<table>
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<tr>
<th><strong>Title</strong></th>
<th>Identification of five structurally unrelated quorum-sensing inhibitors of pseudomonas aeruginosa from a natural-derivative database</th>
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<tr>
<td><strong>Author(s)</strong></td>
<td>Tan, Sean Yang-Yi; Chua, Song-Lin; Chen, Yicai; Rice, Scott A.; Kjelleberg, Staffan; Nielsen, Thomas Eiland; Yang, Liang; Givskov, Michael</td>
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<tr>
<td><strong>Date</strong></td>
<td>2013</td>
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<td><strong>URL</strong></td>
<td><a href="http://hdl.handle.net/10220/26348">http://hdl.handle.net/10220/26348</a></td>
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Bacteria communicate via a phenomenon termed quorum sensing (QS). QS enables bacteria to organize their activities at the population level, including the coordinated secretion of virulence factors. Certain small-molecule compounds, known as quorum-sensing inhibitors (QSIs), have been shown to effectively block QS and subsequently attenuate the virulence of *Pseudomonas aeruginosa*, as well as increasing its susceptibility to both antibiotics and the immune system. In this study, a structure-based virtual screening (SB-VS) approach was used for the discovery of novel QSI candidates. Three-dimensional structures of 3,040 natural compounds and their derivatives were obtained, after which molecular docking was performed using the QS receptor LasR as a target. Based on docking scores and molecular masses, 22 compounds were purchased to determine their efficacies as quorum-sensing inhibitors. Using a live reporter assay for quorum sensing, 5 compounds were found to be able to inhibit QS-regulated gene expression in *P. aeruginosa* in a dose-dependent manner. The most promising compound, G1, was evaluated by isobaric tag for relative and absolute quantitation (iTRAQ)-based proteomic analysis, and it was found to significantly affect the abundance of 46 proteins (19 were upregulated; 27 were downregulated) in *P. aeruginosa* PAO1. It specifically reduced the expression of several quorum-sensing-regulated virulence factors, such as protease IV, chitinase, and pyoverdine synthetases. G1 was also able to reduce extracellular DNA release and inhibited the secretion of the virulence factor, elastase, whose expression is regulated by LasR. These results demonstrate the utility of SB-VS for the discovery of target-specific QSIs.

The virulence of pathogenic bacteria. During the last decade, the QS system has been proposed as a target for developing next-generation antimicrobial agents. The rationale for interrupting bacterial communication rather than inhibiting growth is that QS inhibitors (QSIs), by targeting nonessential processes, are shown to not exert strong selective pressure for the evolution of resistance mechanisms compared to the conventional growth-inhibitory compounds (14, 15).

**References**

The conventional approach to identifying QSIs is to use biosensor systems which often fuse a QS-regulated promoter to the lux, gfp, or lacZ reporter gene (16, 17, 18). A wide range of QSIs were identified by the use of these biosensor systems (19). However, QSIs identified through the use of biosensors might not be target specific and have some potential risk in their application. QS regulation is integrated into the complex bacterial regulation networks which also include nucleotide signaling (e.g., cAMP and c-di-GMP) (20), iron signaling (21), phosphate signaling (22), and so on. Thus, QSIs identified through the use of biosensor systems might actually target other regulators which also affect QS. This brings the risk that these QSIs might be able to induce virulence factors regulated by other regulation networks even though they can inhibit QS. Therefore, a computer-aided method for the rational identification of QSIs may provide a means of discovering QSIs with increased target specificity.

In contrast to the conventional lab-based screens, some have utilized a computer-based approach to drug screening known as structure-based virtual screening (SB-VS). SB-VS can be defined as a method to computationally screen large compound libraries for molecules that bind targets of known structure and then test experimentally those predicted to bind well (23). Recent successes of this approach include inhibitors against the apoptosis regulator Bcl-2, Hsp90, G-protein-coupled receptors, and metalloenzymes (24).

With the recent availability of crystal structures of bacterial QS receptor proteins, such as LasR of *Pseudomonas aeruginosa* (25) and TraR of *Agrobacterium tumefaciens* (26), SB-VS has become a viable option for QSI discovery. Yang et al. (27) used an SB-VS approach in the search for novel inhibitors of the LasR protein of *P. aeruginosa* (27). A total of 147 compounds from the Super-Natural (28) and SuperDrug (29) databases were selected for the screening through a rational approach, based on two-dimensional (2D) structure similarity to known QSIs. These compounds were then subjected to molecular docking against the ligand-binding domain of LasR, and the top-scoring “hits” were then tested for biological activity. Several recognized drugs, such as salicylic acid, were identified as QSIs.

In this study, we investigated the use of a computer-aided approach for the discovery of novel QS inhibitors of the *P. aeruginosa* LasR protein. The DG-AMMOS software program (30) was used to convert 2D chemical structures into 3-dimensional (3D) conformations based on distance geometry. The entire library of compounds from TimTec’s Natural Derivatives Library, 3,040 in all, was converted to 3D structures through the use of AMMOS (30), was used to convert the entire library of 2D chemical structures into 3D conformations based on distance geometry and automated molecular mechanics optimization. Thus, the entire library of compounds from TimTec’s Natural Derivatives Library, 3,040 in all, was converted to 3D structures and used for the subsequent molecular docking procedures. 2D structures of reference compounds were drawn manually using the software program Marvin Sketch v. 5.9.0 (ChemAxon Ltd., Hungary) and then saved as 2D SDF files. The SDF files were merged into a single SDF file using the program OpenBabel v. 2.3.1 (OpenEye Scientific Software) and likewise converted to 3D structures through the use of DG-AMMOS.

**Molecular docking.** The Molegro Virtual Docker (MVD) v. 5.0.0 software program (31) (Molegro ApS, Denmark) was used for the automated docking procedure. MVD uses the MOLDOCK algorithm, which was able to correctly identify the binding mode of ligands with 87% accuracy, which was higher than that of the modern docking programs GLIDE (32) and Surflex (33). From the LasR PDB structure file, only the E monomer LBD in a complex with the OdDHL ligand, OHN 1169 [E], was imported into the MVD workspace. Water molecules were not imported, and default parameters were used for the import process.

For the docking process, a docking template was first generated based on the interaction of the native OdDHL ligand with the LasR ligand-binding domain. This template included steric, hydrogen donor, hydrogen acceptor, and ring contributions. Template docking was then carried out using Ligand Evaluator for scoring of the poses and Moldock Optimizer for the search algorithm. The docking was constrained to a sphere of 9-Å radius centered on the OdDHL ligand and its corresponding cavity. For molecular docking, 3,040 ligands from the Natural Derivative Library and 7 reference ligands (OdDHL reference ligand along with 7 known QSIs) were used. Each compound was docked for 10 iterations in order to obtain an energy-minimized protein-ligand conformation. One pose was generated by each run, and the resulting poses were ranked. The parameters used for import of molecules and docking in MVD are specified in Table S3 in the supplemental material. The software program LIGPLOT v. 4.5.3 (34) was used to map the interactions between QSI lead compounds and the residues within the LasR protein LBD. PyMOL v. 1.4 (Schrodinger, LLC) was used for preparation of 3D images.

**Selection of QSI candidates.** Compounds from the TimTec Natural Derivative Library were docked against the LasR LBD in MVD and subsequently ranked according to their rerank score, molecular mass in Daltons, and calculated ligand efficiency (computed as rerank score divided by the number of heavy atoms in a compound). In order to select for small-molecule QSI candidates, only compounds having a molecular mass less than 200 Da and a rerank score below ~60 were selected. The rerank score value of ~60 was used as a cutoff because known QSIs that were docked in MVD generally had a score below ~60. A total of 22 QSI candidates were selected and were purchased from TimTec, Inc. (TimTec LLC, Newark, DE). Compounds were shipped in glass vials in powder form, and these were dissolved in dimethyl sulfoxide (DMSO) prior to *in vitro* experiments. The purity (>90%) and identity of all compounds were confirmed by high-performance liquid chromatography-mass spectrometry (electrospray ionization) [HPLC-MS (ESI)] on a Waters Aquity reverse-phase ultraperformance liquid chromatography (RP-UPLC) system equipped with a diode array detector using an Aquity UPLC BEH C18 column (diameter, 1.7 μm; 2.1 by 50 mm; column temperature, 65°C;
As such, unstable degradation by housekeeping proteases and therefore to have a short half-life. (ASV) allows for monitoring of temporal QS-regulated gene expression (35). For proteomic analysis, we used the sequenced E. coli MT102 wild-type strain obtained from the Pseudomonas Genetic Stock Center (PAO0001).

Growth media and conditions. The bacteria strains were grown in either ABTGC (see below) or Luria-Bertani (LB) medium.

**ABTGC medium.** ABTGC medium is AB minimal medium (64) containing 2.5 mg/liter thiamine, supplemented with 0.2% (wt/vol) glucose and 0.2% (wt/vol) Casamino Acids; LB medium contains 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl, adjusted to pH 7.0. Overnight cultures were grown for 16 h at 37°C and shaken at 180 rpm. Selective media were supplemented with ampicillin (100 mg liter \(^{-1}\)) or gentamicin (60 mg liter \(^{-1}\)) where appropriate.

**P. aeruginosa QS inhibition assays.** Test compounds were dissolved in 100% DMSO and mixed with ABTGC medium, after which they were added to the first column of wells of a 96-well microtiter plate (Nunc) to give a final concentration of 100 μM in a final volume of 200 μl. One hundred microliters of ABTGC medium was then added to the remaining wells in the plate, and serial 2-fold dilutions of the inhibitors were made by adding 100 μl of the preceding inhibitor-containing well to the subsequent one. The final column was left without inhibitor as a control. Next, an overnight culture of the P. aeruginosa lasB-gfp (ASV) strain, grown in LB medium at 37°C with shaking, was diluted to an optical density at 600 nm (OD\(_{600}\)) of 0.2, and 100 μl of bacterial suspension was added to each well of the microtiter plate. Hence, inhibitor concentrations ranged from 50 μM to 0.78125 μM across the plate, in a volume of 200 μl. The microtiter plate was incubated at 37°C in a Tecan Infinite 200 Pro plate reader (Tecan Group Ltd., Mannedorf, Switzerland). GFP fluorescence (excitation at 485 nm, emission at 535 nm) and cell density (OD\(_{600}\)) measurements were collected at 15-min intervals for at least 14 h. The P. aeruginosa Rhl and Pqs inhibition assays were performed in a manner similar to that for the LasR inhibition assay.

**E. coli competition assay between QS inhibitors and ODDHL.** The E. coli lasB-gfp (ASV) reporter strain was used for the competition assay, which was performed in a manner similar to that for the P. aeruginosa LasR inhibition assay. OdDHL and the QSIs to be studied were added to

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**TABLE 1.** P. aeruginosa and E. coli strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Name and genotype</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>PAO1</td>
<td>Wild type</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>PAO1-gfp</td>
<td>GFP-tagged</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>PAO1 lasB-gfp</td>
<td>Gmr; PAO1 containing lasB-gfp</td>
<td>59</td>
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<tr>
<td></td>
<td>PAO1 rhlA-gfp</td>
<td>Gmr; PAO1, rhlA-gfp (ASV) reporter fusion</td>
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</tr>
<tr>
<td></td>
<td>PAO1 pqsA-gfp</td>
<td>Gmr; PAO1, pqsA-gfp (ASV) reporter fusion</td>
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<tr>
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<td>PAO1 ΔlasR</td>
<td>PAO1 lasR mutant</td>
<td>14</td>
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<tr>
<td></td>
<td>PAO1 ΔrhlA-gfp</td>
<td>Gmr (Carb); PAO1, rhlA-gfp (ASV) reporter fusion</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>PAO1 ΔpqsA-gfp</td>
<td>Gmr (Carb); PAO1, pqsA-gfp (ASV) reporter fusion</td>
<td>27</td>
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<tr>
<td><em>E. coli</em></td>
<td>MT102</td>
<td>Wild type</td>
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</tr>
<tr>
<td></td>
<td>MT102 lasB-gfp</td>
<td>MT102 containing lasB-gfp (ASV) reporter fusion</td>
<td>59</td>
</tr>
</tbody>
</table>

*Description of the strains’ antibiotic resistance. Gmr, gentamicin resistance; Carb*, carbenicillin resistance.

References describing the sources of the respective strains.

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flow, 0.6 ml/min). Eluents A (0.1% HCO\(_2\)H in H\(_2\)O) and B (0.1% HCO\(_2\)H in acetonitrile [MeCN]) were used in a linear gradient (5% B to 100% B) in a total run time of 2.6 min. The liquid chromatography (LC) system was coupled to an SQD (single quadrupole detector) mass spectrometer.

**Bacterial strains.** To determine the QSI activity of the compounds selected from the virtual screening, the following *P. aeruginosa* and *E. coli* monitor strains were used (Table 1). These monitor strains have their respective promoters fused to an unstable GFP (green fluorescent protein) that has a C-terminal oligopeptide extension containing the amino acids ASV [gfp(ASV)]; this causes the GFP to be more susceptible to degradation by housekeeping proteases and therefore to have a short half-life. As such, unstable gfp(ASV) allows for monitoring of temporal QS-regulated gene expression (35).

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**FIG 1.** The chemical structures of the reference ligand, OdDHL, and other known QS inhibitors used as comparisons for the structure-based virtual screening. References are given after for each compound. (A) 3-Oxo-C12-HSL (OdDHL) (7); (B) patulin (49); (C) salicylic acid (27); (D) 3-oxo-C12-(2-aminophenol) (63); (E) furanone C30 (14); (F) 4-nitropyridine-N-oxide (18); (G) nifuroxazide (27); (H) chloroxazone (27).
C1

F1

G1

H1

F2

404

FIG 2 Structures of five QSI candidates and an additional compound found to be structurally similar to C1 and G1. Five QSIs are shown: 6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (C1), 2-amino-3-(3-fluorophenyl)propanoic acid (F1), 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (G1), and 2-amino-3-hydroxy-3-phenylpropanoic acid (H1) and indole-3-carboxylic acid (F2). Compounds F1 and H1 are racemic, and asterisks within the structure denote the stereogenic centers within these compounds. Purine-2,6-diamine (404) was found through a structural similarity search of compounds with structures similar to that of G1.

The wells of a microtiter plate containing ABTGC medium. Wells without OdDHL and/or QSI were included as controls. An overnight culture of the E. coli lasR-gfp (ASV) strain, grown in LB medium at 37°C with shaking, was diluted to an OD600 of 0.2, and 100 μl of bacterial suspension was added to each well of the microtiter plate. Hence, the plate contained OdDHL at concentrations ranging from 20 nM to 320 nM and QSI at concentrations ranging from 3.125 μM to 50 μM (including control wells without OdDHL, QSI, or both). GFP and OD600 readings were obtained as described above for the P. aeruginosa LasR inhibition assay.

Inhibition of the rhl and pqs QS systems in wild-type PAO1 and a PAO1 lasR mutant. Wild-type P. aeruginosa PAO1 strains harboring either the rhlA-gfp (ASV) reporter or the pqsA-gfp (ASV) reporter and a P. aeruginosa PAO1 lasR mutant harboring either the rhlA-gfp (ASV) reporter or the pqsA-gfp (ASV) reporter were used for this experiment (see Table 1 for strain information). Overnight cultures of these four strains were diluted 100-fold in ABTGC medium within 96-well microtiter plates to a final volume of 200 μl per well. Each of the five QSIs was added in triplicate to a final concentration of 50 μM. DMSO at 0.5% was used as a negative control. The microtiter plate was incubated at 37°C in a Tecan Infinite 200 Pro plate reader, where GFP and OD600 readings were measured at 15-min intervals.

Glass-slide biofilm assay for observation of DNA release. The glass-slide biofilm assay was performed as previously reported (36). Briefly, gfp-tagged P. aeruginosa PAO1 biofilms were cultivated in 50 ml BD Falcon tubes containing 15 ml ABTGC medium. A sterile 24-mm by 60-mm glass cover slide was inserted into each Falcon tube for supporting biofilm growth. G1 (10 μM) was added to the biofilm medium to examine its impact on P. aeruginosa PAO1 biofilm formation. DMSO was added alone to the medium as a control. Biofilms were incubated at 37°C without shaking. A 2 μM concentration of propidium iodide (Sigma-Aldrich) was added to biofilm cultures to stain extracellular DNA (eDNA) for 5 min after 24-h growth. After that, biofilm-attached glass slides were observed by confocal laser scanning microscopy (CLSM).

iTRAQ-based proteomics analyses for G1. Isobaric tag for relative and absolute quantitation (iTRAQ)-based proteomic analysis was used to study the changes in protein expression of the P. aeruginosa PAO1 strain in response to the addition of 25 μM G1. Proteomics experiments were performed at the Proteomic Core Facility of the Biological Research Center, School of Biological Sciences, Nanyang Technological University, Singapore. A full description of the proteomics work flow is included as supplemental material.

Elastase assay. P. aeruginosa PAO1 wild type and an elastase-negative las rhl mutant were cultivated in LB medium overnight at 37°C with shaking. Overnight cultures were diluted 1:100 to 5 ml ABTGC medium and incubated at 37°C with shaking. Compound G1 was supplemented to the PAO1 cultures at final concentrations of 0, 50, and 100 μM. After 2 h of incubation, 0.8-ml culture supernatants were sampled by centrifugation (18,000 × g, 4 min) and filtration through 0.2-μm filters. Elastase activity of P. aeruginosa culture supernatants was measured by using the EnzChekElastase assay kit (Invitrogen), which uses BODIPY fluorophore (FL)-labeled DQ elastin conjugate as the substrate of elastase. The BODIPY FL-labeled DQ elastin conjugate, when cleaved by elastase enzyme, yields highly fluorescent fragments. Fluorescence was recorded every 6 min for 180 min by using a Tecan Infinite 200 Pro plate reader (excitation at 490 nm, emission at 520 nm).

RESULTS

Structure-based virtual screening for QSIs. Molecular docking was first performed using the reference ligand OdDHL and several known LasR inhibitors against the ligand-binding domain of LasR (PDB ID 2UV0) in MVD. These compounds and their structures are shown in Fig. 1, and their docking scores (see Table S1 in the supplemental material) provide a comparison for the selection of potential QSI candidates from the library compound screening.

A 3D structural database containing 3,040 structures of compounds from TimTec’s Natural Derivatives Library was created using DG-AMMOS (30), after which it was docked against the ligand binding domain of LasR. Twenty-two compounds having a rerank score below –60 and having a molecular mass less than 200
Da were selected as QSI candidates (these 22 structures are shown in Table S2 in the supplemental material). The cutoff value for the rerank score was set as $60$ because most of the known QSI that were docked earlier had scores below that value. The molecular mass cutoff value of 200 Da was arbitrarily determined in order to select for the most effective small-molecule inhibitors that can easily penetrate the bacteria cell. Thus, the 22 compounds selected were obtained and tested for its inhibition in vitro.

**Inhibition assay with the P. aeruginosa lasB-gfp(ASV) strain.** In the preliminary screen, the 22 selected QSI candidates were screened for their ability to inhibit QS-controlled green fluorescent protein (GFP) expression in the P. aeruginosa lasB-gfp (ASV) strain. Elastase (encoded by the lasB gene) is a virulence factor that is controlled by LasR and is therefore a good indicator for LasR activity (13). Five compounds, code named C1, F1, G1, H1, and F2 (Fig. 2), were found to inhibit LasR-controlled GFP expression in a dose-dependent manner without affecting cell growth. For ease of identification, each compound was designated with a short compound identification code based on its well position in the shipment in place of its standard IUPAC name.

The dose-response curves of these 5 QSI candidates when incubated with the P. aeruginosa PAO1 lasB-gfp (ASV) strain are shown in Fig. 3. GFP expression, which was measured in relative fluorescence units, was normalized by dividing the GFP value by control.

**FIG 3** Dose-response curves of 6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (C1) (A), 2-amino-3-(3-fluorophenyl)propanoic acid (F1) (B), 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (G1) (C), 2-amino-3-hydroxy-3-phenylpropanoic acid (H1) (D), and indole-3-carboxylic acid (F2) (E) when incubated with the P. aeruginosa PAO1 lasB-gfp(ASV) strain. The key shows the concentrations of the respective QSI used. The experiments were performed in triplicate; a representative experiment is shown.
FIG 4 Interaction maps between residues within the LasR LBD and the following compounds: the native acyl homoserine lactone ligand, OdDHL (A); 6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (C1) (B); 2-amino-3-(3-fluorophenyl)propanoic acid (F1) (C); 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (G1) (D); 2-amino-3-hydroxy-3-phenylpropanoic acid (H1) (E); and indole-3-carboxylic acid (F2) (F).

Key

- **Ligand bond**
- **Non-ligand bond**
- **Hydrogen bond and its length**
- **Non-ligand residues involved in hydrophobic contact(s)**
- **Corresponding atoms involved in hydrophobic contact(s)**
the corresponding OD_{600} value measured at that time point. “Control” refers to the PAO1 strain grown without the presence of QSI, and as expected, it had the highest GFP-per-OD values. For these five compounds, dose-dependent inhibition of lasB-gfp expression was observed, i.e., the higher the concentration of QSI that was present, the greater the inhibition of gfp expression.

In order to map the interactions between the five QSI compounds and the residues within the LasR protein ligand-binding site, the software program LIGPLOT v. 4.5.3 (Fig. 4) (34) was used. This program provides a 2-dimensional map showing the hydrogen-bonding and hydrophobic interactions between atoms in the ligand and that of the binding partner. PyMOL was also used for 3D representations of these interaction maps (see Fig. S1 in the supplemental material). Table 2 summarizes the interactions between our 5 QSI compounds and residues within the LasR ligand binding pocket (LBD). In a recent study, LasR was crystallized with the OdDHL LBD and other AHL agonists (37), which showed that LasR and pqsA residues within the LasR ligand binding domain in the supplemental material).

Table 2 Key residues within the LasR ligand binding pocket having hydrogen bonding interactions with OdDHL and the corresponding 5 QSI molecules

<table>
<thead>
<tr>
<th>Compound</th>
<th>Residues within LasR ligand binding pocket having H-bonding interactions with ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>OdDHL</td>
<td>Tyr 56, Trp 60, Asp 73, Ser 129</td>
</tr>
<tr>
<td>C1</td>
<td>Thr 75, Tyr 93</td>
</tr>
<tr>
<td>F1</td>
<td>Thr 75, Tyr 93</td>
</tr>
<tr>
<td>G1</td>
<td>Trp 60, Thr 75, Tyr 93</td>
</tr>
<tr>
<td>H1</td>
<td>Tyr 56, Thr 75, Ser 129</td>
</tr>
<tr>
<td>F2</td>
<td>Tyr 56, Ser 129</td>
</tr>
</tbody>
</table>

Of these 5 QSI candidates, only one compound, G1, was found to specifically inhibit LasR activity in the competition assay. The other four compounds did not show specific inhibition in the E. coli lasB-gfp(ASV) inhibition assay (see Fig. S2A to D in the supplemental material). Figure 7 shows the relative fluorescences of the E. coli lasB-gfp(ASV) strain in response to various concentrations of G1 and OdDHL. We see that increasing levels of OdDHL increase lasB-gfp expression, while increasing levels of QSI decrease it. The largest amount of relative fluorescence was observed for the condition of no G1 and 320 nM OdDHL (value = 1,340.4), and the smallest amount of relative fluorescence was seen for the condition of 100 μM G1 and no OdDHL (value = 438.1). The relative fluorescence values for the condition of 320 nM OdDHL with 100 μM G1 and the condition without OdDHL or G1 are 711.5 and 627.6, respectively. In the presence of 320 nM OdDHL, we have 46.9% inhibition by 100 μM G1 (compared to the value for the control without G1), while in the absence of OdDHL, we have only 30.2% inhibition by 100 μM G1 (compared to the control without G1).

The results also show that even in the presence of 320 nM OdDHL, 1.56 μM G1 was sufficient in inhibiting OdDHL-LasR induction of gfp expression. If the OdDHL concentration were increased further, an outcompetition of G1 by OdDHL would eventually be seen, where lower levels of G1 would be ineffective in inhibiting gfp expression and only higher levels of G1 would be able to inhibit gfp expression. The 50% effective concentration (EC_{50}) of OdDHL for LasR activation has been previously determined to be 10 nM (38), and therefore the range of OdDHL concentrations used for this assay is considered relatively high.

Effects of QSIs on rhl and pqs quorum-sensing systems. In order to address the problem of the specificity of our compounds, our five QSI compounds were tested to see if they had any effect on the rhl and pqs systems. The five QSIs were tested against a PAO1 wild type and a PAO1 lasR mutant harboring either the rhlA-gfp(ASV) bioreporter or the pqsA-gfp(ASV) bioreporter. The rhlA system is dependent on the RhlR/V system. By doing so, we were able to determine if a QSI is able to inhibit the other two QS systems (i.e., rhl and pqs). The P. aeruginosa rhl Q5 system uses the corresponding OD_{600} value measured at that time point. “Control” refers to the PAO1 strain grown without the presence of QSI, and as expected, it had the highest GFP-per-OD values. For these five compounds, dose-dependent inhibition of lasB-gfp expression was observed, i.e., the higher the concentration of QSI that was present, the greater the inhibition of gfp expression.

In order to map the interactions between the five QSI compounds and the residues within the LasR protein ligand-binding site, the software program LIGPLOT v. 4.5.3 (Fig. 4) (34) was used. This program provides a 2-dimensional map showing the hydrogen-bonding and hydrophobic interactions between atoms in the ligand and that of the binding partner. PyMOL was also used for 3D representations of these interaction maps (see Fig. S1 in the supplemental material). Table 2 summarizes the interactions between our 5 QSI compounds and residues within the LasR ligand binding pocket (LBD). In a recent study, LasR was crystallized with the OdDHL LBD and other AHL agonists (37), which showed that LasR and OdDHL appeared to interact at residues Tyr 56, Trp 60, Arg 61, Asp 73, and Ser 129 (Fig. 5). Those results agree with the residues identified by LIGPLOT as determined in this study (Table 2).

IC_{50} value comparisons of the five QSI candidates. The slope of the curve for each QSI was calculated based on its respective dose-response curves (from Fig. 3) and plotted against the log inhibitor concentration. The slope is indicative of the biosynthesis rate of GFP due to AHL induction. The half-maximal inhibitory concentrations (IC_{50}s) of the five QSI candidates were calculated using the Graphpad Prism 6 software package (GraphPad Software Inc., California, USA). The IC_{50}s of the 5 QSIs were mainly in the low micromolar range, with two compounds (C1 and G1) having values in the high nanomolar range (Fig. 6).

G1 had the lowest IC_{50} at 0.64 μM, meaning that this inhibitor was able to inhibit lasB-gfp expression with high efficiency. However, these compounds were not necessarily interacting directly with LasR, and therefore we used the heterologous E. coli strain containing the lasB-gfp(ASV) reporter to test for LasR-specific inhibition.

AHL competition assay using the heterologous E. coli lasB-gfp(ASV) strain. The five QSI candidates were tested for their specificities in inhibiting the LasR receptor by incubating these QSIs with the E. coli lasB-gfp(ASV) reporter strain. LasR is under the control of the Lac promoter and is constitutively expressed, and only LasR can activate gfp expression in this system. P. aeruginosa-based QS systems do not exist in E. coli, so exogenous OdDHL has to be added. Hence, this heterologous system provides a way to eliminate the contribution of QSI interaction with higher levels of control.

FIG 5 Crystal structure model of the interactions between residues in the LasR ligand-binding pocket with the autoinducer molecule OdDHL. The wire mesh shows the electron density map. The image was adapted from reference 37 with permission of the publisher.
a signal molecule, BHL, which is structurally similar to OdDHL of the las QS system, to regulate gene expression. Hence, it is likely that G1 as an inhibitor of LasR could also inhibit RhlR in the absence of LasR. Our results suggest that G1 has a higher binding specificity for LasR than for RhlR.

G1 was observed to strongly inhibit pqsA-gfp expression in wild-type PAO1 (57.5% inhibition) and less strongly in the lasR mutant (24.4% inhibition) (Fig. 8B). Because the pqs QS system is positively regulated by the las system (39), inhibition of the las system by G1 would result in downregulation of pqs expression. This shows that G1 inhibits the las QS system specifically, and inhibition of pqs is through a LasR-dependent mechanism.

Interestingly, F1 was able to inhibit both the rhl and pqs systems in a LasR-independent manner. F1 inhibited rhlA-gfp expression in wild-type PAO1 by 61.7%, and in the lasR mutant it showed inhibition of 63.1% (Fig. 8A). F1 was also found to inhibit pqsA-gfp expression by 25.4% in the wild type and 39.4% in the lasR mutant (Fig. 8B). Our results suggest that F1 has a higher binding specificity for RhlR than for LasR.

**Effect of G1 on extracellular DNA release in P. aeruginosa biofilms.** The pqs QS system regulates release of extracellular DNA (eDNA), which is an important structure component for P. aeruginosa biofilms (40, 41). Using a slide biofilm assay, G1 was tested for its ability to reduce eDNA release in P. aeruginosa biofilms. Propidium iodide (PI) staining was used to stain and visualize eDNA. PI can stain both eDNA and dead cells; however, eDNA appears as string-like structures rather than the circular structures indicative of dead cells. A large amount of eDNA was observed in the substratum of P. aeruginosa PAO1 biofilms cultivated in ABTG medium (Fig. 9A and C), while less eDNA was

**FIG 6** The half-maximal inhibitory concentration (IC$_{50}$) of 6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (C1) (A), 2-amino-3-(3-fluorophenyl)propanoic acid (F1) (B), 3-amino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (G1) (C), 2-amino-3-hydroxy-3-phenylpropanoic acid (H1) (D), or indole-3-carboxylic acid (F2) (E). The minimum concentration of inhibitor was arbitrarily designated by the Graphpad PRISM software program to have a log[inhibitor] value of −10 (log) μM, since the logarithm of zero is undefined.
observed in PAO1 biofilms cultivated in ABTG medium containing 10 μM G1 (Fig. 9B and D). This indicates that G1 was able to reduce eDNA release in P. aeruginosa.

**iTRAQ-based quantitative proteomic analysis.** In order to study the proteins whose expression was downregulated in P. aeruginosa PAO1 as a result of G1 addition, iTRAQ was used as the labeling strategy for comparative quantitative proteomic analysis (performed with a false discovery rate below 1%). The following cutoffs were used for protein identification: unused protein score of at least 2 (i.e., 99% confidence of identification) and having more than 1 peptide identified. Using these cutoffs, 2,258 proteins were identified. Using a \( P \) value (115:114; (ratio of the protein’s abundance in the G1-treated sample [115] to that in the untreated control [114]) cutoff of 0.05, 46 proteins were found to be significantly affected by G1; the abundance of 19 proteins was upregulated, while the abundance of 27 proteins was downregulated. In our study, upregulation was defined as an abundance (115:114 score) of at least 1.5, and downregulation was defined as an abundance value (115:114 score) below 0.66. Table 3 shows the 27 proteins whose abundances were significantly decreased in the G1-treated PAO1 strain versus those in the control PAO1 strain without G1 addition. Of these 27 proteins, 10 had been previously found to be QS regulated: protease IV, chitinase, hypothetical protein PA0572, pyoverdine synthetase D, pyoverdine chromophore synthetase PvdL, AmbE, probable nonribosomal peptide synthetase, chitin-binding protein CbpD precursor, conserved hypothetical protein PA0588, and cystathionine beta-synthase.

Nouvens et al. (42) performed a proteomic analysis of the extracellular proteins regulated by the las and rhl systems in P. aeruginosa (42). In that study, they found the expression of protease IV (PA4175) to be significantly downregulated in a las mutant; however, the amount of downregulation was not quantified. In our study, the abundance of protease IV (PA4175) was found to be 0.29 (fold change, \(-1.79\)). Protease IV is an extracellular protease that causes tissue damage in P. aeruginosa infections (43), and hence reducing the expression of this virulence factor may attenuate P. aeruginosa virulence. Garlic extract and 4-nitropyridine-N-oxide (4-NPO) were also found to reduce the expression of protease IV, by \( 6.9-\) and \( 20.7\)-fold, respectively (18).

Protease IV, also known as PrpL, is regulated by PvdS, which is an alternative sigma factor that regulates genes involved in siderophore biosynthesis (44). pvdS gene expression is regulated by the iron-sensing Fur repressor protein, such that pyoverdine is produced only under iron-limiting conditions (45, 46). Therefore, G1 may act through the interaction with the Fur protein to inhibit PvdS-regulated induction of pyoverdine synthesis genes.

In support of this idea, the abundances of two pyoverdine synthetases were found to be significantly reduced: pyoverdine synthetase D (pvdD; PA2399) by \(-0.89\)-fold and pyoverdine chromophore synthetase (pvdL; PA2424) by \(-0.73\)-fold. Pyoverdine is a siderophore that is required for iron acquisition, and siderophore-mediated signaling regulates the expression of several virulence factors (47). In a recent study by Taguchi et al. (48), a pvdL mutant of the Pseudomonas syringae pv. tabaci 6605 strain exhibited reduced virulence on host tobacco plants (48). The produc-
duction was significantly reduced with the addition of G1. Surprisingly not to find elastase on the list of downregulated proteins. P. aeruginosa metalloprotease elastase B is a lasI rhlI mutant is deficient in quorum-sensing and is used as a negative control. The amount of elastase produced by P. aeruginosa upon exposure to G1 was similar to that for this negative control. This P. aeruginosa PAO1 lasR mutant, elastase expression was decreased to 13-fold (49). Hence, it was rather surprising not to find elastase on the list of downregulated proteins. Thus, we decided to use an enzymatic assay to test if elastase production was significantly reduced with the addition of G1.

Effect of G1 on production of elastase by P. aeruginosa. The metalloprotease elastase B is a las QS system-regulated virulence factor produced and excreted by P. aeruginosa (50). QSIs that inhibit the las QS system should be able to inhibit the production of elastase B. A standard enzymatic assay was used to test whether G1 could inhibit the elastase activities of P. aeruginosa cultures. The result showed that addition of G1 to P. aeruginosa PA01 cultures at 50 and 100 μM could almost abolish elastase production within a 2-h cultivation period (Fig. 10), matching the levels of a P. aeruginosa PA01 las rhlI mutant. This P. aeruginosa PA01 las rhlI mutant is deficient in quorum-sensing and is used as a negative control. The amount of elastase produced by P. aeruginosa upon exposure to G1 was similar to that for this negative control, which indicates that G1 was efficient in inhibiting elastase production.

DISCUSSION

In a previous study by Yang et al. (27), 147 compounds were screened based on structural similarity to the ligand OdDHL. Six top-scoring hits were identified and tested for QSI activity; of these, three were found to have dose-dependent inhibition of QS-related gene expression and associated phenotypes. In the present study, we have used DG-AMMOS to enhance in silico QSI discovery. One advantage of using DG-AMMOS, rather than relying on structural similarity to the ligand of interest, is the avoidance of rational bias in the screening process, therefore allowing the detection of lead compounds that may not be able to be identified rationally. Hence, it is possible that DG-AMMOS can be extended to the conversion of larger compound libraries (e.g., 10,000-compound libraries and combinatorial chemistry libraries) and allows the discovery of new compounds that may have little structural similarity to QSIs or AHLs yet possess QSI properties.

SB-VS has been used extensively in the pharmaceutical industry, famous examples being Relenza, an anti-influenza drug that targets sialidase (51), and Virecept, a human immunodeficiency virus protease inhibitor (52). In recent years, SB-VS approaches have also been used in the search for novel QSIs, and here are a few recent examples: (i) discovery of hamamelitannin, a natural compound from Hamamelis virginiana that inhibits QS in drug-resistant Staphylococcus aureus and Staphylococcus epidermidis (53); (ii) identification of novel AI-2 QS inhibitors of Vibrio harveyi by SB-VS with the crystal structure of LuxP (54); (iii) discovery of a compound from Melia dubia bark extract which could inhibit the QS regulator SdiA, present in uropathogenic E. coli (UPEC) (55); (iv) discovery of five QSIs from an SB-VS of 1,920 natural compounds against the LasR and RhlR receptor proteins (56); and (v) discovery of 5 inducers and 3 inhibitors of LasR through a SB-VS of 800,000-plus compounds from the Chembridge library through a pharmacophore-based approach for compounds similar to OdDHL (57).

One major limitation of SB-VS is the problem of false positives and false negatives predicted by the docking software. However, with the development of newer and better algorithms, the problem of false hits may be minimized. Also, the aim may not be to eliminate false positives entirely but to reduce them to a tolerable level, the reason being that false positives may lead to the discovery of novel molecular interactions. As such, the cutoff of a −60 re-rank score that was used in our study for identifying potential QSI candidates might have been too stringent, and a higher value (i.e., less negative) could have been used instead so as to increase the number of potential hits. However, using a less stringent cutoff would increase the number of false positives, and this trade-off between the number of potential leads and the number of false positives must be considered for all SB-VS studies.

On the whole, SB-VS methods provide a faster and cheaper alternative to high-throughput screening (HTS) approaches for several reasons. First, if the search strategy in SB-VS is restricted to commercially available compound libraries, the lead compounds identified through SB-VS can be purchased easily and one does not need to undertake a costly chemical synthesis process. Second, SB-VS can be used to dock known drugs or natural plant derivatives, which would be likely to have lower toxicities than compounds synthesized through combinatorial chemistry. Thus, compounds identified through screening of known drugs/natural product libraries can avoid failure in the in vitro and in vivo testing stages due to
Effect of G1 on the elastase activities of *P. aeruginosa* cultures. Elastase activities of *P. aeruginosa* culture supernatants were measured by using the EnzChekElastase assay kit (Invitrogen). Fluorescence was recorded every 6 min for 180 min by using a Tecan Infinite 200 Pro plate reader (excitation at 490 nm, emission at 520 nm). The *P. aeruginosa* PAO1 las*H* rhl*H* strain served as a negative control.

Toxicity. Last, SB-VS is able to first narrow down the list of compounds to be tested before proceeding with actual *in vitro* tests for efficacy, and this would greatly reduce costs compared to conventional HTS methods, where all compounds have to be tested.

In this study, we have shown that structure-based virtual screening is a viable and effective means for the discovery of novel QS inhibitors. From a library of 3,040 natural compounds, 22 compounds met selection criteria and were tested for biological activity. Five of these compounds were found to have dose-dependent inhibition of the *las* QS system. However, only G1 was shown to have dose-dependent inhibition of *lasB-gfp* in both the *P. aeruginosa* and *E. coli* strains, indicating its specificity for the LasR protein. Among the five QSI candidates, it had the lowest IC$_{50}$ at 0.64 μM.

G1 was also able to delay the induction of the *rhl* QS system (data not shown). G1 showed some inhibition of the *rhl* QS system; perhaps it either has very weak binding affinity or allosteric effects. This may be due to the presence of the homoserine lactone ring present in both the LasR ligand, OdDHL, and the RhlR ligand, BHL. Studies have shown that the lactone ring is important.
for interaction with the LasR binding pocket (38). Presumably, an RhLR structure would be very useful to help understand the differences in the binding pockets and hence the binding of compounds like G1. However, the crystal structure of RhLR is unavailable, so homology modeling may be used to generate a putative structure for docking studies.

Besides the rhl system, G1 was also found to repress the PQS system. Previous studies have shown that the las QS system positively regulates the pqs QS system (39), and the results of our study suggest that G1 represses expression of the PQS system through inhibition of the las QS system. The pqs QS system regulates release of eDNA, which is an important structural component for *P. aeruginosa* biofilms (40, 41). As such, we found that G1 was able to reduce the amount of eDNA being released by *P. aeruginosa*. Previous studies have shown that eDNA is essential for biofilm formation (38), and further investigation is necessary to study the potential of G1 in the prevention of biofilm formation.

It is not yet clear why the other four QSIs (C1, F1, H1, and F2) were able to inhibit gfp expression in the *P. aeruginosa* lasB-gfp system but not in the *E. coli* lasB-gfp system. This is puzzling because they were identified by molecular docking against the LasR LBD and were shown by LIGPLOT to have specific H-bonding interactions with residues within the LasR ligand binding pocket. Therefore, these compounds should in theory specifically target LasR. This could indicate two things: (i) that these four QSIs do not bind specifically to LasR and instead inhibit gfp expression indirectly in the *P. aeruginosa* reporter strain through other pathways that affect the las QS pathway or (ii) that *E. coli* has molecular machinery that is able to either degrade or selectively pump these compounds out of the cell.

These results suggest that the structure of G1 may be important for its ability to interact with and inhibit LasR specifically. If we compare the structures of G1 and C1 (Fig. 2), we see that their structures are very similar, the difference being the presence of an additional imino group on G1. Hence, it is interesting that G1 (but not C1) was able to inhibit lasB-gfp (ASV) in the *E. coli* system despite their structural similarity. In fact, there are other compounds similar to G1 that may have been missed. A structure subgroup search for compounds within the Natural Derivatives Library found the molecule 404 (Fig. 2) to be similar in size and structure to G1. However, molecule 404 had a docking rerank score of ~5.03 and was therefore excluded by the ~60 cutoff score we used. As such, the cutoff rerank score of ~60 that was used may have been too stringent, and we may have missed hits by using that value. Hence, the idea should be to use a cutoff for excluding low-scoring compounds but not to use it for identifying top-scoring hits; a larger starting pool of molecules would then be tested for *in vitro* inhibition efficacy, and this may result in a better hit rate.

Another interesting result was that F1 was able to inhibit both the rhl and pqs systems in a LasR-independent manner. This may mean that F1 may be interacting with a QS component distinct from LasR yet affects the rhl and pqs systems. Further studies will be done to investigate the mechanisms by which F1 inhibits QS in *P. aeruginosa* and its potential as an antivirulence compound.

These results emphasize the application of SB-VS for the discovery of target-specific inhibitors, and future work includes the extension of this method to new molecular targets. A future aim would be to work alongside chemists to develop new QSI compounds and to perform quantitative structure-activity relationship (QSAR) studies to aid in our knowledge of rational drug design.

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**REFERENCES**


