL; IC_{50}: 127 ± 70 μM) into RhlI to identify potential binding sites for acyl-chains in both inhibitors and activators of this enzyme.[37]

The acyl-chain in all of the inhibitory compounds (**31**, **35**, **46**, **55**, **60**, and **64**) was observed to occupy a common pocket directly behind the acyl-chain pocket (referred to as the "inhibition pocket;" see Figure 4) in the enzyme. This pocket is formed by two orthogonal helices, α5 and α6, with β5 and β7 strands covering the lid. The inhibition pocket is both deep and wide, filled with predominantly hydrophobic residues such as L102, Y105, F120, L124, V135, M143, F147, V152, and F173. The thiolactone headgroup in the D-isomer (compound **60**) stacks with two aromatic residues, F28 and W34, while in the L-isomer (compound **46**) the sulfur atom of the thiolactone points toward M143 (Figures 4 and 5). In homoserine lactone compounds (such as compound **36**), the polar oxygen atom in the lactone headgroup points outside toward the surface of the enzyme. The overlay of docking poses in **60** and **46** suggests that the acyl-chains of the two isomers overlap quite well. Therefore, the differences in the positioning of the respective headgroup in RhlI likely contribute to activity differences observed among **36**, **46**, and **60**. The strong edge to face stacking of the D-thiolactone with F28 and W34 may stabilize the headgroup in the RhlI active site, resulting in stronger inhibition relative to the L isomer. In compound **64**, the C3 oxygen atom forms an internal hydrogen bond resulting in a six-membered ring structure, which places the headgroup closer to W34 but not with F28. The intervening methylene groups (C5–C9) in all of the inhibitory compounds are surrounded by a wall of aromatic residues, namely, Y105, F120, F147, and F173. Additionally, a leucine-valine clamp (L124

and V135) holds the terminal methyl group in the 12-carbon chain analogs of **46**, **60**, and **64**. Docking experiments with **35** suggest that the shorter, 3-oxoC8 chain does not go deep enough in the inhibition pocket to reach L124 and V135. Interestingly, a phenylalanine clamp has been reported to grasp the terminal methyl group of 3-oxoC12-HSL substrate in the *P.* aeruginosa AiiA lactonase.[38]

We docked **63** (an activator) on RhlI to understand why this compound activated the enzyme. Compound **63** was found to bind to RhlI in two distinct conformations. In conformation 1, the acyl-chain binds to the inhibition pocket observed for inhibitory compounds. In the second conformation, the acyl-chain loops around the headgroup (like a "U") to bind in a pocket that is diametrically across from the inhibition pocket. The residues that line-up the "activation pocket" are highlighted in Figure 5. Like compound **63**, the two 3-oxoacyl-D-homoserine lactones **34** and **35** bind to RhlI in two modes with acyl-chains occupying both inhibition and activation pockets (Figures S19 and S20). Furthermore, we observe that the shorter chains are likely to populate the activation pocket more than the inhibition pocket as their acyl-chains (also in compounds **34**, **61**, and **62**) are not sufficiently long to reach the leucine-valine clamp. The docking analysis presented in this study, however, can neither predict the relative residence times of the compound's acyl-chain in each of these pockets nor reveal the active conformation of the enzyme to which the ligand binds (receptor is kept rigid). Despite these limitations, the acyl-chains in all of our docked compounds (**31**, **34**, **35**, **36**, **46**, **55**, **60**, **63**, and **64**) bound at either the same inhibition or activation pockets, reaffirming that the binding *pockets* predicted by this analysis are likely to be correct. It is worth noting that the activation pocket contains many polar residues. In general, a nonpolar acyl-chain should fit best in the hydrophobic, inhibition pocket. When a shorter acyl-chain of an AHL analog (such as in compound **34**) is not long enough to bind deeper in the predominantly hydrophobic inhibition pocket, they reorient to bind in the activation pocket of the enzyme (provided the headgroup, 3-oxo, or other moieties assist in binding; see Figures S19–S21). It appears that the extent to which one binding mode is preferred over the other depends on a delicate balance of favorable interactions between the enzyme and the headgroup, 3-oxo, and acyl-chain moieties of a modulator.

SUMMARY

The objective of this study was to investigate the potential of AHL analogs as LuxI-type synthase modulators. We make the following inferences based on the results accrued from studying a small, focused AHL analog library with the RhlI synthase: (a) the "D" stereocenter at the headgroup increased the inhibition potency compared to the "L" enantiomer for both lactones and thiolactones; (b) longer, unsubstituted acyl-chain thiolactones resulted in stronger inhibitors; (c) the thiolactone headgroup attached to short and medium 3-oxoacyl-chains produced more activators than the unsubstituted acyl-chains; and (d) the inhibition potency (IC_{50}) and % maximum inhibition of AHL analogs share an inverse relationship (Figures 3, S1–S7). Despite being a short-chain AHL synthase, our study of AHL analogs supports a novel binding site in RhlI that can accommodate longer acyl-chains. We reason that this inhibition pocket is broad and deep with significant wiggle room for acyl-chains to move around in this site. The acyl-chain of the AHL analog modulators reported in this study are probably too small to fit in this large pocket, which

would increase the off-rate (promote ligand dissociation) and decrease the inhibition potencies of these compounds.

Docking analyses of AHLs into a computationally derived model of RhlI does shed some light on the future development of potent inhibitors for this enzyme. For instance, aryl-chains should pack well with residues that form the aromatic wall (Y105, F120, F147, and F173; see Figure S18), and branched side-chains should fill in the extra space in this pocket, decrease the off-rate, and increase compound potency. Indeed, docking of compound **31** reveals tighter interactions between the phenyl side-chain in the inhibitor and the aromatic cluster in the enzyme (Figure S18). The activation pocket is also wide where the acyl-chains attempt to reach distal residues including L80 and F85 (Figure 5). Since this activation pocket is closer to the protein surface, it makes sense that the pocket is filled with a cluster of polar and charged residues such as R24, K162, E166, and T167 (Figure 5). It is reasonable to assume that a polar activation pocket is not well-suited to stabilize a nonpolar side-chain and thus the acyl-chain moves out of this pocket to interact with distant residues such as L80 and F85. Therefore, increasing the acyl-chain polarity should facilitate interactions of the AHL side chain with these polar residues (through electrostatics and hydrogen bond), stabilize the acyl-chain in the activation pocket, and thus increase the potency of AHL-based activators of the RhlI synthase.

To close, we note that we recently conducted a comprehensive study delineating the SAR of AHL analogs with the RhlR protein, and many of those compounds were evaluated herein. We notice some striking differences in their specificities between the RhlR and RhlI proteins.[22] For instance, the RhlR modulators require an “L” stereocenter in the headgroup, while the RhlI modulators preferentially contain the “D” isomer of the headgroup (Figure 6). Similarly, RhlR shows high selectivity toward shorter acyl-chains, while RhlI prefers longer acyl-chains for modulatory activity. These two studies provide us with the first snapshot of the differences in the SAR of AHL analogs between LuxI- and LuxR-type proteins in *P. aeruginosa*, and to our knowledge, any bacteria utilizing LuxI/R QS. Compounds that modulate either class of protein or both should serve as useful mechanistic probes to interrogate QS pathways. Finally, due to the proof of concept nature of this study, we restricted our choice of compound structures to a relatively narrow chemical space. An expanded library with a larger chemical space should likely yield more potent quorum signal synthase modulators for a wider range of bacteria, and such investigations are ongoing.

MATERIALS AND METHODS

See the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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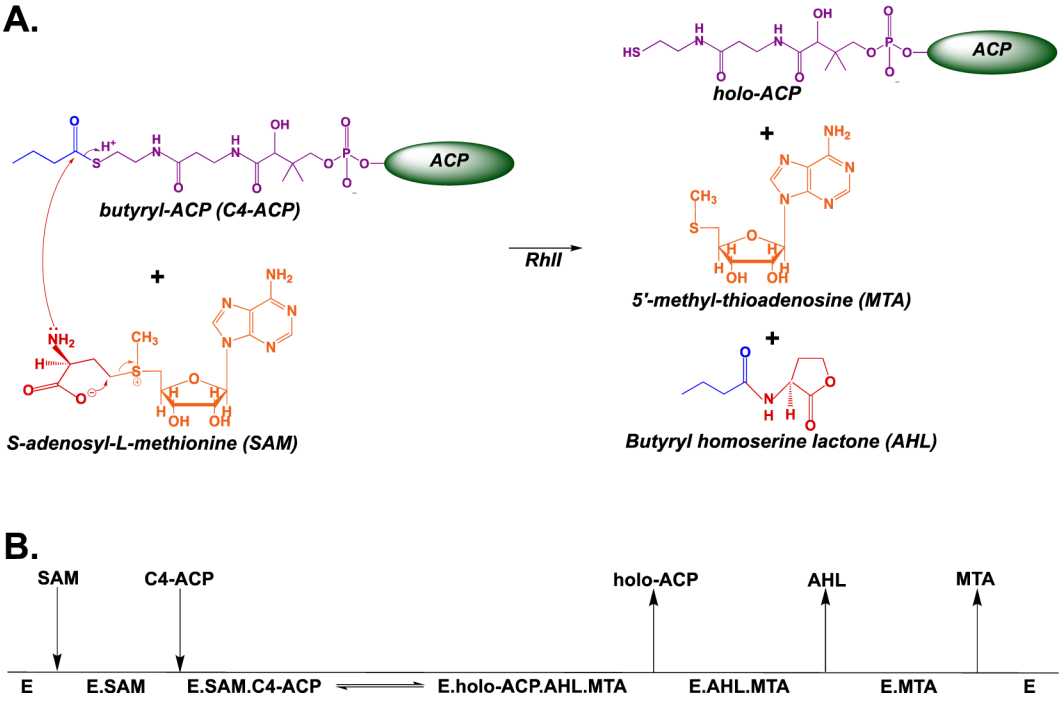

Figure 1.

RhlI catalyzed AHL synthesis. (A) Enzymatic steps in *N*-butyryl L- homoserine lactone autoinducer synthesis. SAM substrate undergoes acylation and lactonization reactions to form butyryl homoserine lactone. The acyl-chain, phosphopantetheine, and ACP are colored, respectively, in blue, purple, and green. The MTA fragment of SAM is shown in orange. (B) Mechanism of substrate addition and product release in RhlI. E in this panel refers to the RhlI enzyme.[21]

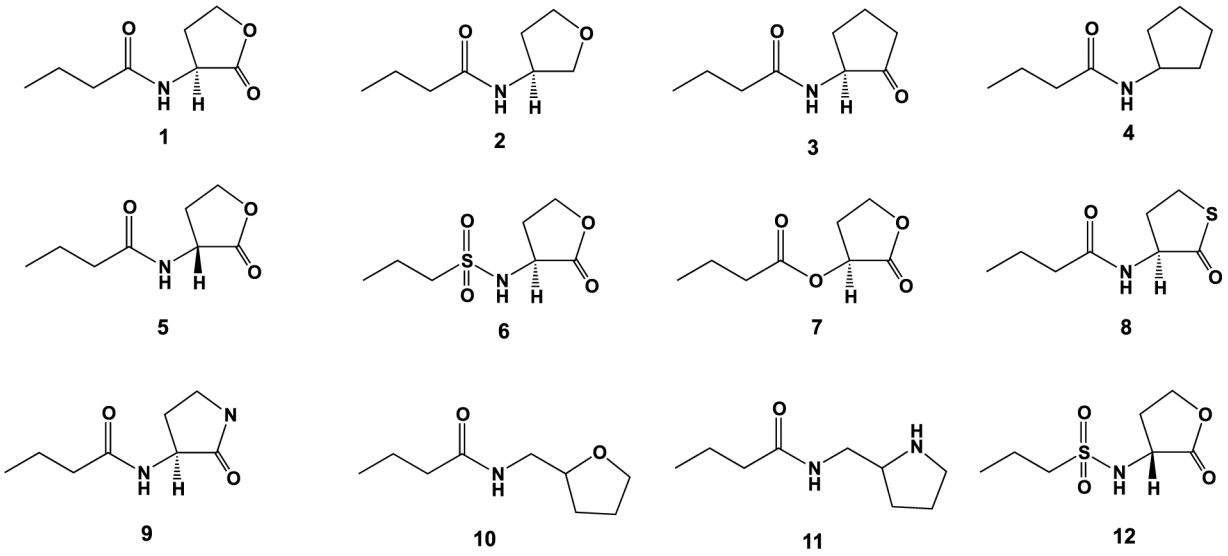

Figure 2.

Headgroup and acyl-chain connectivity in RhlI modulation. Our initial compounds were chosen to evaluate the effect of changes to the stereocenter, chemical functionalities of the headgroup, and acyl-chain-headgroup connectivity for RhlI modulatory activity.

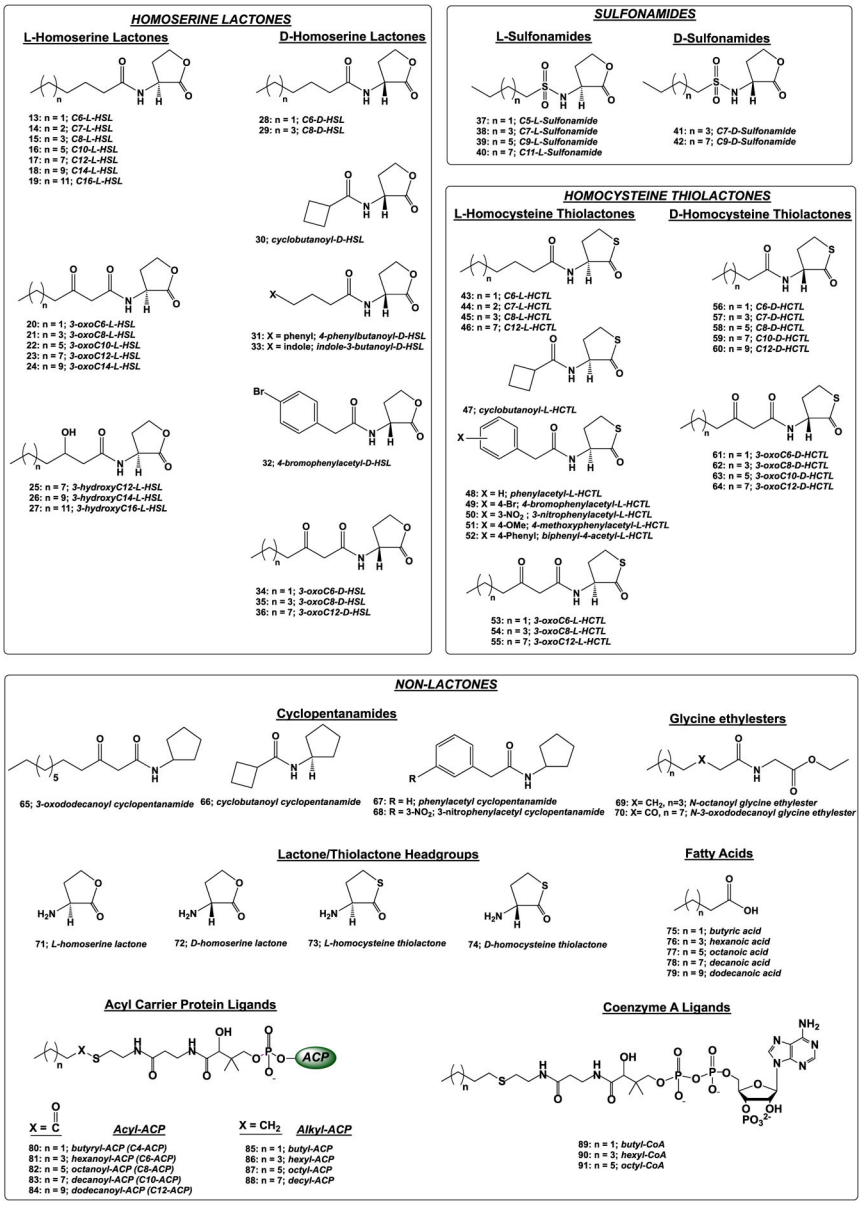

Figure 3.

Chemical structures of compounds used in this study. The four major classes of AHL analogs evaluated in this study are represented in individual boxes. The inert alkyl-ACP/alkyl-CoA analogs were used to determine the relative binding affinities of various alkyl chains with the RhlI enzyme.

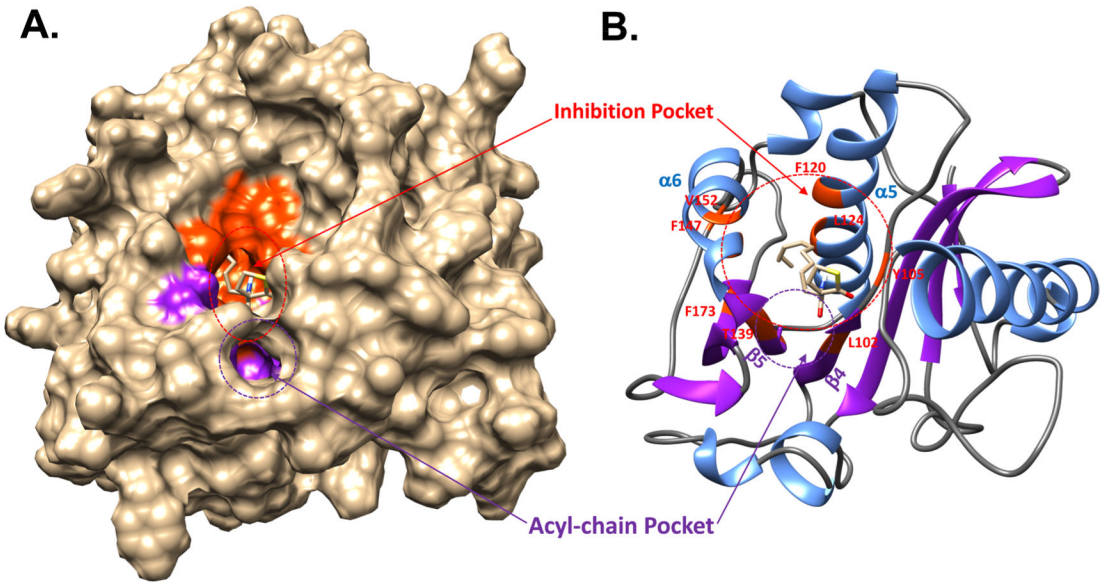

Figure 4.

Inhibition pocket in RhlI. Compound **60** (tan-colored stick model) is docked on RhlI. (A) Surface view of inhibition and acyl-chain pockets. The inhibition and the acyl-chain pockets are colored, respectively, in orange and purple. (B) RhlI homology model. The helices, strands, and coil of RhlI are colored, respectively, in cornflower blue, purple, and gray.

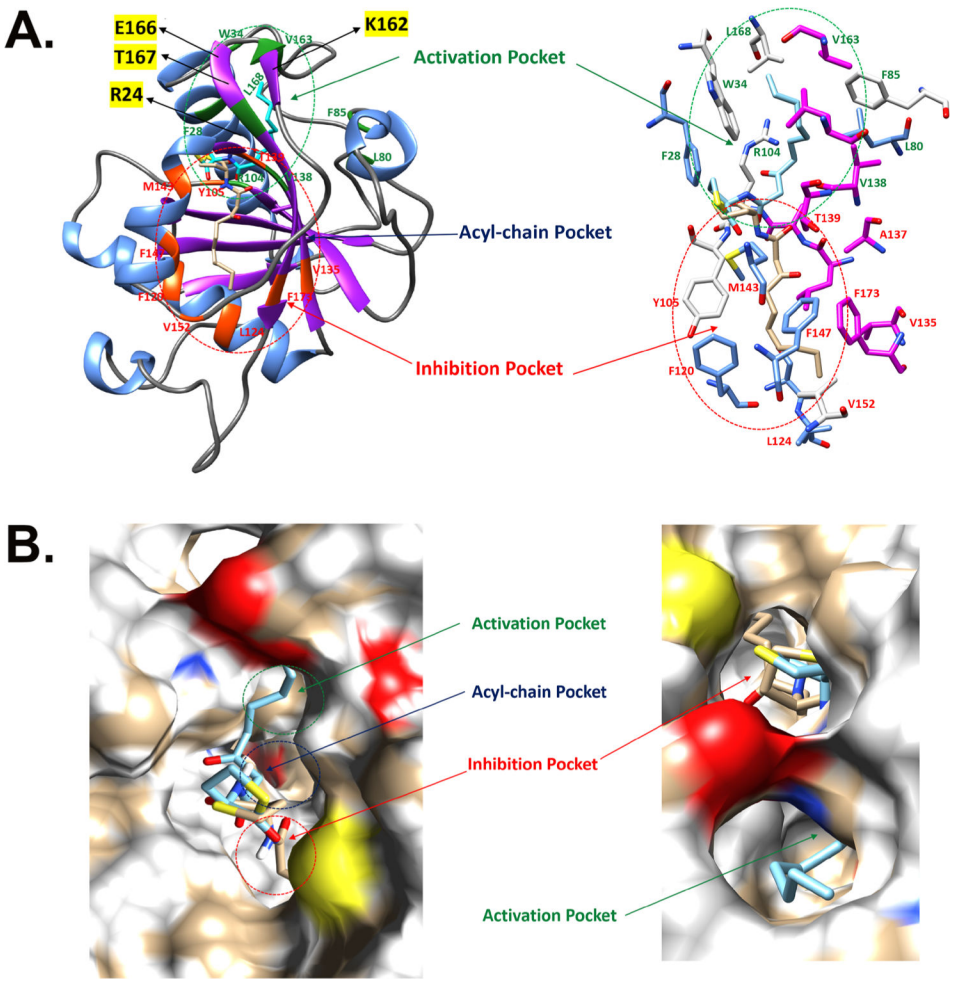

Figure 5.

Activation pocket in RhlI. Compound **63** is docked on RhlI. In general, activators bind to RhlI in two distinct modes. (A) Overlay of two binding modes of compound **63**. Inhibition and activation modes of compound **63** are represented, respectively, in tan and cyan colors. Residues that line up activation and inhibition pockets in RhlI are colored, respectively, in green and red. The helices, strands, and coil of RhlI are colored, respectively, in cornflower blue, purple, and gray. Left panel is a ribbon diagram representation of the two pockets, while the right panel describes the binding mode of compound **63** in each of these pockets. Polar residues in the activation pocket are highlighted in yellow. (B) Surface representation of inhibition, acyl-chain, and activation pockets. Left panel and right panels, respectively, show pocket views looking above the acyl-chain and activation pockets.

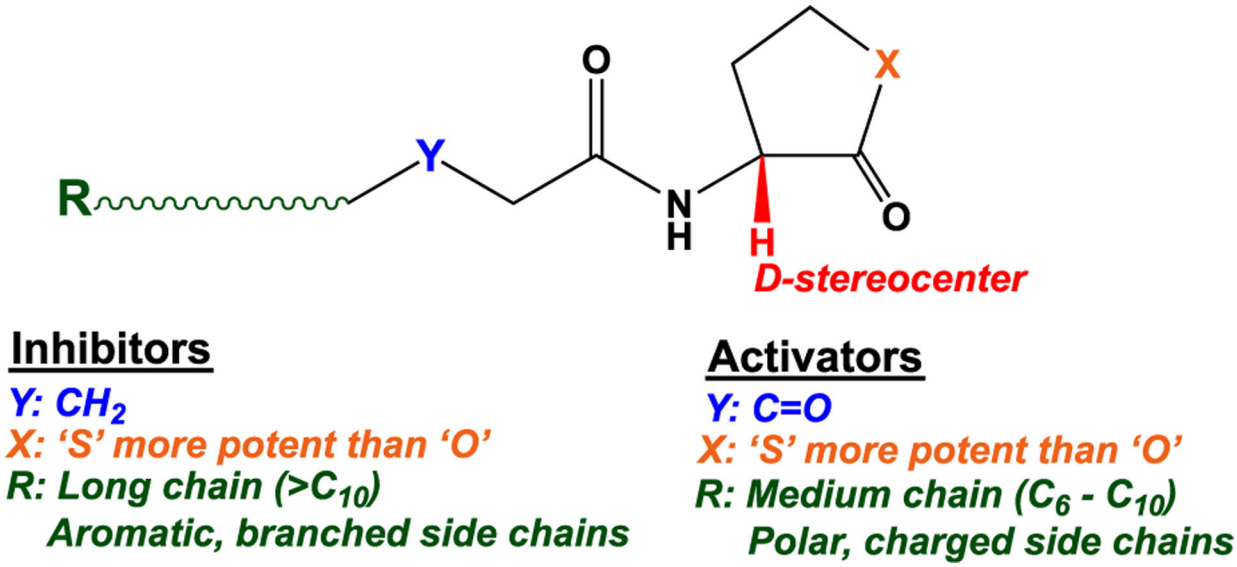

Figure 6.

Molecular features of RhlI modulators. Both activators and inhibitors prefer the "D" stereoisomer over the naturally occurring "L" isomer in the headgroup for modulatory activity.

Table 1.

Inhibition and Activation Data for RhlI Modulators[a]

compound	IC_{50} (μM)	EC_{50} (μM)
5	688 ± 91	
12	171 ± 98	
31	20 ± 10	
34		224 ± 55
35	282 ± 34	
37	345 ± 79	
46	387 ± 89	
55	621 ± 50	
60	11 ± 1	
61		1506 ± 110
62		855 ± 74
63		57 ± 23
64	127 ± 70	

[a]The remaining compounds in the library neither inhibited nor activated RhlI.

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Table 2.

Kinetic Constants for acyl-ACPs with RhlI

substrate	compound	K_m (μM)	k_{cat} (min^{-1})
butyryl-ACP	**80**	7.3 ± 2.1	2.6 ± 0.3
hexanoyl-ACP	**81**	1.2 ± 0.2	0.86 ± 0.02
octanoyl-ACP	**82**	0.40 ± 0.06	0.60 ± 0.02
decanoyl-ACP	**83**	0.14 ± 0.04	0.38 ± 0.01
dodecanoyl-ACP	**84**	0.26 ± 0.03	0.60 ± 0.01

Table 3.

Inhibition Data for alkyl-ACPs and alkyl-CoAs with RhlI

inert substrate	compound	IC_{50} (μM)	K_i (μM)	inhibition mode
butyl-ACP	**85**	9.9 ± 4.0	15.9 ± 0.9	noncompetitive
hexyl-ACP	**86**	0.7 ± 0.4	10.6 ± 1.1	noncompetitive
octyl-ACP	**87**	0.06 ± 0.02	6.5 ± 0.7	noncompetitive
decyl-ACP	**88**	0.10 ± 0.04	4.8 ± 0.3	noncompetitive
butyl-CoA[a]	**89**		ND	ND
hexyl-CoA	**90**	70 ± 10	ND	ND
octyl-CoA	**91**	5.1 ± 0.8	ND	ND

[a]No inhibition was observed until 1 mM butyl-CoA. ND, not determined.