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Library Screening for Synthetic Agonists and Antagonists of a *Pseudomonas aeruginosa* Autoinducer

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Summary

The autoinducer (AI) that initiates the quorum sensing (QS) signaling cascade in *Pseudomonas aeruginosa* is an acyl-homoserine lactone (acyl-HSL). We initiated a study of the requirements for binding of the AI to its protein effector LasR by synthesizing a library of analogs with the HSL moiety replaced with different amines and alcohols. We tested each compound for both agonist and antagonist activity using a QS-controlled reporter gene assay and found several new agonists and antagonists. A representative antagonist was further tested for its ability to inhibit virulence factors. This data progresses our understanding of the LasR-AI interaction toward the rational design of therapeutic inhibitors of QS.

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen that causes chronic, often fatal infections in immunocompromised individuals. This bacterium uses QS to regulate expression of virulence factors required for disease [1, 2]. QS is an intercellular communication system that in Gram-negative bacteria requires a set of two proteins, one which synthesizes a signaling molecule called an autoinducer (AI) and the other which senses and responds to the AI [3]. In *P. aeruginosa*, two homologous sets of QS proteins have been identified, and their genes are arranged in a cascade. The primary level of regulation consists of the transcription factor LasR that is activated by AI1, 3-oxo-C_{12}-homoserine lactone (3-oxo-C_{12}-HSL) (Figure 1) [4, 5]. This occurs at high cell density when the background level of AI1 produced by each cell has accumulated in the culture media above a threshold concentration. The LasR-AI1 complex activates transcription of the *lasR* gene as well as the *lasI* gene encoding the LasI enzyme that synthesizes AI1, creating a positive feedback regulatory system. Activation of LasR by AI1 also induces expression of a secondary QS system comprised of RhlR and RhlI, which are a second transcription factor and synthase of AI2 (C_4-HSL) (Figure 1), respectively [6]. In a manner similar to the *las* regulon, AI2 activates RhlR, and the RhlR-AI2 complex induces the *rhlI* gene [2]. Activation of each QS transcription factor by its respective AI results in induction of a large number of genes involved in pathogenicity [7].

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The management of chronic *P. aeruginosa* infections remains challenging due to the physiology and genetics of the organism. The QS cascade is an excellent target for design of novel drugs that could reduce the pathogenicity of *P. aeruginosa*, because QS is required for expression of virulence factors and establishment of chronic infections. Autoinducer analogs that act as antagonists to disrupt transcription factor activation would inhibit the QS cascade and prevent induction of QS-regulated virulence factors [8–10].

The structure of a homolog of LasR, TraR from *Agrobacterium tumefaciens*, in a complex with its AI (3-oxo-C_8-HSL) and DNA was recently solved [11, 12]. Although some residues in the TraR crystal structure that directly interact with the AI are conserved in LasR, the overall sequence identity between the two proteins is only 17%. Furthermore, the specificity determinants of each protein for its cognate AI are unknown. Unfortunately, the active LasR protein has yet to be purified due to technical challenges, and therefore we have little information about the LasR-AI1 interaction at the molecular level. Hence, any knowledge regarding this interaction gained indirectly from studies, such as this one, would aid in design of potent inhibitors of *P. aeruginosa* QS.

Two primary strategies have been pursued to identify small molecule inhibitors of QS. The initial studies in this field utilized the natural AI as a starting point for structure-activity relationship studies of the *V. fischeri* QS system. It was found that the length of the acyl chain could be changed, and its autoinducing activity was reduced but not eliminated. Similarly, substitution of the 3-oxo group with hydroxyl or methylene reduced activity, as did introduction of an unsaturated bond anywhere into the side chain [13, 14]. Addition of a phenyl group to the acyl chain terminus resulted in compounds with antagonist activity [15]. A compound with the HSL replaced by homocysteine thiolactone also acted as a modest competitive inhibitor of the *V. fischeri* AI. However, other substitutions to the HSL resulted in loss of activity presumably due to loss of LuxR binding [14]. A similar study of *P. aeruginosa*'s AI1 resulted in similar findings with regard to the side chain, but the homocysteine thiolactone compound was a strong agonist and the corresponding lactam was nearly inactive [16]. The other strategy used natural AI antagonists produced by a marine algae [17–19] as a scaffold for antagonist design [9, 10]. The natural antagonists are halogenated furanones, and a derivative of the natural product was shown to antagonize the *P. aeruginosa* QS system. While several antagonists have emerged from these studies, there remains a need to improve their potency.

We report herein the construction and screening of a library of 96 synthetic AI1 analogs in an attempt to find antagonists that would interfere with LasR activation. The library was designed to replace the HSL moiety with a variety of amines and alcohols. We chose to randomize this region of the AI because it is the common structural element of AIs produced by all organisms with a LasI homolog, many of which are important human and plant

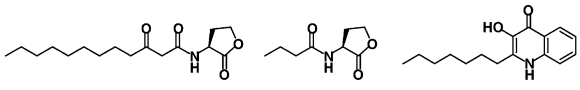

Figure 1. Structure of Three *P. aeruginosa* Autoinducers: AI1, 3-Oxo-C_{12}-HSL; AI2, C_4-HSL; and PQS, *Pesudomonas* Quinolone Signal

pathogens [20, 21]. While our primary goal is to find inhibitors of QS, the library was also screened for agonist activity to gain more insight into the nature of the LasR-AI1 interaction. Examination of agonist structures allows us to infer which AI components are required for LasR activation. We discovered that for strong activation of LasR, a five- or six-membered ring with a hydrogen (H) bond acceptor adjacent to the amine of the HSL is required. Furthermore, inhibition was achieved by compounds that maintained the hydrogen (H) bond acceptor but contained an aromatic ring. A representative antagonist was tested for its QS inhibition activity, and it successfully reduced expression of LasR and RhlR controlled reporter genes and elastase.

Results and Discussion

Library Synthesis

To facilitate generation of the desired library, we immobilized the 3-oxo-C_{12} side chain onto solid phase and rmed coupling with various amines and alcohols in parallel. We modified the terminus of the acyl chain with a hydroxyl group and added an extra carbon atom (see 13-OH-3-oxo-C_{13} in Figure 3) for immobilization to a 3,4-dihydro-2H-pyran (DHP) resin. Based on previous observations that shortening or extending the AI1 side chain one or two carbons did not reduce its activity significantly [16], we were confident that this modification would not interfere with LasR binding. However, a previously identified HSL agonist, homocysteine thiolactone [16], was included in the library to verify this assumption and confirm the ability of our screening techniques to identify agonists. The template molecule **2** (Figure 2) was synthesized in solution phase through a three-step sequence in an overall yield of 38%. The attachment of **2** to the DHP resin and on-resin hydrolysis of the ester moiety proceeded successfully, as evidenced by on-resin FTIR spectroscopy (see Supplemental Data at http://www.chembiol.com/cgi/content/full/10/6/563/DC1). To maximize library diversity, we selected various amines and alcohols with different structural features (the structures are not shown). Parallel coupling of **2** with these molecules was accomplished on a 96-well reactor block. The products were released from the resin by treatment with 95% trifluoroacetic acid, which simultaneously removed the protective ketal group. The individual products were collected into a 96-deep-well plate and dried under vacuum.

Library Screening for Agonists

Screening of individual molecules for their ability to activate LasR was conducted using *P. aeruginosa* PAO-JP2 (*lasI, rhlI*) [2] harboring plasmid p*lasI*-LVAgfp [22]. Since this strain lacks the ability to produce natural AIs due to disruption of both AI synthase genes, activation of LasR relies on the addition of exogenous AI1. Expression of *gfp* (encoding *g*reen *f*luorescent *p*rotein) is under control of the LasR-AI1 inducible *lasI* promoter, allowing rapid screening for LasR agonists by measuring the level of GFP expression in their presence.

Results of screening are shown in Figure 3. Three strong agonists (D11, D12, and H3) and two weaker agonists (C11, E11) were present in the library. The corresponding unconjugated amines were assayed, and no agonist activity was observed (data not shown), indicating that the side chain is crucial for activity. Among the three strong agonists identified is homocysteine thiolactone (D11), which was included in the library as an internal control. This confirmed the reliability of the assay system as well as the assumption that the OH modification at the terminus of the acyl side chain does not interfere with agonist activity. Interestingly, B11 and G12, compounds very similar to D11, were not active. B11 is likely inactive due to the γ-lactam ring structure (which is known to be nearly inactive) [16] and interference of the oxygen substitution with putative hydrophobic interactions between the inducer and LasR. G12, which has the HSL amide bond replaced with an ester, is also inactive. This suggests that the amino group is required for activity in *P. aeruginosa*. This data is supported by the TraR-3-oxo-C_8-HSL crystal structure that shows Asp70 (a residue conserved in LasR) in a H bond with the HSL amino group. Obviously, this putative H bond cannot be maintained by compound G12.

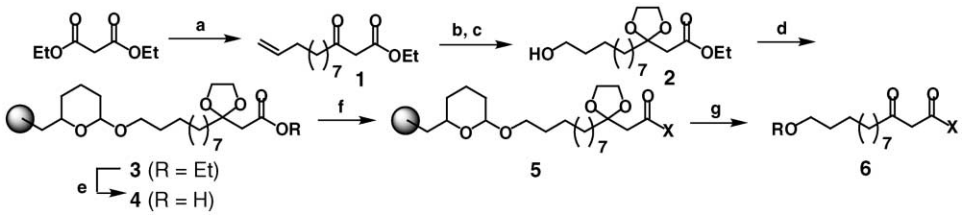

Figure 2. Synthesis of AI1 Analogs on Solid Phase

Reagents and conditions: (a) n-BuLi, THF, −78°C; undecenoyl chloride; (b) $HOCH_2CH_2OH$, *p*-TsOH, benzene, reflux; (c) BH_3·THF, THF; H_2O_2, $NaHCO_3$ aq, 38% for three steps (a–c); (d) DHP resin, PPTS, CH_2Cl_2, room temperature, 27 hr; (e) LiOH in THF/H_2O, 75°C, 20 hr; (f) parallel couplings with amines or alcohols (X), EDC, DMAP, DIPEA, DMF, room temperature, 72 hr; (g) 95% TFA, room temperature, 30 min, R = CF_3CO or H. Note that although the TFA deprotection yielded approximately 50% of product with trifluoroacetate on the 13-OH group, due to its hydrolytic instability, this group readily comes off after dissolving into aqueous media, exposing the 13-OH group.

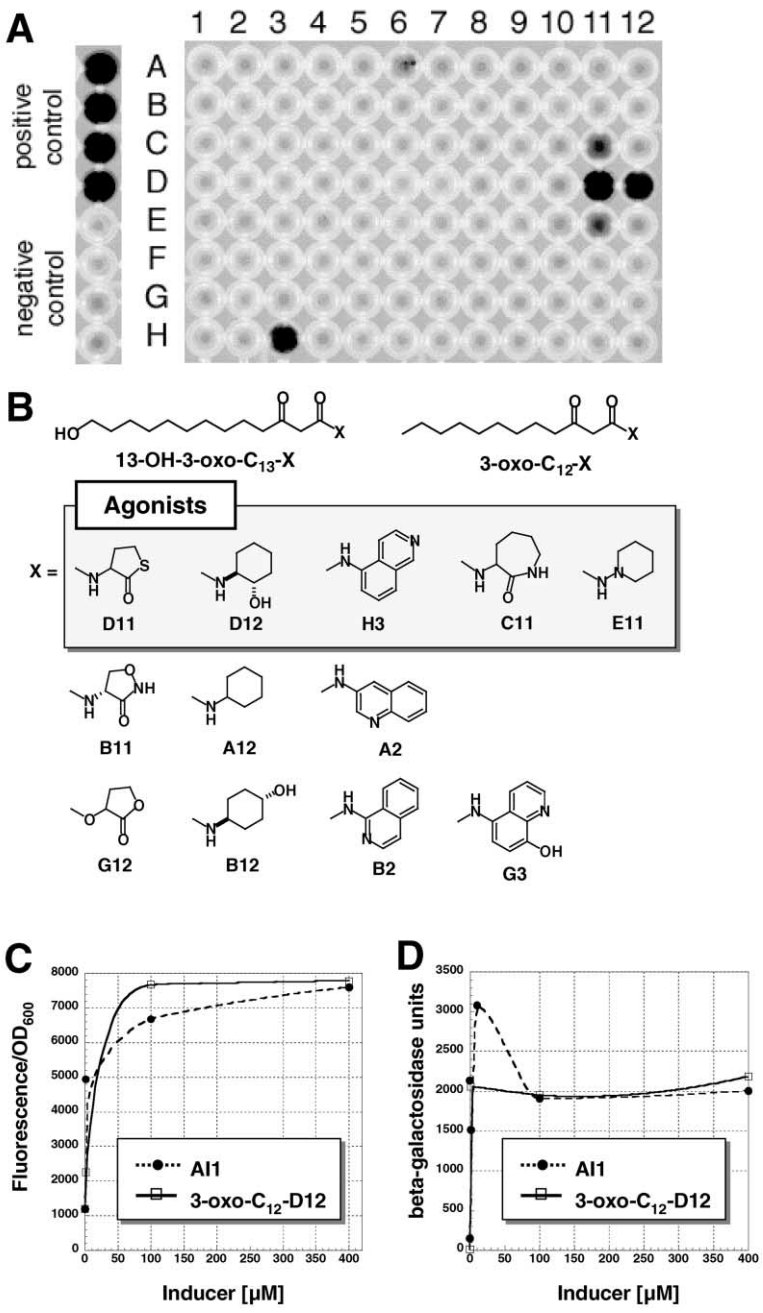

Figure 3. Agonist Reporter Screening and Assays

(A) 96-well plate assay of PAO-JP2 (p*lasI*-LVAgfp) in the presence of library compounds. Negative control is no treatment. Positive control is 1 μM AI1. Each well of the plate contains roughly 400 μM of one library compound.

(B) Structures of compounds in wells with significant GFP expression (in shaded box) and related compounds lacking agonist activity. D12 and B12 are diastereomerically pure but racemic mixtures. D11, G12, and C11 are racemic mixtures.

(C) PAO-JP2 (p*lasI*-LVAgfp) expression of GFP in the presence of increasing amounts of designated compounds.

(D) MG4 (pKDT37) β-galactosidase activity in the presence of increasing amounts of designated compounds.

One of the new agonist structures identified from the library, D12, is 2-aminocyclohexanol (Figure 3). The hydroxyl group adjacent to the amino group in D12 is clearly important for activity, since similar molecules that lack the OH group at this position, such as compounds A12 and B12, show no activity. Interestingly, D12 shares two structural features with AI1: (1) the OH group mimics the carbonyl group of HSL which participates in a hydrogen bond in the TraR crystal structure, with the highly conserved residue Trp57; and (2) the cyclohexane ring mimics the saturated ring structure of HSL, presumably presenting appropriate hydrophobic contacts to LasR. 3-oxo-C_{12}-D12, containing the natural side chain, was synthesized in solution phase and tested with both the *lasI-gfp* reporter as well as a *lasB-lacZ* reporter in *Escherichia coli*. *E. coli* strain MG4 harboring plasmid pKDT37 expresses *lasR* and contains a *lacZ* reporter gene controlled by a LasR-AI1 inducible *lasB* promoter [16]. Both of these reporter genes were activated in the presence of control AI1 or 3-oxo-C_{12}-D12 (Figures 3C and 3D), demonstrating that this agonist has LasR inducing activity comparable to the natural AI1. It also provides strong evidence that the agonist activity in *P. aeruginosa* is LasR dependent.

We were very interested in exploring the nature of D12's strong agonist activity further, so we synthesized a series of compounds with five- or six-membered saturated rings with H bond acceptors (hydroxyl or keto

group) at position 1. We found that 2-aminocyclohexanol and 2-aminocyclopentanone are agonists, but 2-aminocyclohexanone and 2-aminocyclopentanol are antagonists (see Figure 7). These results are presented in previous work [23] and are discussed further below in relation to the current study.

The second strong agonist, H3 (Figure 3), is a quinoline derivative. Comparison of H3 with other quinoline derivatives in the library such as A2, B2, and G3, suggests that the positions of N2 and the 5-exo-amine on the quinoline ring dictate activity. Since H3 shares some structural features with another autoinducer called *Pseudomonas* quinolone signal (PQS, Figure 1) [24], we speculate that it may exert its agonist activity via the PQS pathway. PQS has been shown to strongly induce *rhlI*, and mildly induce both *lasR* and *rhlR*, but not *lasI* [25]. However, both of these studies were done in a *lasR* null background and might be drastically different in the presence of LasR. Other protein(s) involved in PQS signaling have not yet been identified.

Two weaker agonists, C11 and E11, were also identified. Although both compounds exhibited detectable levels of agonist activity, their activities are significantly lower than the other three agonists found in the library. It should be noted that 3-oxo-C_6-C11 was not active in *V. fischeri* reporter gene assays [14].

Library Screening for Antagonists

We tested each compound in the library for antagonist activity using the same reporter strain, PAO-JP2 (p*lasI*-LVAgfp) [2], to find compounds that could compete against AI1 and reduce GFP expression. We identified eight compounds that inhibited reporter gene expression by greater than 50% (Figure 4A). None of the compounds exhibited an obvious affect on growth of the bacteria.

Among these antagonists, D10, H10, C10, F10, and G9 are aniline derivatives. This set of molecules also has an ortho- or meta-substituent of a hydroxyl, carboxyamide, or pyridyl group, which can act as H bond acceptors. The position of these substituents seems important, and depends on the particular substituent, i.e., ortho for hydroxyl or pyridyl and ortho/meta for carboxyamide. Structurally similar compounds differing only in the position of these substituents, E10 and G10 (Figure 4A), are inactive. We speculate that the hydroxyl group in D10 and H10, the pyridyl group in G9, and the carbonyl group in C10 and F10 maintain the putative H bond interaction observed between TraR Trp57 and the *A. tumefaciens* AI by acting as H bond acceptors. Trp57 is conserved in LasR. The importance of the putative H bond is consistent with our observation that agonists D11, D12, C11, and E11 (Figure 3B) all have a potential H bond acceptor group adjacent to the amino group. We therefore conclude that the combination of an aniline structure and H bond acceptor dictates the antagonist activity of this set of molecules.

We also identified F11, G11, and E3 as antagonists, but these molecules do not have obvious structural connections to each other or the aniline antagonists discussed above. Therefore, these individual compounds need to be resynthesized in solution phase, and their activity must be confirmed before hypothesizing about their inhibitory action. These studies are underway in our laboratory.

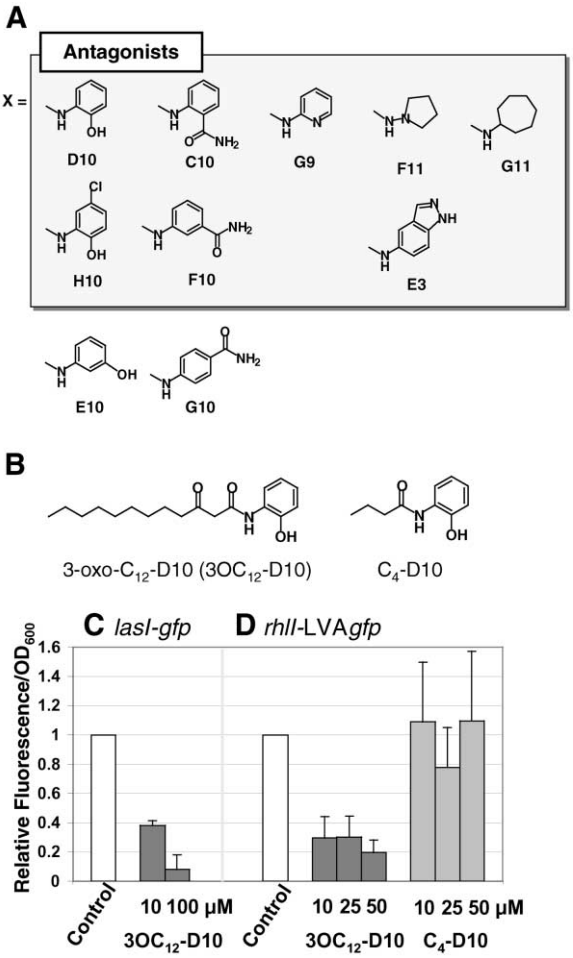

Figure 4. Antagonist Reporter Screening and Assays

(A) Structure of antagonists (in shaded box) and similar compounds with agonist or no activity.

(B) Structure of 3-oxo-C_{12}-D10 and C4-D_{10}.

(C) PAO-JP2 (p*lasI*-LVAgfp) was grown for 6 hr in the presence of 1 μM AI1 alone (white) or with 3-oxo-C_{12}-D10 competitor (dark gray).

(D) PAO-JP2 (p*rhlI*-LVAgfp) was grown for 6 hr in the presence of 1 μM AI1 and 10 μM AI2 alone (white) or with 3-oxo-C_{12}-D10 (dark gray) or C_4-D10 (gray). Fluorescence intensity was divided by OD_{600} of cell culture. Control was set to 1, and all other values are relative.

Antagonist Assays of 3-Oxo-C_{12}-(2-Aminophenol) and C_4-(2-Aminophenol)

Since D10 (2-aminophenol) is the simplest molecule among the aniline-based antagonists (Figure 4A), and most interestingly it differs from the strong agonist D12 only by the aromatic ring (Figure 3B), we focused on D10 for further studies. We synthesized 3-oxo-C_{12}-D10 in solution phase, where the natural side chain was coupled with D10 and purified by chromatography. To determine if the same structural motif can be applied to inhibition of the RhlR-AI2 interaction, C_4-D10 was also synthesized. Assays were performed with both the *lasI-gfp* reporter PAO-JP2 (p*lasI*-LVAgfp) and an RhlR-AI2-

controlled *rhlI-gfp* reporter, PAO-JP2 (p*rhlI*-LVAgfp) [22]. The *lasI-gfp* reporter assay clearly shows a concentration-dependent inhibition of GFP expression induced by 1 μM AI1, resulting in approximately 60% and 90% reduction of GFP expression by the addition of 10 and 100 μM 3-oxo-C_{12}-D10, respectively (Figure 4B). A strong reduction of GFP expression by 3-oxo-C_{12}-D10 was also observed with the *rhlI-gfp* reporter (Figure 4C). Expression induced by 1 μM AI1 and 10 μM AI2 was inhibited by more than 80% in the presence of 50 μM 3-oxo-C_{12}-D10. The compound did not interfere with expression of a *lac-gfp* reporter construct in PAO-JP2 (pTdK-GFP) [22] nor did it inhibit *P. aeruginosa* growth (data not shown). These results suggest that 3-oxo-C_{12}-D10 is a strong and specific inhibitor of QS, and therefore assays of QS controlled virulence factors were further investigated. C_4-D10 did not inhibit *rhlI-gfp* expression, indicating that C_4-D10 does not antagonize the RhlR-AI2 interaction (Figure 4C). This compound was also tested in virulence factor assays but uniformly showed no effect on their expression (data not shown).

To further confirm the ability of 3-oxo-C_{12}-D10 to specifically interfere with QS-controlled gene expression, we tested its ability to reduce expression of elastase, pyocyanin, and biofilm, three *P. aeruginosa* virulence factors controlled by QS [26]. Elastase activity produced by *P. aeruginosa* in the presence of AIs and competing 3-oxo-C_{12}-D10 was determined by measuring breakdown of the substrate elastin Congo red. Using the *lasI rhlI* knockout strain PAO-JP2, 10–100 μM 3-oxo-C_{12}-D10 reduced elastase activity by approximately 50% when competing against 5 μM AI1 and 10 μM AI2 supplied exogenously (Figure 5A). This antagonist also reduced elastase activity produced by wild-type *P. aeruginosa* strain PAO1 (Figure 5B). These results suggest that 3-oxo-C_{12}-D10 inhibits elastase production by *P. aeruginosa*, and provide additional evidence that this compound specifically interferes with QS-controlled gene expression. We also tested 3-oxo-C_{12}-D10 for its effect on pyocyanin production (Figures 5C and 5D), but this compound was unable to interfere with pyocyanin production by either PAO-JP2 (Figure 5C) or PAO1 (Figure 5D).

Finally, we tested the effect of this antagonist on biofilm development using a static biofilm assay with PAO-JP2 and PAO1 harboring plasmid pTdK-GFP [22] which encodes a constitutively expressed GFP construct for visualization of cells by scanning confocal laser microscopy (Figures 6A–6E). In PAO-JP2, formation of biofilm was dependent on the presence of exogenous AIs (Figures 6A and 6B). To our surprise, when 3-oxo-C_{12}-D10 was added to PAO-JP2 in combination with AIs (Figure 6C), biofilm formation was slightly enhanced rather than inhibited. Similarly, 3-oxo-C_{12}-D10 could not interfere with biofilm formation by wild-type PAO1 cells (Figures 6D and 6E). However, the molecule clearly had an effect on biofilm morphology, resulting in a biofilm that appeared "fluffy." Therefore, although inhibition of biofilm formation was not observed, it seems to have some impact on biofilm architecture. Additional studies utilizing more sophisticated biofilm assays are being pursued in our laboratory to further explore the effect of 3-oxo-C_{12}-D10 on biofilm formation.

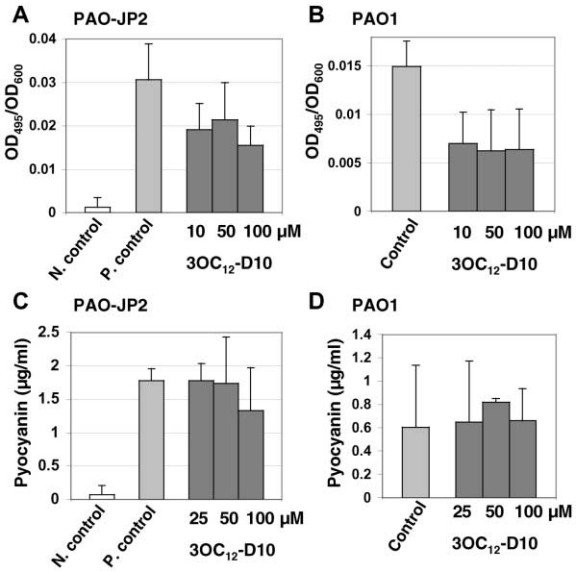

Figure 5. Virulence Factor Assays

(A) PAO-JP2 was grown for 6 hr in the absence (negative control, white) or presence (positive control, gray) of 5 μM AI1 and 10 μM AI2 or in combination with competing 3-oxo-C_{12}-D10 (dark gray), and elastase activity was determined.

(B) Wild-type strain PAO1 was grown for 6 hr without (gray) or with antagonist (dark gray) and elastase activity was determined.

(C) PAO-JP2 was grown for 18 hr in the absence (negative control, white) or presence (positive control, gray) of 25 μM AI1 and 25 μM AI2 or in combination with competing 3-oxo-C_{12}-D10 (dark gray), then assayed for pyocyanin production.

(D) PAO1 was grown without (gray) or with antagonist (dark gray), then assayed for pyocyanin production.

Comparison between 3-Oxo-C_{12}-D10 and 3-Oxo-C_{12}-(2-Aminocyclohexanone)

Our two studies described here and in a previous report [23] yielded four new compounds that agonize or antagonize the *P. aeruginosa* QS circuit (Figure 7). Interestingly, these molecules are structurally related to each other: all compounds have a hydroxyl or keto group adjacent to the amino group and a five- or six-membered ring structure. A particularly intriguing discovery is that a very subtle change to the agonist 3-oxo-C_{12}-D12 makes it an antagonist: the hydroxyl group to ketone (3-oxo-C_{12}-acHone), saturated ring to aromatic benzene ring (3-oxo-C_{12}-D10), and six- to five-membered ring (3-oxo-C_{12}-acPol).

Despite the structural similarity of antagonists (Figure 7), the observed activities are different. 3-oxo-C_{12}-acHone exhibits strong inhibition of both reporter genes, nearly complete inhibition of pyocyanin production and biofilm formation, but moderate inhibition of elastase activity. However, 3-oxo-C_{12}-D10 shows very strong inhibition of the reporter genes (stronger than 3-oxo-C_{12}-acHone) and moderate inhibition of elastase, but no inhibition of pyocyanin or biofilm. In a similar manner, 3-oxo-C_{12}-acPol inhibited the *las* reporter and pyocyanin expression from PAO-JP2, but could not inhibit the *rhl* reporter or elastase.

How can we explain these observations? It is clear that the effect of QS transcription factors is dependent

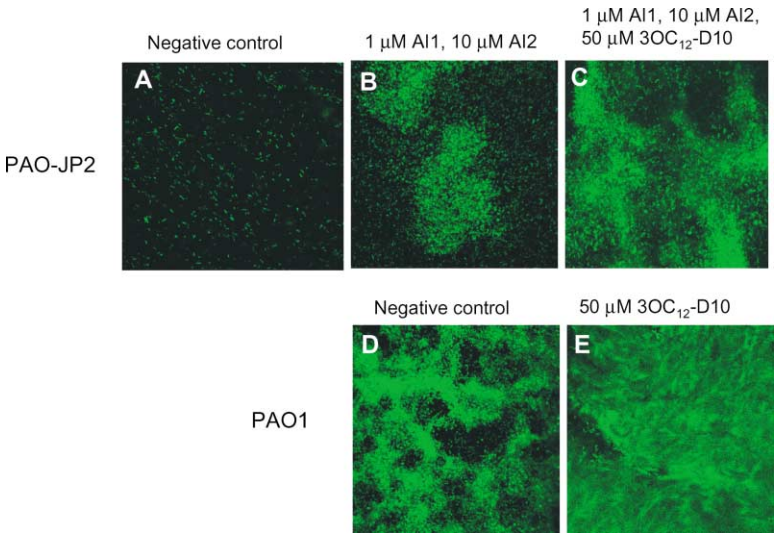

Figure 6. Static Biofilm Assay

(A–C) PAO-JP2 (pTdK-GFP) biofilm development in the presence of designated compounds.

(D–E) PAO-1 (pTdK-GFP) biofilm development in the absence (negative control) or presence of antagonist. Images are representative of three independent experiments, each with three replicate biofilms.

on the individual promoters, due to the affinity of the promoter sequence for LasR and RhlR [27], and any number of other regulatory proteins influencing the level of activation of each gene. This would explain why a specific promoter would be more or less susceptible to inhibition. The difference between the capacity of each compound to inhibit specific virulence factors, however, is less clear. We propose it is due to the ability of 3-oxo-C_{12}-acHone to antagonize both LasR-AI1 and RhlR-AI2. 3-oxo-C_{12}-D10 and 3-oxo-C_{12}-acPol, in contrast, can only disrupt the LasR-AI1 interaction. Therefore, the *rhl* regulon can still be activated even though the *las* regulon is significantly inhibited, resulting in induction of virulence factors. Evidence supporting this hypothesis is provided by agonist assays of AI2 analogs with the corresponding ring structures, and data from the native AIs with HSL rings. It has been shown that AI1 competitively inhibits binding of AI2 to RhlR in *E. coli*; AI1 reduced binding of AI2 to RhlR by 86% when present in only 4-fold excess [2]. Apparently, the long chain-HSL binds the same site as the short chain-HSL, but does not activate RhlR. The aminocylohexanone ring, like HSL, also fits into the AI binding site of both LasR and RhlR. This is supported by our finding that C_4-(2-aminocylcohexanone) (C_4-acHone) is a strong agonist of AI2, and 3-oxo-C_{12}-acHone is a strong antagonist of AI1 [23]. We speculate that 3-oxo-C_{12}-acHone may also antagonize the RhlR-AI2 interaction. This is a plausible hypothesis, since this compound inhibited all the virulence factors tested. Since the genes required for expression of elastase, pyocyanin, and biofilm are controlled by both LasR-AI1 and RhlR-AI2 [2, 28, 29], it is likely that both R proteins need to be affected to see the strong inhibition we observed in the presence of 3-oxo-C_{12}-acHone.

In contrast, both 3-oxo-C_{12}-D10 and 3-oxo-C_{12}-acPol inhibited the reporter genes but were less effective at inhibiting the virulence factors assayed. We suspect these two compounds elicit their effects through LasR alone and do not interact with RhlR. This is supported by our observation that neither C_4-(2-aminocylcopentanol) [23] nor C_4-D10 (data not shown) could activate the *rhlI-gfp* reporter. Since these particular HSL analogs could not activate RhlR, it seems less likely that the same analog with a 3-oxo-C_{12} side chain could enter the RhlR AI binding site to act as an inhibitor.

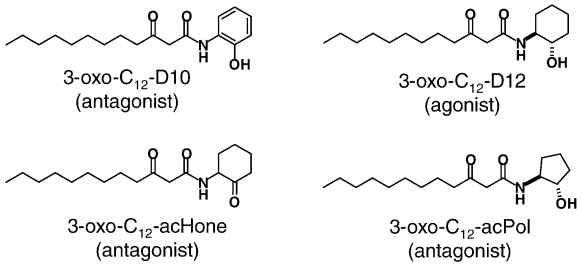

Figure 7. Agonists and Antagonists

3-oxo-C_{12}-D10 and 3-oxo-C_{12}-D12 are antagonist and agonist, respectively. Both 3-oxo-C_{12}-acHone and 3-oxo-C_{12}-acPol are antagonists found in the previous study [23]. Abbreviations: acHone, aminocyclohexanone; acPol, aminocyclopentanol.

Conclusions and Outlook

We have developed an efficient synthetic sequence to generate a library of AI1 analogs. New analog libraries can be easily and rapidly synthesized and screened for activity. Screening of our initial library identified new agonists that follow the same trend as previously discovered structures, allowing us to conclude that the HSL keto group and saturated carbons on the ring are involved in the interaction between the inducer and LasR. This result is consistent with the X-ray structure of a LasR homolog, TraR, that identified an H bond interaction between TraR and the HSL keto group [11, 12]. We also identified several new antagonists of the QS system. We have shown that a common structural feature for antagonists is an aniline ring with an appropriate H bond acceptor at the ortho or meta position. It is striking that the only difference between the agonist 3-oxo-C_{12}-D12 (2-aminocyclohexanol) and the antagonist 3-oxo-C_{12}-D10 (2-aminophenol) is an aromatic ring. This is especially interesting when looking at other natu-

rally occurring antagonists. The halogenated furanones produced by *Delissea pulchra* that interfere with QS in related bacteria have five-membered rings containing unsaturated bonds [17, 19], as do derivatives of these compounds that inhibit *P. aeruginosa* QS [9]. We speculate that an aromatic ring somehow interferes with the ability of the antagonists to activate the R protein for DNA binding.

The antagonist 3-oxo-C_{12}-D10 displays potent inhibitory activity in reporter gene assays and interferes with elastase activity and normal biofilm formation. The promoter-specific differences in inhibitory activity result from the complexity of the *P. aeruginosa* QS signaling network. Knowledge gained from this study is currently being used to design new analog libraries in order to discover more potent QS inhibitors. To our knowledge, no short chain antagonists of AI2 have been discovered. In fact, we found that none of the C_4 derivatives of the antagonist structural elements in Figure 7 exhibit strong inhibition of the *rhl* circuit. The importance of finding an AI2 antagonist is emphasized by our results showing only partial inhibition of virulence factor production when LasR but not RhlR is inhibited. We are currently synthesizing a large library of AI2 analogs in order to find inhibitors specific to the *rhl* regulon that can be used in combination with the identified AI1 antagonists.

Significance

The potential benefits of controlling QS are very significant both medically and economically. The parallel synthesis of AI analogs allowing discovery of several new analogs that activate or inhibit QS provides a significant advancement in our understanding of the LasR-AI1 interaction. The new structural elements identified in this study for both agonists and antagonists are currently being used to design new focused libraries of analogs that should contain more potent antagonists. This may eventually lead to alternative therapeutic options for the treatment of chronic *P. aeruginosa* infections.

Experimental Procedures

Chemical Synthesis

3,4-dihydro-2H-pyran-2-ylmethoxymethyl polystyrene (DHP resin) was purchased from Novabiochem (Laufelfingen, SWI). All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) or Acros. All the solvents were anhydrous grade or were distilled before use. All reactions were carried out in oven-dried glassware under an argon atmosphere, except the ones containing water. Anhydrous $MgSO_4$ was used as drying agent for all products. Analytical thin layer chromatography was performed on EM Reagent 0.25 mm silica gel 60-F plate. Flash chromatography was performed using EM silica gel 60 (230–400 mesh). Parallel synthesis was performed using a reactor block from Robbins Scientific Inc. (Sunnyvale, CA). Mass spectra were recorded on a Finnigan MAT95 XL spectrometer (San Jose, CA). NMR spectra were taken on a Varian Gemini 300, Inova 400, or Inova 500 MHz spectrometer (Palo Alto, CA). ^{1}H chemical shifts were referenced to tetramethylsilane (0.00 ppm). ^{13}C chemical shifts were referenced to CDCl3 (77.0 ppm). IR spectra were recorded on a Perkin-Elmer 1760 infrared spectrometer (Shelton, CT). AI1 [4] and AI2 [13] were synthesized according to literature procedures.

Synthesis of the 13-Hydroxyl-Acyl Side Chain

Ethyl 3-Oxo-12-Tridecenoate 1

To a stirred solution of 10-undecenoic acid (5.06 ml, 25 mmol) in CH_2Cl_2 (50 ml) was slowly added $(COCl)_2$ (4.36 ml, 50 mmol). The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure to give 10-undecenoyl chloride (25 mmol). The product was used for the next reaction without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.29–1.48 (m, 10H), 1.71 (m, 2H), 2.05 (m, 2H), 2.88 (t, J = 7 Hz, 2H), 4.98 (m, 2H), 5.80 (m, 1H).

A stirred solution of monoethyl malonate (5.3 g, 40 mmol) in anhydrous THF (150 ml) was cooled to −78°C under an argon atmosphere, and n-BuLi (2.5 M, 32 ml, 80 mmol) was added dropwise via an air-tight syringe. After the addition, the temperature was raised to 0°C, and the stirring was continued for 1 hr. The reaction mixture was then recooled to −78°C, and 10-undecenoyl chloride (25 mmol) was added dropwise via an air-tight syringe. The mixture was stirred for 1 hr at −78°C, 30 min at 0°C, and 30 min at room temperature. The solvent was removed under reduced pressure, and the residue was redissolved in 50 ml ethyl acetate. The solution was washed with 1 M HCl (100 ml), saturated aqueous $NaHCO_3$ (50 ml), and brine (50 ml). The organic layer was dried over $MgSO_4$ and concentrated to give **1** (6.27 g). The crude product was used for the next reaction without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28–1.40 (m, 13H), 1.58 (m, 2H), 2.03(m, 2H), 2.53 (t, J = 7 Hz, 2H), 3.43 (s, 2H), 4.21 (q, J = 7 Hz, 2H), 4.97 (m, 2H), 5.80 (m, 1H).

Ethyl 3,3-Ethylenedioxo-13-Hydroxy-Tridecanoate 2

The mixture of **1** (6.27 g, ca. 24.7 mmol), ethylene glycol (13.7 ml, 247 mmol) and a catalytic amount of p-TsOH (475 mg, 2.5 mmol) in benzene (120 ml) was refluxed at 110°C under an argon atmosphere overnight. The solvent was removed, and the residue was diluted with 50 ml ethyl acetate. The solution was washed with saturated aqueous $NaHCO_3$ (50 ml) and brine (50 ml), and the organic phase was dried ($MgSO_4$) and concentrated to give ethyl 3,3-ethylenedioxo-12-tridecenoate (6.97 g). The crude product was used for the next reaction without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26–1.50 (m, 15H), 1.80 (m, 2H), 2.02 (m, 2H), 2.64 (s, 2H), 3.98 (m, 4H), 4.16 (q, J = 7 Hz, 2H), 4.96 (m, 2H), 5.79 (m, 1H).

A stirred solution of ethyl 3,3-ethylenedioxo-12-tridecenoate (6.97 g, ca. 23.4 mmol) in anhydrous THF (100 ml) was cooled to 0°C under an argon atmosphere. BH_3·THF (1 M, 117 ml, 117 mmol) was added dropwise via an air-tight syringe. The mixture was stirred at 0°C for 3 hr. Then, aqueous $NaHCO_3$ (2 M, 70 ml, 140 mmol) and 30% H_2O_2 (4.4 ml, 140 mmol) were added to the reaction slowly, and the mixture was stirred at room temperature for 1.5 hr. The organic solvent was evaporated under reduced pressure, and the residue was extracted with ethyl acetate (2 × 100 ml). The combined organic layers were washed with brine, then dried over $MgSO_4$ and concentrated. The residue was purified by flash chromatography (hexane/EtOAc 2:1, Rf = 0.21) to give **2** (3.00 g, 9.5 mmol) as a colorless liquid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.26–1.40 (m, 17H), 1.56 (m, 2H), 1.79 (m, 2H), 2.65 (s, 2H), 3.63 (t, J = 7 Hz, 2H), 3.96 (m, 4H), 4.16 (q, J = 7 Hz, 2H). The overall yield of the above three steps was 38%, and ^{1}H NMR data were consistent with the literature data [4], except for the functionalities (olefin or hydroxyl group) newly introduced into the terminus of the acyl chain.

Solid Phase Reactions

DHP resin (0.98 mmol/g, 5.1 g, 5 mmol) was soaked in 50 ml CH_2Cl_2 for 1 hr. Then, **2** (2.3 g, 7.28 mmol) and PPTS (1.83 g, 7.29 mmol) were added. The mixture was stirred gently at room temperature for 27 hr. The resin was washed with an excess amount of CH_2Cl_2 and dried under vacuum to give **3**. The mixture of **3** (ca. 5 mmol) and LiOH·H_2O (21 g, 500 mmol) in 100 ml THF/H_2O (1:1) was gently stirred and heated at 75°C for 20 hr. The resin was sequentially washed with an excess amount of water, THF, and CH_2Cl_2 and dried under vacuum to give **4**. The parallel couplings of **4** with 96 molecules (the list of molecules is available upon a request to the corresponding author) were performed on a 96-well reactor block by mixing the following compounds in each well: **4** (0.05 mmol), amine or alcohol (0.5 mmol), EDC (0.5 mmol), DMAP (0.5 mmol), iPr_2NEt (0.5 mmol), and DMF (1.5 ml). The reactor block was assembled and rotated end-to-end at room temperature for 70 hr to afford coupling products **5**. DHP resin, the intermediates **3** and **4**, and four coupling

products arbitrarily chosen were characterized by on-resin FT-IR. In each case, a small amount of dried resin (2 mg) was mixed with anhydrous KBr (75 mg) to make a pellet, and FT-IR spectroscopy was recorded to confirm the product (see Supplemental Data at http://www.chembiol.com/cgi/content/full/10/6/563/DC1).

After the coupling reaction, the reactor block was disassembled and connected to a vacuum line. The resin was washed with a large amount of DMF and CH_2Cl_2 to remove excess reactants, coupling reagents, and undesired byproducts. The vacuum was applied overnight to dry the resin. 1 ml TFA/H_2O (95:5) was added into each well, and the reactor block was reassembled and rotated at room temperature for 30 min. The cleavage products **6** were collected into a 96-deep-well plate. 1 ml CH_2Cl_2 was added to each resin to wash off the products into the collection plate. The solvents were evaporated with a water pump, and the products were dried under vacuum. Four products were arbitrarily chosen and characterized by ^{1}H NMR and FAB mass spectrometry to confirm the formation of the desired product (see Supplemental Data at http://www.chembiol.com/cgi/content/full/10/6/563/DC1).

Synthesis of N-(*trans*-2-Hydroxycyclohexyl)-3-Oxododecanamide (3-Oxo-C_{12}-D12)

The same procedure for the preparation of AI1 was used. 38 mg crude product was obtained from *trans*-2-aminocyclohexanol hydrochloride. Flash chromatography (EtOAc) gave 3-oxo-C_{12}-D12 in the ketone form (23 mg, 0.074 mmol, Rf = 0.29). Ketone form: IR (KBr) 3274, 2927, 1715, 1652, 1617, 1564 cm^{-1}; ^{1}H NMR (500 MHz, CDCl3) δ 0.88 (t, J = 7.5 Hz, 3H), 1.18–1.40 (m, 16H), 1.58 (m, 2H), 1.72 (m, 2H), 1.90–2.10 (m, 2H), 2.53 (t, J = 7.5, 2H), 3.35 (m, 1H), 3.43 (s, 2H), 3.67 (m, 1H), 7.19 (d, J = 5 Hz, 1H); ^{13}C NMR (125 MHz, CDCl3) δ 14.1, 22.6, 23.3, 23.9, 24.5, 28.9, 29.21, 29.31, 29.34, 31.2, 31.8, 34.2, 44.0, 48.3, 55.7, 75.2, 167.3, 207.5; EI-HRMS calculated for $C_{18}H_{33}O_3N$ (M^+) 311.2455, found 311.2463.

GFP Reporter Assays

P. aeruginosa strain PAO-JP2 (*lasI*, *rhlI*) [2] harboring p*lasI*-LVAgfp [22] was grown at 37°C in LB with 300 μg/ml carbenicillin. For agonist assays, an overnight culture was diluted to an OD_{600} of 0.1 and transferred to wells of a 96-well plate on which test compounds had previously been added and dried. The final concentration of each analog tested was roughly 400 μM, although this is an overestimate due to purity issues. Cells were then incubated for 6 hr at 37°C with vigorous shaking. GFP expression was detected with a Molecular Imager (BioRad) (488 nm excitation and 695 nm bandpass filter) and quantified with ImageQuant software. The OD_{600} of cultures was determined to normalize GFP expression to cell density. For antagonist assays, the methods were the same except that 1 μM AI1 was added to each well in combination with the library compound. The fluorescence/OD_{600} of each compound competing against AI1 was reported relative to the fluorescence/OD_{600} of the positive control of AI1 alone, which was set to 1. When strain PAO-JP2 (p*rhlI*-LVAgfp) was used, the positive control was 1 μM AI1 and 10 μM AI2.

lacZ Reporter Assays

E. coli strain MG4 (pKDT37) was grown overnight in supplemented A media with 100 μg/ml ampicillin at 30°C and diluted 1:100 in the same media. 1 ml of this culture was added to a test tube containing AI1 or 3-oxo-C_{12}-D12 that had been previously aliquoted and dried. Following a 6 hr incubation at 30°C, β-galactosidase activity was determined using standard methods [30].

Virulence Factor Assays

Pyocyanin, elastase, and biofilm were assayed as previously described [23].

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