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Covalent Inhibition of Bacterial Quorum Sensing

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Abstract: Chemical coordination of gene expression among bacteria as a function of population density is regulated by a mechanism known as 'quorum sensing' (QS). QS in *Pseudomonas aeruginosa*, an opportunistic pathogen that causes disease in immunocompromised patients, is mediated by binding of the transcriptional activator, LasR, to its ligand, 3-oxo-C_{12}-HSL, leading to population-wide secretion of virulence factors and biofilm formation. We have targeted QS in *P. aeruginosa* with a set of electrophilic probes designed to covalently bind Cys79 in the LasR binding pocket, leading to specific inhibition of QS-regulated gene expression and concomitant reduction of virulence factor secretion and biofilm formation. This first example of covalent modification of a QS receptor provides a new tool to study molecular mechanisms of bacterial group behavior and could lead to new strategies for targeting bacterial virulence.

Introduction

Cell-to-cell communication enables single cell organisms to coordinate their behavior so as to adapt to changing environments, allowing them to compete, as well as coexist, with multicellular organisms.[1] Examples of QS-controlled behaviors include biofilm formation, virulence factor expression, antibiotic production, and induction of bioluminescence. These processes are beneficial to a bacterial population only when carried out simultaneously. For example, bioluminescence produced by the marine bacterium *Vibrio fischeri* is beneficial to a number of organisms that host this species but only if a sufficient number of bacteria synchronize their light emission. While various QS signaling systems have been discovered, more proteins and small molecules involved in QS remain to be described.[2−4]

The importance of QS in bacteria and its effect on human health is significant, especially when one considers that the total microbial population ($\sim 10^{14}$) in the human adult is estimated to exceed the number of mammalian cells by at least a factor of 10.[5] The gastrointestinal tract alone contains 500−1000 different species presenting great genetic diversity, and since most of these species have not yet been cultured in vitro, this population has barely been characterized. Intra- and interspecies QS may very well aid this commensal population in coordinating important processes, such as maintenance of population size and aiding or preventing pathogenic bacterial colonization.[2,6]

QS is regulated by autoinducers that can be categorized into several classes, depending on shared molecular features (Figure 1a).[3,4] More than 70 species of Gram-negative bacteria employ *N*-acyl homoserine lactones (AHLs) as autoinducers, with differences within this class of QS signals occurring in the length and oxidation state of the acyl side chain. Various AHLs from different species have been shown to play important roles in bacterial infections. An important example is the Gram-negative bacterium *Pseudomonas aeruginosa*. This common environmental microorganism is an opportunistic human pathogen, being prominent, for example, in patients suffering from cystic fibrosis, a common and lethal inherited genetic disorder, where patients often die due to impaired lung defense functions.[7] A key factor contributing to the pathogenesis and antibiotic resistance of *P. aeruginosa* lies in its ability to form a biofilm, a microbially derived sessile community of cells that attach either to an interface or to each other, inhabit an extracellular polymeric matrix, and exhibit a phenotype distinct from that of planktonic cells with respect to growth, gene expression, and protein production.[8] Although the formation and specific architecture of biofilms are regulated by various QS systems[9] as well as other factors, such as cyclic di-GMP,[10] it has been

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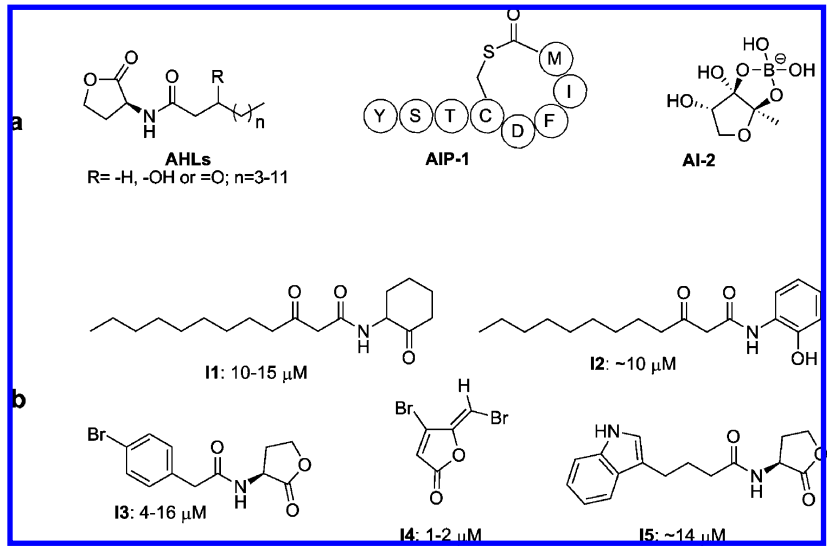

Figure 1. Bacterial autoinducers and inhibitors. (A) Examples of bacterial autoinducers belonging to distinct structural classes. (B) Examples of synthetic QS inhibitors in *P. aeruginosa* (**I1**−**I5**). Approximate IC_{50} values (from different reporter assays) are listed below the compounds. Efficacies of **I1** and **I2** were reported by Smith et al.,[11,12] **I3** and **I5** by Geske et al.,[13] and **I4** by Hentzer et al.[14]

shown that inhibition of even a single QS regulator can lead to a significant decrease in overall biofilm formation.

The primary QS system in *P. aeruginosa* is regulated through the synthesis and secretion of 3-oxo-C_{12}-HSL, which, upon reaching a threshold concentration, binds the transcriptional activator LasR. This interaction has been proposed to lead to correct folding, followed by dimerization and binding of the LasR dimer to its target DNA, resulting in gene expression. In addition, several other small molecules have been found to play a role in the regulation of gene expression (e.g., C_4−HSL, PQS), although the signaling events initiated by 3-oxo-C_{12}-HSL recognition appear to be at the top of the QS hierarchy.[15] Because of its medical importance, QS in *P. aeruginosa* has been extensively studied. One notable breakthrough in this field came with the determination of the crystal structure of LasR bound to its natural ligand (3-oxo-C_{12}-HSL), recently reported by Bottomley et al.[16]

Interfering with QS signaling has been explored in recent years as a novel approach to combat pathogenesis, with strategies ranging from receptor antagonism,[4,17,18] signaling molecule binding and degradation,[19] to inhibition of signaling molecule biosynthesis.[20] Several groups have identified compounds showing significant inhibition of QS in *P. aeruginosa*,[12,14,18,21,22] although the number of strong inhibitors resulting from such efforts remains low. Examples of moderately potent inhibitors, with their IC_{50} values, are shown in Figure 1b.

One obstacle in the design of more potent *P. aeruginosa* QS inhibitors is the apparently high affinity and specificity with which LasR binds the primary QS molecule, 3-oxo-C_{12}-HSL. Consequently, small changes in the structure of the ligand can lead to a large loss in affinity. A potential solution to this problem lies in the design of structures that present only a minimal deviation from the parent autoinducer and contain a small reactive moiety that can covalently bind a residue in the LasR binding pocket. Such covalent probes would be expected to compete effectively with 3-oxo-C_{12}-HSL for binding to LasR, such that their slightly altered occupation of the binding pocket upon conjugation would likely result in a conformational change that is less than optimal for effective binding of the transcriptional activator to its target DNA. Use of this type of probe could also severely affect the regulation and recycling of both LasR and 3-oxo-C_{12}-HSL. Covalent probes have been widely used to target, identify, and investigate proteins, enzymes, and receptors.[23] In recent years, the field of activity-based protein

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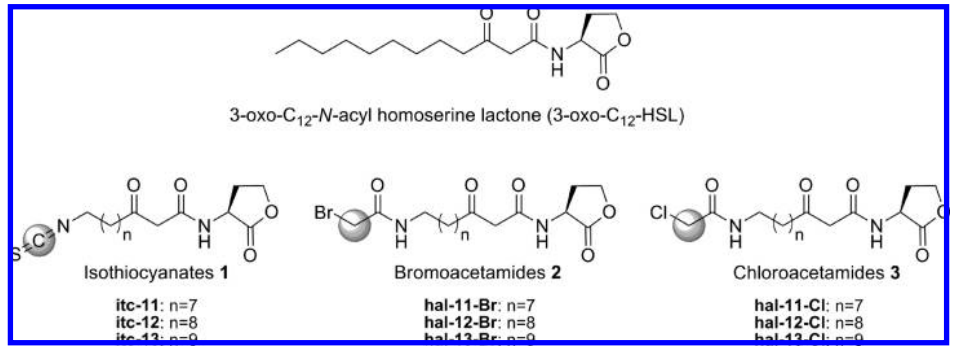

Figure 2. Structures of the natural autoinducer of *P. aeruginosa*, 3-oxo-C12-*N*-acyl homoserine lactone (3-oxo-C12-HSL), and nine synthetic analogues classified as isothiocyanates (**1**) bromoacetamides (**2**), or chloroacetamides (**3**). Electrophilic carbons are marked (gray circles) for each reactive group.

profiling has yielded a wealth of information on the activity of numerous previously unknown or uncharacterized enzymes.[24] Most activity-based probes comprise a reactive group, usually an electrophile capable of forming a covalent bond with a nucleophile in the active site of its target protein, without reacting nonspecifically with other proteins. Recognition of the covalent probe is dependent on its ability to interact noncovalently and with high affinity and specificity with the target binding site, upon which nucleophilic attack by an appropriately positioned residue can take place.[25]

We decided to exploit the presence of a nucleophilic cysteine residue in the binding pocket of LasR to develop reactive QS probes. We, therefore, designed a series of electrophiles with different functional groups and different alkyl chain lengths (isothiocyanates **1**, bromoacetamides **2**, chloroacetamides **3**, Figure 2). The main challenge is to design a probe that would be sufficiently reactive so as to react with the nucleophilic cysteine but not so overly reactive that unwanted reactions would take place with other residues before the probe reaches the binding pocket.

We evaluated the ability of our series of covalent probes to specifically target QS receptors and interfere with QS-mediated bacterial group behavior. As a model organism, we focused on *P. aeruginosa*, with the design of our probes being facilitated by the recently solved LasR structure.[16] Relying on this structural information, we were able to design probes that alkylate a specific nucleophile in the LasR binding pocket and, consequently, attenuate QS in *P. aeruginosa*.

Experimental Methods

Chemical Synthesis. Syntheses of isothiocyanates itc-11,12,13 (**7a**−**c**) and haloacetamides hal-11,12,13-Br (**11a**−**c**) and hal-11,12,13-Cl (**11d**−**f**) are shown in Scheme 1 and described in the Supporting Information.

Mass Spectrometry. All MS analyses were performed on a LCQ Fleet mass spectrometer (Thermo Scientific) with an ESI source. Spectra were collected in positive ion mode and analyzed by Xcalibur and Promass software (Thermo Scientific). For LC/MS analyses, a Surveyor Plus HPLC System (Thermo Scientific) was used, equipped with a Luna C18, 5 μm (150 × 4.6 mm) column at a flow rate of 0.5 mL/min, using a mobile phase linear gradient of 0.1% aqueous formic acid (solvent A) and CH_3CN containing 0.1% formic acid (solvent B).

Expression of LasR-LBD. The expression of full length LasR was previously found to yield largely insoluble protein in the presence or absence of the native ligand, 3-oxo-C_{12}-HSL.[16] Therefore, expression was performed using an *E. coli* strain transformed with a pETM-11 vector encoding for a shortened, His_6-tagged LasR construct, LasR-LBD (ligand-binding domain), spanning residues Met-1 to Lys-173. The plasmid was transferred into *E. coli* BL21, and cells were plated on LB agar plates containing kanamycin (50 μg/mL). Proteins were expressed in the presence of either native 3-oxo-C_{12}-HSL or different inhibitors and purified by Ni^{2+} affinity chromatography as previously described,[16] yielding ∼70 mg of purified protein per liter of LB medium using large scale expression conditions, and ∼0.5−1 mg of purified protein from 50 mL of LB medium using small scale expression conditions. The purification process was monitored by SDS-PAGE electrophoresis, and the molecular mass of the purified proteins was confirmed by mass spectrometry.

Large Scale Expression. One milliliter of an overnight grown cell culture was used to inoculate 1 L of rich LB medium containing kanamycin (50 μg/mL) and 10−100 μM of 3-oxo-C_{12}-HSL or inhibitors **7a**−**c** and **11a**−**f**. Cells were grown to an optical density at 600 nm (OD_{600}) of 0.4, after which expression was induced at 21 °C by addition of 0.2 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) and an additional amount of ligand/inhibitor was added to the media. After reaching an $OD_{600\ nm}$ of 1.4 (approximately 6−8 h), cells were centrifuged at 6000 rpm, washed, and resuspended in lysis buffer containing 5 mM imidazole, 300 mM NaCl, 50 mM Tris-HCl, pH 8. Cells were ultrasonicated for 2 min at 70% amplitude for two cycles. The lysate was centrifuged at 12 000 rpm for 30 min, and the supernatants were purified by Ni^{2+} affinity chromatography.

Small Scale Expression. The previous procedure was followed but in 50 mL volume. Cells were harvested by chemical lysis, adding 1 mL of lysis buffer (5 mM imidazole, 300 mM NaCl, 0.2% (v/v) Triton X-100, 0.75 μg/mL DNase-I, 0.05 mM $MgCl_2$, 0.01 mM $CaCl_2$, 50 mM Tris-HCl, pH 8, and 0.01% (v/v) protease inhibitor cocktail (Sigma-Aldrich)), and incubated for 60 min at 37 °C. Cell debris was removed by centrifugation at 4000 rpm for 15 min. The supernatants were purified using Ni-NTA spin columns (QIAGEN).

***P. aeruginosa* Wild-Type Strain (PA01) QS Agonist/Antagonist Assays.** Either a *P. aeruginosa* PA01 wild type strain or a PAO-JP2 (*lasI*/*rhlI*-deleted) strain, harboring plasmid pKD201 containing

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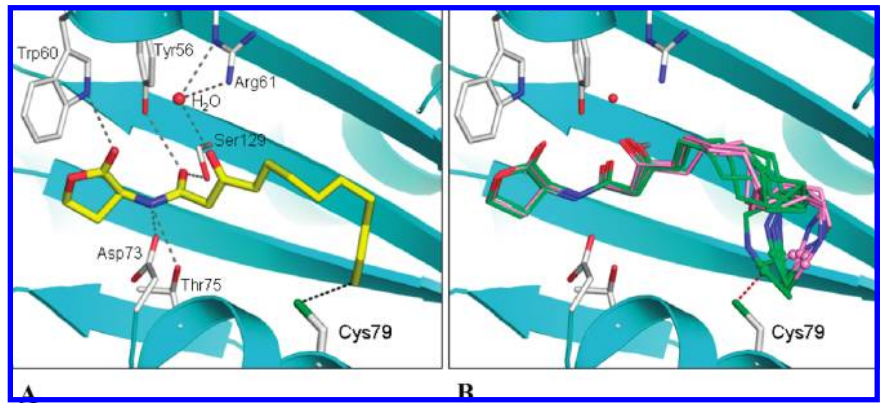

Figure 3. The ligand-binding domain of LasR (A) with its bound ligand, 3-oxo-C_{12}-HSL, as elucidated by Bottomley et al.[16] RCSB PDB ID: 2UV0. Cys^{79} is highlighted to show its vicinity (4.3 A) to the terminal carbon of 3-oxo-C_{12}-HSL. The illustration was prepared using PyMol. (B) The 10 lowest energy structures of itc-12 bound to LasR cluster into two groups. Computational methods are detailed in the Supporting Information. One group (green) is well positioned for attack by Cys79 (at a distance of 3.9−4.3 Å from the electrophilic carbon atom), while the other group (pink) is poorly positioned (4.7−5.6 Å, S → C). Green/pink spheres indicate the electrophilic carbon. Assuming that Cys79 is not fixed, the S → C distance could be shorter. However, in the case of the pink cluster, the electrophilic carbon is blocked by the sulfur atom of itc-12, i.e., the orientation is not suitable for nucleophilic attack by the thiol on the carbon.

a *LasI* reporter coupled to the *luxCDABE* luminescence system,[26] was incubated overnight in LB medium containing 300 μg/mL of trimethoprim. A 96-well black microtiter plate (Greiner) was prepared with the desired concentrations of inhibitors (up to 1 mM, above which growth inhibition was observed), and bacteria were added to reach a final OD_{600} of 0.015. The plate was then incubated for a period of 12 h at 37 °C. During this time, luminescence measurements were performed at 10 min intervals. The relative luminescence was then plotted against the added inhibitor concentration; IC_{50} values were calculated using Grafit 6.0 (Erithacus Software). For antagonist experiments, a final concentration of 50 nM 3-oxo-C_{12}-HSL was used.

***E. coli* DH5α LasR Agonist/Antagonist Assay.** *E. coli* DH5α harboring the LasR expression vector, pJN105L, and a plasmid-borne PlasI-lacZ fusion (pSC11)[27] was used to quantify quorum sensing inhibition by measuring expression levels of β-galactosidase. Bacteria were incubated overnight in LB medium containing 100 μg/mL of ampicillin and 15 μg/mL of gentamicin. The culture was diluted at a 1:10 ratio by volume with fresh medium and further incubated until an OD_{600} of 0.3 was reached. A 96-well microtiter plate (Greiner) was prepared with the desired concentrations of inhibitors and bacteria were added to reach a final OD_{600} of 0.3. Expression was induced on the edition of L-(+)-arabinose (4 mg/mL), and the plates were incubated at 37 °C for a period of 4 h (OD_{600} of 0.45−0.5). The cultures were then assayed for β-galactosidase activity according to the Miller assay method:[28] 200 μL aliquots were transferred to clear 96-well microtiter plates and the OD_{600} was recorded. A 100 μL aliquot from each well was then added to a polypropylene-based 96-well microtiter plate containing 200 μL of Z-Buffer, 10 μL of chloroform, and 5 μL of 0.1% SDS (w/v). Wells were thoroughly rinsed with a pipet, after which the chloroform was allowed to settle. A 100 μL aliquot of the aqueous upper layer was transferred to a fresh 96-well microtiter plate, and 20 μL of *o*-nitrophenyl-β-D-galactopyranoside (ONPG, 4 mg/mL in phosphate buffer of pH 7) was added. The plates were incubated 35 min at 28 °C. The reaction was terminated with the addition of 80 μL of 1 M sodium carbonate solution, and absorption at two wavelengths (550 nm, 429 nm) was recorded. Miller units were calculated using standard methods.[28] For antagonist experiments, a final concentration of 50 nM 3-oxo-C_{12}-HSL was used.

Biofilm Formation and Pyocyanin Production Assays. The effects of inhibitors on biofilm formation and pyocyanin production of the *P. aeruginosa* PAO1 wild-type strain were measured following standard procedures described by Smith et al.[11] and Kaufmann et al.[19a]

Results

Covalent Binding to the Ligand-Binding Domain of LasR. The 1.8 Å-resolution crystal structure of the ligand-binding domain of LasR reveals that there is a cysteine residue (Cys79) in close proximity to the end of the alkyl chain of the bound natural ligand, 3-oxo-C_{12}-HSL (Figure 3). As the nucleophilic thiol of cysteine has been shown to be reactive toward various electrophiles, such as isothiocyanates and haloacetamides, we decided to synthesize the following covalent probes: isothiocyanates itc-11−13, bromoacetamides hal-11−13-Br and chloroacetamides hal-11−13-Cl (Scheme 1 and Supporting Information).

To examine our proposed modification of LasR, we set out to determine whether a covalent bond indeed forms between the covalent probes and LasR Cys79. Accordingly, we overexpressed and purified the LasR ligand-binding domain (LasR-LBD) from *E. coli* BL-21 cells. Both Greenberg et al.[29] and Bottomley et al.[16] have reported that LasR assumes a soluble and stable conformation only when its natural ligand is correctly bound. These authors proposed that several known inhibitors act by interfering with the correct folding of LasR, resulting in a loss of soluble LasR, leading, in turn, to a decrease in transcription of the target DNA. In contrast, we found that upon incubation of bacteria expressing LasR with several of our probes, soluble LasR-LBD could be obtained (Figure 4a). Importantly, in the absence of probe or 3-oxo-C_{12}-HSL, no soluble LasR-LBD was observed, while overexpression of LasR in the presence of most of the haloacetamides resulted in the appearance of only minor amounts of soluble LasR-LBD. Similarly, when cells were incubated with 4-Br-PHL (compound I3 in Figure 1), no soluble LasR was observed (Supporting Information), confirming the earlier findings of Bottomley et al.[16]

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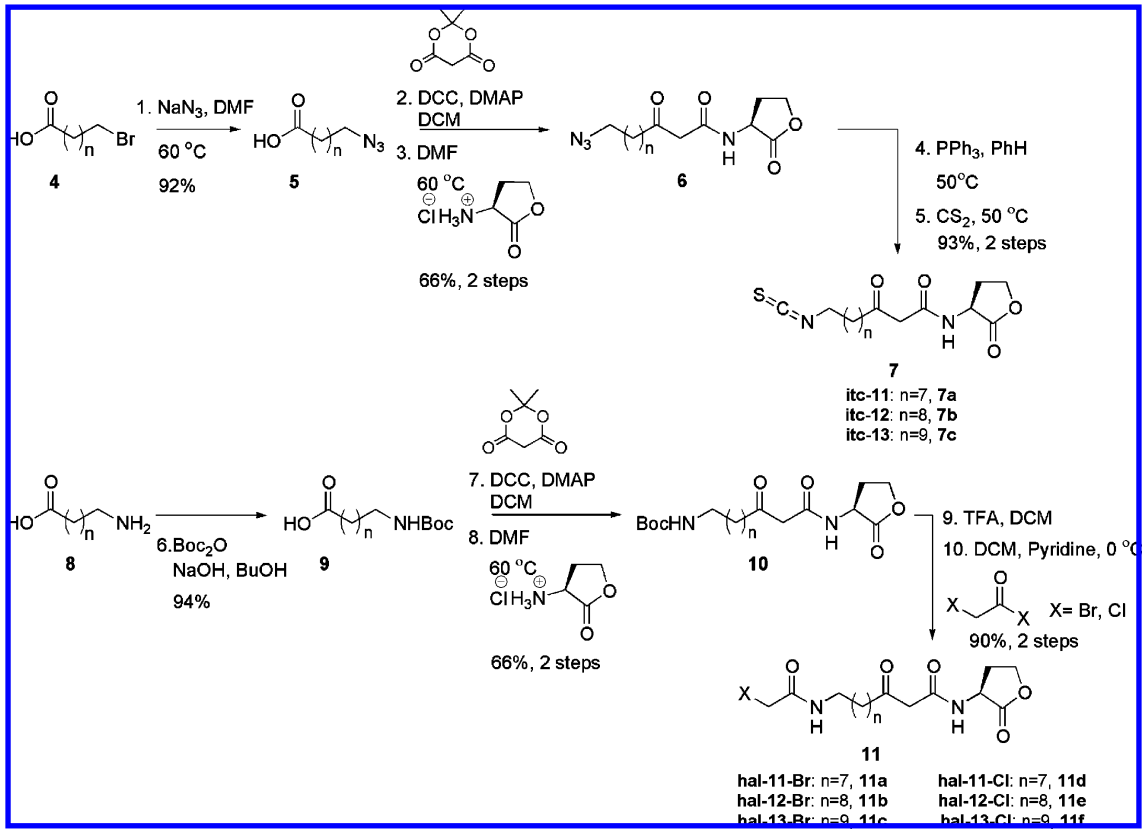
Scheme 1. Synthesis of the Nine Covalent Probes[a]



[a] DMF, dimethylformamide; DCC, *N,N′*-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; DCM, dichloromethane; TFA, trifluoroacetic acid.

LC-MS measurements revealed that the purified LasR-LBD (MW 22,430 Da, Figure 4b) could be covalently modified with itc-11 (MW 340 Da) and itc-12 (MW itc-12 354 Da) (Figure 4c,d), with calculated masses being in good agreement with measured masses (22 770 Da vs 22 770 Da and 22 784 Da vs 22 783 Da, respectively). Importantly, even though a large excess of probes was used (10−100 μM in the bacterial growth culture, leading to expression of 0.5−3.5 μM LasR-LBD), no more than one unit of covalently attached probe could be observed, indicating the reaction to be sufficiently specific at the concentrations used. No such covalent modifications were observed upon purification of LasR-LBD from cells incubated with any of the haloacetamides. From these results, we deduced that either no covalent reaction had taken place between the haloacetamides and LasR, meaning that their inhibitory effect is mediated in a manner similar to other strong noncovalent inhibitors (i.e., binding nascent LasR followed by misfolding and precipitation), or that a covalent reaction had occurred; yet, because of insolubility of the protein, we were unable to observe the product.

Covalent Binders React Specifically with Cys79 in the LasR Binding Pocket. Next, we sought to determine whether Cys79 is labeled with the probes, as predicted. LasR-LBD was expressed in the presence of either 3-oxo-C_{12}-HSL or itc-12 (or itc-11), followed by protein purification and trypsin digestion. The cysteine-containing fragment (72-VDPTVSHCTQSVLPIF-WEPSIYQTR-96) was identified by LC/MS as a single peak (2903.4 Da), while a modified peptide with increased retention time and a mass gain corresponding to itc-12 (or itc-11) attachment was also identified (Figures S18−S24 in the Supporting Information). Tandem MS/MS measurements on both modified and unmodified LasR-LBD confirmed that indeed Cys79 had reacted with the covalent probes (Figures S25−S27 in the Supporting Information).

In addition, we introduced two point mutations (Cys→Ala or Cys→Ser) to examine whether LasR-LBD is still covalently modified in the absence of a reactive thiol moiety in its binding pocket. As expected, upon overexpression of the LasR-LBD Cys79Ala mutant in bacteria incubated with itc-12, no covalent modification was detected (Figures 13−16 in the Supporting Information). Soluble protein was, however, obtained, indicating that the mutant LasR was able to recognize the isothiocyanate probe as a substrate that induces correct folding. Likewise, the Cys79Ser mutation yielded expression of soluble protein in the presence of itc-12, despite no covalent modification being observed. These observations strengthen our hypothesis that a reaction can take place only upon correct binding of the isothiocyanate probe in close proximity to a sufficiently strong nucleophile.

Notably, when native LasR-LBD was expressed in the presence of itc-11 and itc-12, covalent labeling often appeared incomplete and resulted in significant amounts of soluble, nonlabeled LasR-LBD (25−40%, depending on conditions), indicating that an alternative binding mode for the isothiocyanates may exist in which the reactive carbon atom is located sufficiently far from Cys79 so as to prevent a reaction.

Computational Analysis of LasR−Isothiocyanate Interactions. We sought to complement our experimental data by computational conformational analyses and docking calculations simulating the binding of the isothiocyanates to LasR. As a control to validate the docking procedure, the natural 3-oxo-C_{12}-HSL ligand was removed from its binding site and successfully redocked, i.e., the conformation corresponding to the ligand in the crystal structure was very highly ranked among

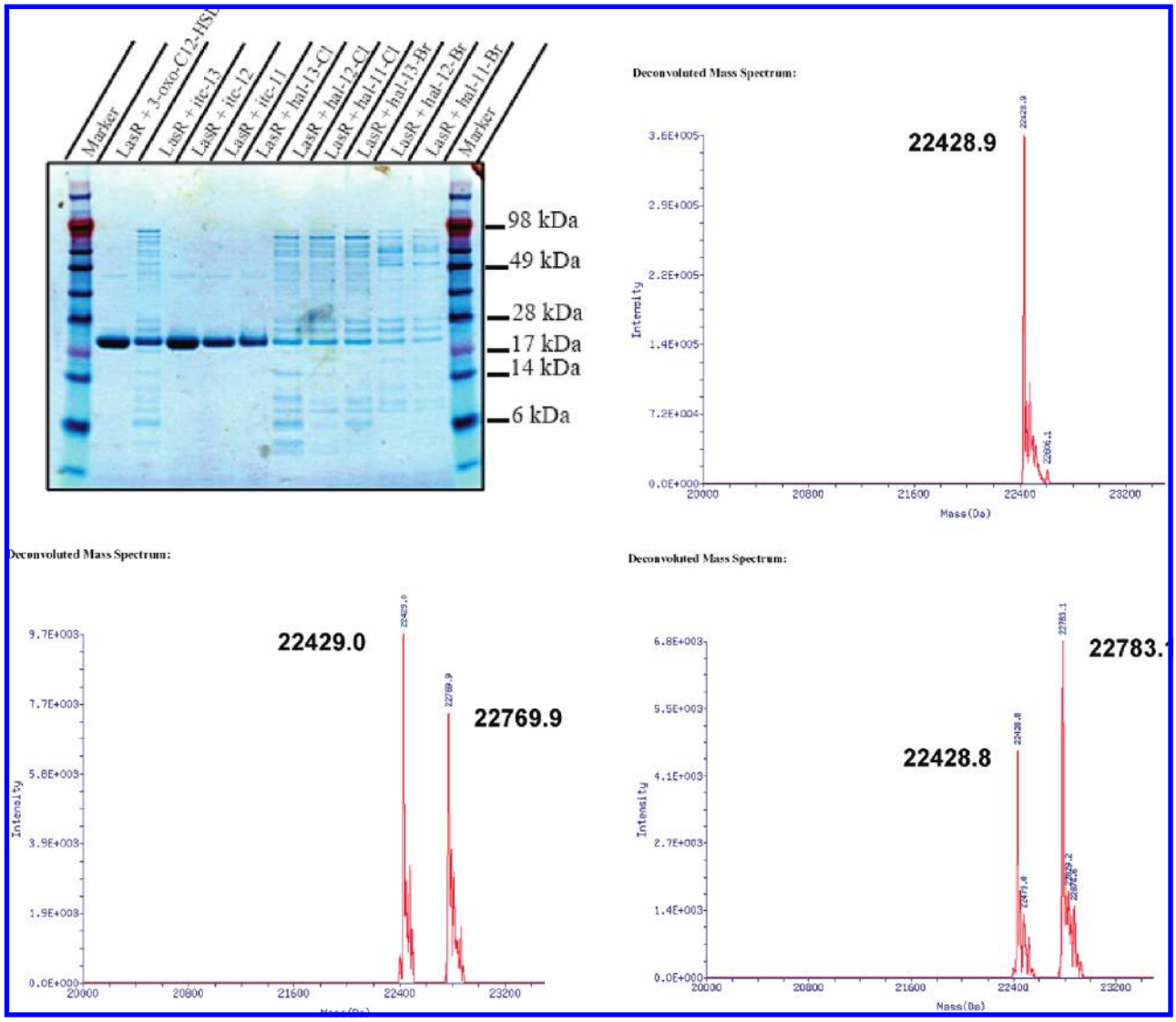


Figure 4. Covalent binding of itc-11 and itc-12 to LasR-LBD: (A) SDS-PAGE of purified LasR-LBD, expressed in the presence of 3-oxo-C_{12}-HSL and nine reactive probes. (B) deconvoluted mass spectrum of LasR-LBD expressed in the presence of 3-oxo-C_{12}-HSL. (C) Deconvoluted MS of LasR-LBD expressed in the presence of itc-11. (D) Deconvoluted MS of LasR-LBD expressed in the presence of itc-12. Full spectral data can be found in the Supporting Information.

the output poses, with an rmsd < 0.2 Å for all non-hydrogen atoms. The three isothiocyanate compounds were then docked into the LasR-binding site. The most highly ranked pose for each ligand was then submitted to an extensive conformational analysis in the context of the protein, which was considered as a rigid body. This analysis revealed that the longest isothiocyanate, i.e., itc-13, cannot be accommodated in the binding site without disrupting the interactions of the polar headgroup with the protein. In contrast, the shorter compounds, namely itc-11 and itc-12, can be accommodated while maintaining all favorable polar interactions with the protein. Interestingly, the energy-minimized conformers observed for both itc-11 and itc-12 clustered into two groups, differing significantly only in the orientation of their isothiocyanate group (Figure 3b). One orientation presents an ideal preorganization for nucleophilic attack by the sulfur atom of Cys79, whereas the other orientation is suboptimal for this reaction. For itc-11, the conformer population was equally divided (50/50), whereas for itc-12, approximately 66% of the population adopted the conformation suitable for the reaction. The nucleophilic attack would be enhanced by reorientation of the Cys79 side chain toward the itc compounds; the LasR crystal structure suggests this rotamer would be permitted.

Reactive Probes Inhibit QS in *P. aeruginosa.* The activities of the covalent probes were evaluated using several reporter strains, namely the luminescent PAO1-*luxABCDE* wild type strain and a PAO1 *lasI-rhlI* double mutant (PAO-JP2-*luxABCDE*), as well as an *E. coli* β-galactosidase-LasR-based reporter strain. Several isothiocyanates and bromoacetamides strongly inhibited luminescense in the wild type strain (Figure 5a,b), while some of the probes displayed both agonist and antagonist activity in assays performed with the PAO-JP2- and *E. coli* strains (Figure 5c,d). To compare our data with those reported for known strong QS inhibitors, we synthesized a control antagonist, 2-(4-bromophenyl)-*N*-(2-oxotetrahydrofuran-3-yl)acetamide (4-Br-PHL), identified by Blackwell and co-workers as one of the most active *P. aeruginosa* QS antagonists.[21] In the *E. coli*-based LasR antagonist studies (Figure 5c,d), we obtained an IC_{50} value for 4-Br-PHL (4.8 $\pm$ 0.5 μM) similar to that reported by Geske et al. (3.9 μM).[21] Of the nine probes

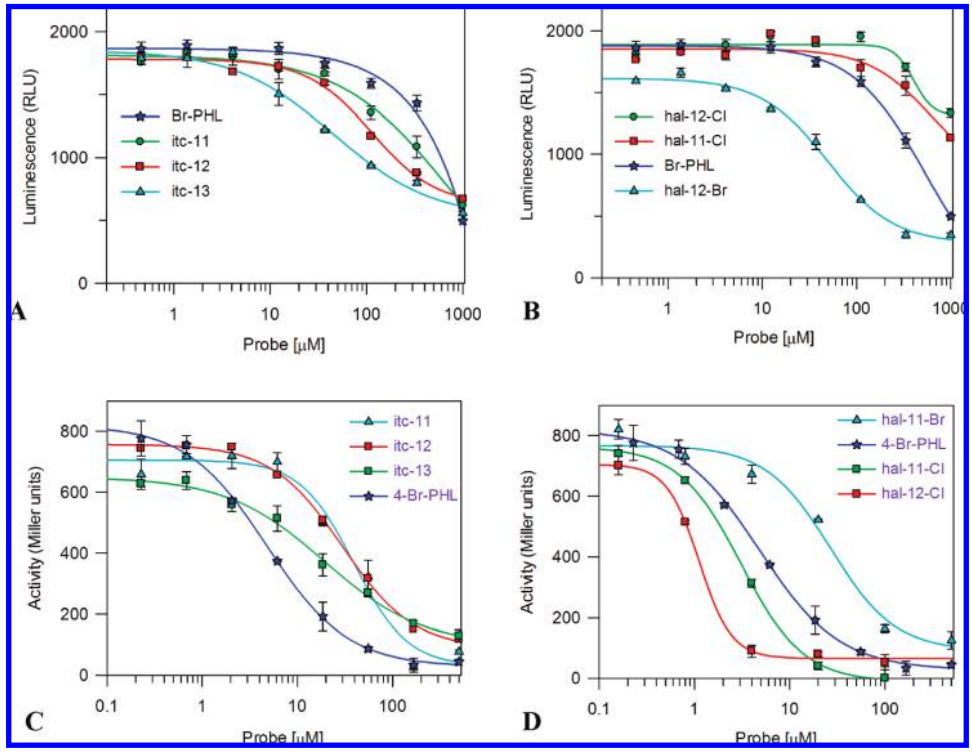


Figure 5. Reporter gene assays. PAO1 QS inhibition by isothiocyanates (A) and haloacetamides (B); antagonism of LasR activation by 50 nM 3-oxo-C_{12}-HSL in *E. coli* (this reporter strain does not produce 3-oxo-C_{12}-HSL) by isothiocyanates (C) and haloacetamides (D). Each point represents the average of three experiments ± SD.

screened in our assays, the chloroacetamide, hal-12-Cl, appeared to be the best antagonist (IC_{50}: 1.1 ± 0.1 μM), followed by hal-11-Cl and hal-11-Br (IC_{50}: 3.1 ± 0.1 μM and 26.8 ± 1.3 μM, respectively) and the three isothiocyanates, itc-11−13 (IC_{50}: 39.1 ± 9.4, 29.8 ± 0.5, 19.2 ± 3.9 μM, respectively). Surprisingly, one of the bromoacetamides (i.e., hal-13-Br) showed a strong enhancement of LasR activation at higher concentrations in our assay, while its shorter analogues acted as strong inhibitors.

In our inhibition assays relying on the wild type PAO1 reporter strain, we observed quite different behaviors for the tested analogues. The strongest inhibitors of luminescence appeared to be itc-13 and hal-12-Br, followed by itc-12, itc-11, and 4-Br-PHL (Figure 5a,b; IC_{50}s: itc-13: 45.2 ± 0.7 μM, hal-12-Br: 100 ± 7 μM; itc-12, 113 ± 19 μM; itc-11: ~300 μM). Strikingly, 4-Br-PHL displayed much weaker LasR antagonism (IC_{50}: ~250 μM) in the wild-type PAO1 reporter strain than in the *E. coli* reporter.

In addition to studies relying on the *E. coli* reporter strain, we performed experiments using a PAO1 mutant that does not produce 3-oxo-C_{12}-HSL (i.e., strain PAO-JP2) to verify whether the various inhibitors showed specific 3-oxo-C_{12}-HSL antagonist activity (Figure 6a,b). Of the nine probes considered, itc-13 (IC_{50}: 30 ± 7 μM), hal-12-Cl (70 ± 27 μM), hal-12-Br (85 ± 1 μM), itc-12 (134 ± 6 μM) and 4-Br-PHL (~200 μM) displayed significant antagonism. We entertained the counter-intuitive possibility that the mode of active inhibition was noncovalent and, accordingly, performed experiments with an azido isostere analogue of itc-12 (azido-C12) unable to react with Cys79. The inhibitory activity of this analogue was significantly lower than that of itc-12 (Figures S28 in the Supporting Information), with no covalently labeled product being observed in MS measurements of purified LasR-LBD expressed in the presence of azido-C12 (Figures S17 in the Supporting Information).

We also examined whether the inhibitory effects of the covalent QS inhibitors could be attributed to partial agonism, since several of the inhibitors (in particular, the isothiocyanates) showed agonism using the PAO-JP2-based reporter (Figure 6c), albeit to markedly reduced levels, as compared to the natural autoinducer. We focused on itc-12 since this probe consistently displayed strong activity in all assays. When compared to the other inhibitors, itc-12 appeared to induce the expression of larger amounts of soluble LasR-LBD. Blackwell and co-workers recently showed that several of their inhibitors displayed characteristic partial agonism patterns.[21] Our data also display partial agonism patterns (Figure 6d), although at high concentrations of itc-12, marked differences in the effects elicited by these other inhibitors and itc-12 was noted. Our explanation for this phenomenon is that the observed differences can be explained by the covalent binding mode of the reactive itc-12 probe.

Isothiocyanate-Based Probes Inhibit QS-Regulated Activities. To assess whether the reactive probes inhibit QS-regulated activities, such as biofilm formation and pyocyanin production, we incubated the wild type *P. aeruginosa* PAO1 strain in the presence of itc-12 and 4-Br-PHL (both at 50 μM), or DMSO, as a control, in microtiter plates that allow analysis of 24 h biofilm formation and in vials allowing measurement of 36 h pyocyanin production. As shown in Figure 7a,b, both activities were significantly inhibited in the presence of the isothiocyanate probe, as well as the known QS inhibitor, 4-Br-PHL. Full inhibition of either phenotype is rarely seen, suggesting regulation by QS-associated mechanisms to be only partial. However, even a partial reduction in biofilm formation may be sufficient to render the bacteria vulnerable to host responses, as not only is total biofilm mass affected upon disruption of QS but also

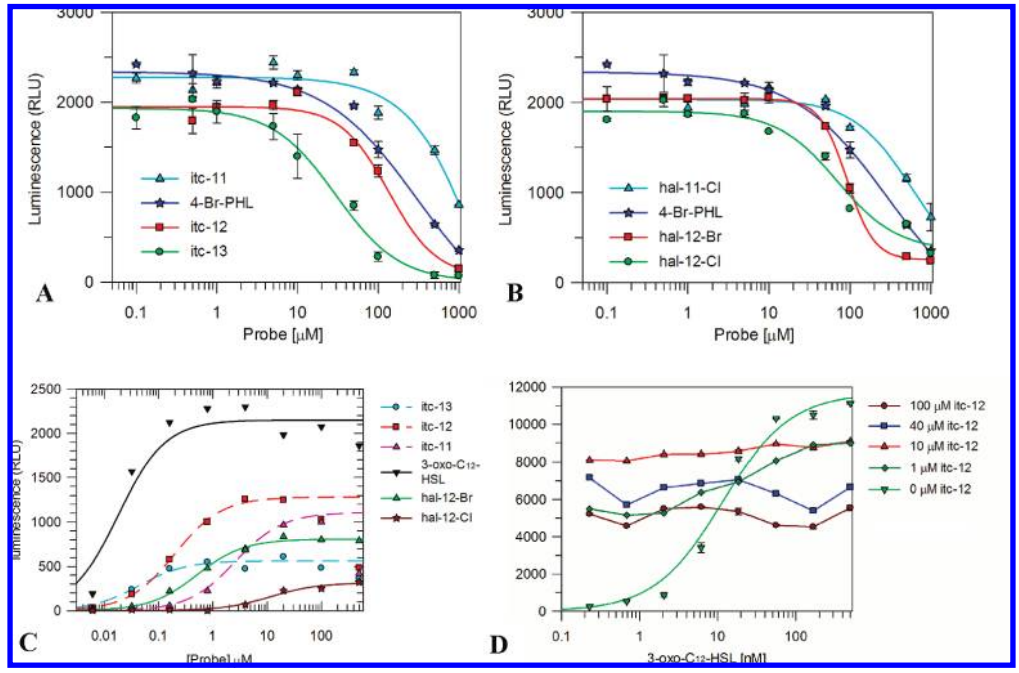


Figure 6. PAO-JP2-based antagonist (A and B), agonist (C), and partial agonist (D) assays. The curve shapes in the partial agonist assay can be attributed to the covalent binding mode of itc-12, as detailed further in the Supporting Information. Each point represents the average of three experiments ± SD.

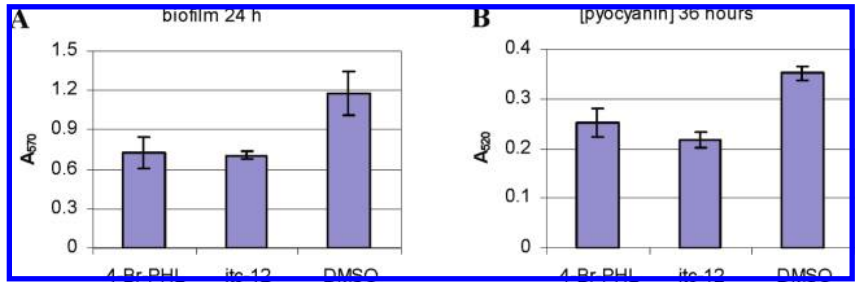


Figure 7. Inhibition of biofilm formation (a) after 24 h and pyocyanin production (b) after 36 h, upon incubation of wild type *P. aeruginosa* strain PAO1 with 50 μM 4-Br-PHL, itc-12 or DMSO. Each bar represents the average of three experiments ± SD.

its architecture, its degree of porosity, and its extent of flexibility and robustness.[30]

Discussion

Chemical attenuation of bacterial quorum sensing has become an increasingly studied topic. In this study, we have explored a new mode of QS inhibition. With a set of reactive probes, we were able to target the *P. aeruginosa* QS regulator, LasR, and examine whether QS can be inhibited through covalent binding of this protein. We determined that our isothiocyanate-based probes covalently and selectively bound Cys79, found in the LasR binding pocket. Furthermore, through the use of several well-characterized reporter strains, we were able to evaluate the influence of the nine synthetic inhibitors on *P. aeruginosa* quorum sensing-related gene expression. Although differences in measured activity between reporter assays were noted, strong inhibition of QS was observed for the isothiocyanate analogues.

Ambiguous effects were seen for the haloacetamides, with bromoacetamide hal-12-Br showing strong activity. No covalent interactions between any of the haloacetamides and LasR were, however, observed. From these results, we deduced that no covalent reaction had taken place between the haloacetamides and LasR, meaning that their inhibitory effect may be mediated in a manner similar to other strong inhibitors, namely via binding nascent LasR followed by protein misfolding and precipitation. When compared to 4-Br-PHL, the isothiocyanates showed similar activity overall, with low micromolar IC_{50} values being measured for itc-12 and itc-13 in assays using the *E. coli* reporter strain. Perhaps most striking is the large difference in activity between itc-13 and 4-Br-PHL in the PAO-JP2-based antagonist assay. It should be noted though that comparison of IC_{50} values of different compounds obtained through the use of different strains and reporter assays is problematic, as differences in membrane composition, secondary regulation of gene expression, competing ligands, etc., may all have large effects on the observed inhibition. Therefore, it is difficult to draw absolute conclusions with respect to the extent of inhibition of specific QS systems by certain compounds. Nevertheless, a compound that shows good and specific inhibition in a reporter assay, as well as phenotypical inhibition in a wild type strain, can be regarded as a good candidate for further QS inhibition and mechanistic studies. The isothiocyanates showed significant inhibition of QS at low concentrations in all assays. As such, we decided to study the efficacy and mode of action of one such compound, itc-12, in more detail. In assays with the wild

(30) (a) Davies, D. *Nat. Rev. Drug Discovery* **2003**, *2*, 114–122. (b) Hentzer, M.; Eberl, L.; Nielsen, J.; Givskov, M. *BioDrugs* **2003**, *17*, 241–50.

type PAO1 strain, itc-12 showed significant inhibition of QS-controlled virulence factor expression, as well as biofilm formation.

We propose the following model to describe the mode of action of the covalent isothiocyanate-based inhibitors, based on our experimental findings. Because of the close isosteric relation between these compounds and the natural LasR ligand, 3-oxo-C_{12}-HSL, the designed isothiocyanates are able to compete with 3-oxo-C_{12}-HSL for access to the binding pocket. Upon binding, however, two or more binding modes are favored, one of which leads to a covalent bond with Cys79. LC/MS analysis of purified LasR-LBD revealed that a portion of LasR was alkylated, while part was not. Indeed, unreacted itc-12 could be extracted from the protein after purification (0.05−0.25 mol equiv). Our experiments with azido-C12, serving as a control isostere, indicated that the main inhibitory effects of these reactive probes involved covalent binding. The noncovalent binding mode results in the formation of folded and soluble protein, as observed by MS experiments.

Conformational analyses of the isothiocyanate/LasR-LBD interaction revealed two main sets of conformers of itc-11 and itc-12 that could occupy the ligand-binding pocket of LasR. Of these two conformations, only one is well-positioned for nucleophilic attack by the sulfur atom of Cys79. The itc-13 probe was not readily accommodated in the binding pocket, suggesting that binding of its headgroup would prevent stable folding of the protein. These models are compatible with our ability to purify stable forms of LasR-LBD bound to itc-11 and itc-12 but not to itc-13. The failure to accommodate the larger isothiocyanate (itc-13) reflects the high degree of ligand specificity exhibited by LasR-LBD, as also demonstrated by its failure to form stable complexes with shorter noncognate ligands, such as 3-oxo-C_8−, 3-oxo-C_6−, or 3-oxo-C_4−HSL, or the brominated furanone, C30.[16]

Our models suggest that if the isothiocyanates are accommodated concomitantly with protein folding, there would be little possibility for interconversion of conformers belonging to the two different clusters. Consequently, chemical modifications of itc-12 that promote its initial accommodation in the reactive conformation would be expected to increase the potency of this class of probe. The noncovalent binding mode is likely to compete poorly with binding of the natural ligand to LasR. On the basis of our model, this may partially explain the relatively large excess of itc-12 needed (2 to 3 orders of magnitude) to achieve significant inhibition of QS, as well as the results indicating that our isothiocyanates are partial agonists.[31] While itc-12, when positioned correctly and aided by an irreversible reaction with Cys79, is expected to effectively compete with 3-oxo-C_{12}-HSL for occupation of the LasR binding pocket, a significant proportion of the inhibitor molecules is not so positioned, resulting in an overall decrease in efficacy. As these are the first, rationally designed covalent QS inhibitors, we believe that further structural refinement and optimization of these compounds will lead to increased efficacies.

An interesting question that could, in principle, be addressed through the use of covalent probes asks whether the selection of 3-oxo-*N*-acyl homoserine lactones by Gram-negative bacteria as autoinducers has been driven purely by molecular recognition of the active species, or whether the degradation of these molecules plays an active role in recycling the transcriptional activator. At neutral pH, AHLs are hydrolyzed to their ring-open forms with rate constants of 4.8×10^{-6} s^{-1}. Upon hydrolysis of the lactone ring, 3-oxo-C_{12}-HSL is expected to lose affinity for LasR, such that concomitant release of its substrate will result in a conformational change in LasR that will cause disruption of dimer interactions and removal of the activator from its target DNA. In a detailed study by Greenberg and co-workers on the DNA binding of purified LasR,[29] it was demonstrated that 3-oxo-C_{12}-HSL is bound sufficiently tight to LasR, in a 1:1 molar ratio, so as to resist release when extensively dialyzed at 4 °C. At room temperature, however, there was apparent release of 3-oxo-C_{12}-HSL, resulting in loss of LasR activity, a finding that supports a mechanism whereby release of the autoinducer is mediated by accelerated hydrolysis of the lactone ring. In the PAO-JP2-based reporter assay, maximal activation of QS (or QS-regulated gene expression) by 3-oxo-C_{12}-HSL can be seen after 8 h of incubation, followed by a sharp decrease in gene expression. When the same strain was incubated in the presence of itc-12, activation was slower and markedly reduced but was sustained for many hours (Figure S29 in the Supporting Information), suggesting that covalent activation of LasR had rendered it into a permanently active state. This may actually be detrimental to the bacteria, since it will interfere with the ability of the colony to coordinate gene expression optimally as a function of population density.

Conclusion

We have shown that the *las* quorum sensing system of *P. aeruginosa* can be targeted by covalent probes, resulting in specific inhibition of the transcriptional activator, LasR. This is, to the best of our knowledge, the first example of covalent modification of a quorum sensing receptor and the study of its effects on bacterial communication. Several of the inhibitors were shown to specifically label LasR inside the binding pocket, as predicted. Moreover, through detailed analysis of the mode of binding of one of the reactive probes, we have demonstrated that LasR Cys79 is labeled covalently. The most potent inhibitors were shown to be partial agonists, effectively inhibiting pyocyanin production, as well as biofilm formation, at moderate concentrations. Together, our experimental and modeling studies provide a basis for future structural adjustments to the itc-based probes introduced here that may lead to the preparation of more potent and effective inhibitors of bacterial pathogenicity. It should be noted that isothiocyanates are relatively stable at low concentrations, are found at micromolar concentrations in various plants, and, moreover, have shown significant bioactivity in animal models (e.g., inhibition of carcinogenesis),[32] pointing to their stability in vivo.

Because of increasing bacterial resistance to new antibiotics, inhibition of bacterial virulence has been proposed as a viable new therapeutic target. Such a strategy may yield desired results without inducing resistance to drugs targeting virulence, in contrast to drugs targeting bacterial growth. Furthermore, covalent probes that target LasR (or its homologues in other bacteria, as well as structurally characterized receptors for

(31) Zhu, B. T. *Biomed Pharmacother* **2005**, *59*, 76–89. See the Supporting Information for further discussion on the extent of predicted and measured partial agonism for these inhibitors.

(32) (a) Mi, L.; Xiao, Z.; Hood, B. L.; Dakshanamurthy, S.; Wang, X.; Govind, S.; Conrads, T. P.; Veenstra, T. D.; Chung, F. L. *J. Biol. Chem.* **2008**, *283*, 22136–46. (b) Pan, M. H.; Ho, C. T. *Chem. Soc. Rev.* **2008**, *37*, 2558–74.

other classes of QS molecules) may be used as molecular tools to provide novel insight into the mechanisms of activation and deactivation of bacterial quorum sensing.

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Supporting Information Available: Additional methods, including descriptions of reagents, synthetic procedures, computational analysis, and biofilm assay; NMR and MS data and spectra. Complete ref 14. This material is available free of charge via the Internet at http://pubs.acs.org/.

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