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Novel *Pseudomonas aeruginosa* Quorum-Sensing Inhibitors Identified in an Ultra-High-Throughput Screen▽†

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The opportunistic pathogen *Pseudomonas aeruginosa* has two complete acyl-homoserine lactone (acyl-HSL) signaling systems, LasR-LasI and RhlR-RhlI. LasI catalyzes the synthesis of *N*-3-oxododecanoyl homoserine lactone (3OC12-HSL), and LasR is a transcription factor that requires 3OC12-HSL as a ligand. RhlI catalyzes the synthesis of *N*-butanoyl homoserine lactone (C_4), and RhlR is a transcription factor that responds to C_4. LasR and RhlR control the transcription of hundreds of *P. aeruginosa* genes, many of which are critical virulence determinants, and LasR is required for RhlR function. We developed an ultra-high-throughput cell-based assay to screen a library of approximately 200,000 compounds for inhibitors of LasR-dependent gene expression. Although the library contained a large variety of chemical structures, the two best inhibitors resembled the acyl-homoserine lactone molecule that normally binds to LasR. One compound, a tetrazole with a 12-carbon alkyl tail designated PD12, had a 50% inhibitory concentration (IC_{50}) of 30 nM. The second compound, V-06-018, had an IC_{50} of 10 μM and is a phenyl ring with a 12-carbon alkyl tail. A microarray analysis showed that both compounds were general inhibitors of quorum sensing, i.e., the expression levels of most LasR-dependent genes were affected. Both compounds also inhibited the production of two quorum-sensing-dependent virulence factors, elastase and pyocyanin. These compounds should be useful for studies of LasR-dependent gene regulation and might serve as scaffolds for the identification of new quorum-sensing modulators.

The opportunistic human pathogen *Pseudomonas aeruginosa* can sense its own population density by using an intercellular signaling system. Such systems have been termed quorum-sensing and response systems (11, 47). We have focused on a *P. aeruginosa* quorum-sensing signal that has been shown to be involved in the regulation of a battery of virulence genes. This signal, *N*-3-oxo-L-homoserine lactone (3OC12-HSL), is produced by a synthase called LasI (encoded by *lasI*) (23). The 3OC12-HSL signal affects gene expression by binding to a specific signal receptor called LasR (encoded by *lasR*), which in turn modulates transcription of effector genes (13). LasI and LasR are members of conserved families of synthases and receptors that have been found in dozens of different species of *Proteobacteria*. Acyl-homoserine lactone (acyl-HSL) signals produced by homologs of LasI differ in their acyl side chains, and the receptors have differing specificities for the various acyl-HSLs (11, 42).

The LasRI system controls the expression of many genes, several of which encode virulence factors (36, 41). Mutants with defects in quorum sensing (*rhlI*, *lasI*, and *lasR*) have substantially reduced virulence in a variety of animal models (reference 40 and references therein). Furthermore, there is evidence that inhibitors of 3OC12-HSL quorum sensing can reduce the severity and duration of *P. aeruginosa* lung infections in rodents (14, 48). The second acyl-HSL signaling system in *P. aeruginosa*, the RhlR-RhlI system (encoded by *rhlR* and *rhlI*) (24), also controls the expression of multiple genes (36, 41). Production of RhlR and RhlI requires activation by the LasR-LasI system (16, 28). Thus, LasR-LasI is at the top of the acyl-HSL signaling cascade in *P. aeruginosa*. There is also a third LasR homolog called QscR (2) which lacks a cognate I protein. Instead, like LasR, it responds to 3OC12-HSL and regulates a set of genes that overlaps with the LasR- and RhlR-activated genes (17, 18).

Molecules that target quorum sensing have been proposed as an antivirulence strategy that could be added to the existing armamentarium in treating *P. aeruginosa* infections. Efforts to identify small molecule inhibitors of quorum sensing were reviewed recently (26). Three approaches have been used to identify quorum-sensing inhibitors: (i) chemical synthesis of compounds modeled on the natural acyl-HSL signals (reference 33 and references therein), (ii) characterization of natural products (14, 27), and (iii) screening for naturally occurring enzymes, such as lactonases (7) and acylases (19), involved in the degradation of acyl-HSLs in bacteria and mammalian tissue (3, 8). Here, we describe a fourth approach, screening a large library of synthetic molecules. We have developed a high-throughput cell-based screen that utilizes nanowell technology (3,456 wells per plate) and used it to screen a library of approximately 200,000 small molecules. We describe the characterization of two inhibitors identified in this screen.

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MATERIALS AND METHODS

Chemical library. The compound library was a combination of diverse, commercial compound collections sourced from multiple vendors combined with a diverse combinatorial chemistry library based on 23 core scaffolds plus a focused set of known, biologically active compounds.

TABLE 1. *P. aeruginosa* strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
PAO1	Wild type	31
PAO-MW1	PAO1 *rhlI*::Tn*501 lasI*::*tetA*	45
PAO *lasR rhlR*	(Δ*lasR*::Tc^r Δ*rhlR*::Gm^r) of PAO1	31
Plasmids		
pQF50	Broad-host-range plasmid for promoter analysis	10
pMW312	pQF50 carrying the *rsaL* promoter from −82 to +29 relative to the translation start of *rsaL*; Ap^r	44
pUC18	Cloning vector	49
pUM11	*yfp* with T7gene10 ribosome binding site in pUC18	This study
pUM15	*rsaL*::*yfp* transcriptional fusion, derived from pUM11 and pMW312; Ap^r	This study
pRSET(B)	Expression vector	Invitrogen Corporation
pRSET(B)-10Bnh	*yfp* cloned into pRSET(B)	This study

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are described in Table 1. Unless otherwise noted, bacteria were grown in Luria-Bertani (LB) broth or on LB agar with 0.4% sodium chloride. For plasmid maintenance or selection, we used 300 μg carbenicillin per ml for *P. aeruginosa*. For high-throughput screening, we used LB plus 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, and 300 μg carbenicillin per ml. For transcription profiling experiments and measurements of elastase and pyocyanin, we used LB plus 50 mM MOPS, pH 7.0.

Plasmid construction. Standard cloning techniques (34) were used to construct plasmids as follows: pRSET(B)-10Bnh contains *yfp* and was obtained by cloning the coding region of wild-type green fluorescent protein from *Aequorea victoria* into pRSET(B) (Invitrogen) and introducing the mutations F64L, S65G, Q80R, and T203Y (30). To construct the *rsaL-yfp* reporter pUM15, we amplified *yfp* from pRSET(B)-10Bnh by PCR. The forward primer was complementary to the first 16 bases of the open reading frame and introduced a HindIII site and the T7gene10 Shine-Dalgarno sequence. The reverse primer was complementary to the stop codon and the last 12 bases of the *yfp* open reading frame followed by an AatII site. The resulting PCR product was digested with HindIII and AatII and ligated with AatII-HindIII-digested pUC18 to produce pUM11. This same fragment plus some flanking DNA was then excised from pUM11 with HindIII and ScaI and ligated to HindIII-ScaI-digested pMW312 to produce pUM15. This last cloning step replaced the *lacZ* reporter in pMW312 with *yfp* and a T7gene10 Shine-Dalgarno sequence.

Ultra-high-throughput and high-throughput assays for quorum-sensing inhibitors. Compounds were seeded into nanoplates (3,456-well format) in a volume of 25 nl from 2 mM stocks in 75% dimethyl sulfoxide (DMSO) by using a Piezo sample distribution robot (Vertex Pharmaceuticals, Inc., San Diego, CA) (29). Cells from a single colony of *P. aeruginosa* MW1 containing pUM15 were used to inoculate a 3-ml starter culture, which was grown with shaking at 30°C to mid-log phase. Cells were subcultured to an optical density at 600 nm of 0.05 and grown with shaking at 37°C for 1 to 2 h. These mid-logarithmic cultures were then added to 3OC12-HSL to achieve a final inducer concentration of 0.3 μM. Bacterial cell cultures (1.5 μl) were added to each well with a flying reagent dispenser (Vertex Pharmaceuticals). Controls included wells without compound and wells without 3OC12-HSL. Plates were sealed in a humidified container and incubated at 37°C for 8 to 12 h. Fluorescence was then measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm by using a topography-compensating plate reader (Vertex Pharmaceuticals). To evaluate growth in the presence of compound, the absorbance at 620 nm was recorded.

Compounds that reduced fluorescence by 50% without affecting growth were subjected to a dose-response analysis in the nanoplate format. A typical dose response included 11 compound concentrations from 20 mM to 10 nM in triplicate wells. Candidates were then retested in a 96-well format dose-response assay.

Cells for the 96-well format assay were grown as described for the nanowell plate assay. Each well was inoculated with 50 μl, and the plates were incubated for 6 to 8 h. Fluorescence in individual wells was measured with a SpectroFluor Plus plate reader (Tecan, Durham, NC). The absorbance at 620 nm was recorded to monitor growth effects.

The statistical robustness of the assays was tested by comparing the signals from induced and uninduced cultures. From the data, we calculated the screening window parameter Z′ (50) to be 0.84 for the nanoplate format and 0.57 for the 96-well plate format. The parameter Z′ reflects both the assay signal dynamic range and the data variation. The calculated values of 0.84 and 0.57 indicate that the assay signal is roughly 18-fold (nanoplate format) and 7-fold (96-well format) higher than the combined standard deviation of signal and baseline. Hence, the applied criterion for inhibition (50%) was significantly outside the variation of the assay.

Synthesis of tetrazole library. Alkylated tetrazoles were synthesized based on a previously described procedure (22, 43). Purity was assessed by liquid chromatography-mass spectrometry.

Transcript profiling. Cells grown to mid-logarithmic phase were used to inoculate 3 ml of warm LB plus MOPS in 18-mm borosilicate tubes (initial density, 0.01 at 600 nm). Reagents (0.3 μM 3OC12-HSL, 10 μM PD12, and 100 μM V-06-018) were added as indicated. In all cases, 0.75% DMSO was present and incubation was carried out at 37°C with shaking. When the optical density (600 nm) reached 2, a sample containing 2×10^9 CFU was mixed with RNA Protect bacteria reagent (QIAGEN Inc., Valencia, Ca.). RNA was then isolated and processed as described previously (36). We used Affymetrix *Pseudomonas* GeneChips in our analyses. All experiments were performed in duplicate. Affymetrix Microarray Suite 5.0 was used to scale and normalize signal intensities and to score each transcript as present, marginal, or absent. The software also calculates a signal log ratio for each gene, which is the change in transcript relative to a baseline value. Microarray Suite software uses statistical criteria to calculate whether a signal change is considered a decrease or an increase, as well as the corresponding *P* value (15, 20). The criterion for deciding which genes were induced or repressed was a statistically significant change, as determined by Microarray Suite software (default parameters), in at least one of two replicates.

Virulence factor assays. Elastase activity was measured as described by Pearson et al. (25), and pyocyanin activity was measured as described by Essar et al. (9). Mid-logarithmic-phase cultures were either subcultured directly (MW1) or washed twice with fresh LB plus MOPS (PAO1), and these cells were used as an inoculum (initial optical density, 0.01). The culture volume was 1 ml, and incubation was carried out in 16-mm tubes at 37°C and 250 rpm with 3OC12-HSL, with inhibitors added as indicated. For elastase activity, cultures were grown for 6 h, cells were removed by centrifugation, and the culture fluid was filtered through a 0.2-μm filter (Millex GP, 0.22 μM; Millipore, Ireland). A 100-μl volume of filtered culture fluid was added to 900 μl of 5 mg per ml Elastin Congo red (Elastin Products Company; Owensville, MO) in 1 mM $CaCl_2$ and 100 mM Tris buffer, pH 7.2. Reaction mixtures were incubated at 37°C and 250 rpm for 18 h. Reactions were stopped by the addition of 100 μl of 0.12 M EDTA, undigested substrate was removed by centrifugation, and absorbance was read at 495 nm. To measure pyocyanin, cultures were grown for 9.5 h. Pyocyanin was extracted in 1 ml chloroform, followed by a second extraction into 150 μl 0.2 N HCl. The absorbance of this solution was measured at 520 nm.

LasR Western blotting. *P. aeruginosa* strain MW1 containing pUM15 was grown in the presence or absence of test compound as indicated. A 625-μl sample of cell culture was pelleted, resuspended in 350 μl Laemmli buffer, and sonicated with a microtip for 5 to 7 s (Branson Sonifier 450) at an output of 40% power. Ten microliters of lysate was separated on 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by standard Western blotting techniques with ECL detection reagent (Amersham Biosciences) and a LasR polyclonal antibody (37). Quantitation of LasR was performed by densitometry scanning of the developed film.

RESULTS

Screening for inhibitors of *P. aeruginosa* 3OC12-HSL reception. The screen was performed in *P. aeruginosa* MW1, which lacks the acyl-HSL synthases, LasI and RhlI, and thus does not produce acyl-HSLs. The screening strain also harbored the reporter plasmid, pUM15. This plasmid has a gene for the yellow fluorescent protein (*yfp*) under the control of the LasR-dependent promoter *prsaL*. We chose to target LasR rather than RhlR in our screen because LasR precedes the Rhl circuit in the quorum-sensing hierarchy of *P. aeruginosa*, and thus, inhibition of LasR should result in inhibition of expression of

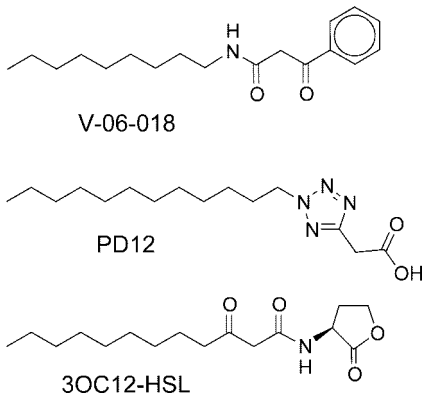


FIG. 1. Inhibitors (PD12 and V-06-018) of *P. aeruginosa* quorum sensing. The native Las-dependent signal molecule, 3OC12-HSL, is shown for comparison.

both LasR- and RhlR-regulated genes (29). The *rsaL* promoter was chosen because, as shown by reporter gene technology (5, 44) and confirmed by transcription profiling, (36), this promoter is induced several hundredfold to high levels in the presence of 3OC12-HSL. Growth of *P. aeruginosa* MW1 (pUM15) in the presence of 3OC12-HSL results in high fluorescence, and any compound that interferes with acyl-HSL reception should reduce the fluorescence. The level of $3OC_{12}$-HSL provided in the culture medium (0.3 μM) results in approximately half-maximal induction of *yfp*. For comparison, a fully grown wild-type *P. aeruginosa* culture in rich medium contains about 10 μM 3OC12-HSL (46). As the 50% effective concentration for 3OC12-HSL is 0.3 μM, only a very strong inhibitor capable of lowering the endogenous signal about 30-fold would be detected in fully grown cultures in a wild-type background.

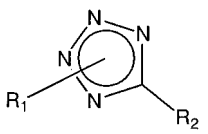
A library of approximately 200,000 compounds was screened in nanowell plates at a compound concentration of 33 μM, as described in Materials and Methods. From this screening process, we identified 20 compounds, which is about 0.01% of the compounds screened. This low percentage of active compounds is likely a reflection of the fact that we performed a cell-based screen with *P. aeruginosa*, which has a substantial permeability barrier for small molecules. Based on potency (determined by secondary assays as described in Materials and Methods) and chemical stability, two quorum-sensing inhibitors were chosen for further study: V-06-018 (75% inhibition; 50% inhibitory concentration (IC_{50}) of 10 μM) and PD12 (80% inhibition; IC_{50} of 30 nM; first identified with a more labor-intensive predecessor of the current assay) (unpublished data). Interestingly, both inhibitors resemble the native *P. aeruginosa* LasI-generated signal molecule, 3OC12-HSL (Fig. 1). Both inhibitors have a 12-carbon aliphatic tail which is attached to a tetrazole in the case of PD12 and a phenyl in the case of V-06-018. That neither V-06-018 nor PD12 affected bacterial growth is consistent with their targeting quorum sensing because null mutations in LasR or LasI do not affect the growth of *P. aeruginosa*. Both PD12 and V-06-018 were stable upon storage in DMSO at −20°C for many months. Neither compound contains moieties predictive of chemical reactivity.

Screening a focused tetrazole library. A library of 66 compounds was synthesized to explore the structure activity relationship of PD12. Compounds were designed to investigate alkyl chain length and regioisomeric substitution at either the N-1 or the N-2 position of the tetrazole. Variations at the C-5 position of the tetrazole included acetic acid, the ethyl ester thereof, and several aryl moieties with a urea spacer.

None of the compounds with aryl moieties and a urea spacer inhibited reporter fluorescence at a concentration less than that which inhibited growth (data not shown). Inhibition of *yfp* reporter expression was seen with either an acetic acid or the ethyl ester at C-5. Because the ethyl ester may not be stable for extended periods of time in the assay medium, it is possible that all ethyl ester compounds were present as the acetic acid derivatives. An alkyl chain length of at least C_{12} or C_{14} was optimal for inhibition (Table 2). A C_{10} side chain was not tested, and C_8 showed intermediary levels of inhibition. In all cases, the 2,5 tetrazole derivatives were better inhibitors than the 1,5 tetrazole derivatives. In summary, the optimal tetrazole inhibitor carries an acetic acid moiety at C-5 and is substituted at N-2 with an alkyl side chain of C_{12} (PD12) or C_{14}. The data suggest that these compounds might function as inhibitors by interacting directly with the 3OC12-HSL binding site on LasR. The data also suggest that inhibition by PD12 and compound 3 is not due to a nonspecific detergent effect, since a large number of compounds with an equally hydrophobic side chain did not result in a similar inhibition.

Transcript profiling indicates that the inhibitors of *rsaL-yfp* expression serve as general quorum-sensing inhibitors. If PD12 and V-06-018 inhibited *rsaL-yfp* expression as a consequence of blocking LasR-mediated gene expression, the expression of other LasR quorum-sensing genes should be affected as well. We used microarrays to test whether this was true. Previous microarray studies (14, 36, 41) have shown that the number and identity of quorum-sensing-dependent genes depend on the growth conditions and the statistical criteria applied in the analysis. We analyzed global gene expression under conditions similar to those used in our inhibitor screen.

TABLE 2. Structure-function relationship of select tetrazole analogs



Compound	Molecule at:		% Inhibition[a]
	R1 (R1 position)	R2	
1	C_6H_{13} (N-2)	CH_2COOH	−17
2	C_8H_{17} (N-2)	CH_2COOH	45
PD12	$C_{12}H_{25}$ (N-2)	CH_2COOH	76
3	$C_{14}H_{29}$ (N-2)	CH_2COOH	77
4	C_6H_{13} (N-1)	CH_2COOH	10
5	C_8H_{17} (N-1)	CH_2COOH	−18
6	$C_{12}H_{25}$ (N-1)	CH_2COOH	−4
7	$C_{14}H_{29}$ (N-1)	CH_2COOH	20

[a] Inhibition was measured with the *prsaL*-dependent reporter plasmid in the *P. aeruginosa* signal mutant MW1. Negative numbers indicate fluorescence higher than that of the negative control, i.e. no inhibition.

TABLE 3. Inhibition of select LasR-controlled genes in *P. aeruginosa* MW1[a]

Gene number	Gene name	Fold induction in:		% Inhibition by:			
				V-06-018		PD12	
		MW1	PAO1	MW1	PAO1	MW1	PAO1
PA1001	*phnA*	73	39	98	55	8	5
PA1002	*phnB*	17	8	93	39	7	−13
PA1431	*rsaL*	333	659	28	33	46	24
PA1894		53	16	5	19	44	2
PA1897		81	30	−25	24	21	20
PA2302		8	20	10	32	27	14
PA2303		34	79	30	34	38	16
PA2587		6	7	38	25	47	22
PA2592		5	10	56	31	38	−14
PA3477	*rhlR*	4	8	5	24	19	−12
PA3724	*lasB*	91	294	68	58	74	29
PA3907		9	18	57	26	26	29

[a] The concentration of $3OC_{12}$-HSL in the growth medium for *P. aeruginosa* MW1 was 0.3 μM. A negative value indicates percent activation.

We first studied *P. aeruginosa* MW1 and compared the gene expression profiles of cells grown without added signal or inhibitor and cells grown in the presence of 0.3 μM 3OC12-HSL. Ninety-three 3OC12-HSL-induced genes were identified, that is, the genes were expressed ≥4-fold higher in cells exposed to 3OC12-HSL (a subset of previously characterized LasR-dependent genes is shown in Table 3) (see Table S1 in the supplemental material). All but one of these genes was previously identified as quorum-sensing controlled (*qsc*) (36). The exception, PA1892, is in an operon with several other known *qsc* genes, and thus, we believe that it is a *qsc* gene that was previously not recognized as such.

We then examined the effects of V-06-018 and PD12 on the transcriptional profile of *P. aeruginosa* MW1 grown in the presence of 0.3 μM 3OC12-HSL. Of the 93 3OC12-HSL-induced genes, 49 showed 50% or greater inhibition by 100 μM V-06-018 and 19 by 1 μM PD12 (Table 3 and see Table S2 in the supplemental material). At a 25% inhibition level, 76 were affected by V-06-018 and 59 by PD12. These transcriptome data support the conclusion that the two inhibitors function by targeting 3OC12-HSL signal reception.

To further characterize the inhibitors, we examined their influence on quorum-sensing gene expression in the wild-type *P. aeruginosa* strain PAO1, a strain which makes relatively high levels of acyl-HSLs under the growth conditions used in our experiments. We identified 293 genes that were induced fourfold or more in strain PAO1 compared to strain MW1 grown without exogenous 3OC12-HSL. This finding of more quorum-controlled genes in the wild type was expected since the amount of 3OC12-HSL we added to strain MW1 was limiting, whereas the wild type produces higher levels of 3OC12-HSL. Of the 293 *qsc* genes in PAO1, 221 had previously been described as quorum-sensing induced (36, 41). The additional 72 genes are likely due to different statistical criteria used. Out of the 293 induced genes, V-06-018 inhibited expression of 129 while PD12 inhibited expression of only 7 with a cutoff of 50% inhibition or greater (Table 3 and see Table S3 in the supplemental material). All of our microarray experiments showed that V-06-018 functioned as a better general inhibitor of quorum sensing than did PD12. We conclude that although the IC_{50} for PD12 is lower than for V-06-018, at IC_{50}, V-06-018 is the broader quorum-sensing inhibitor.

Analysis of the microarray data showed that each compound inhibited (by 50% or more) the expression of fewer than 50 genes that are not on the list of quorum-sensing-induced genes defined by our criteria (data not shown). Almost all of these genes were previously described as quorum-sensing induced (36, 41). The inhibited genes that have not been identified as quorum controlled previously may be inhibited due to a non-quorum-sensing mechanism of the inhibitors, they may represent the small number of statistically predicted false positives expected from this analysis, or they may be quorum-sensing genes that have not been identified by previous analyses.

We show inhibition of select LasR-dependent genes by the two inhibitors in Table 3. The selected genes have either been extensively characterized (5, 12, 23, 24) or been shown to be under LasR-dependent control using gene fusions (45). Transcription of all of these genes was decreased by at least one of the inhibitors. While many are inhibited to similar extents by both compounds, several operons responded more strongly to V-06-018 (e.g., the *phnAB* operon) and others more strongly to PD12 (e.g., PA1894). Of note, recently PA1897 was shown to be controlled by QscR directly (17) and not controlled by LasR. PA1897 transcription is only marginally inhibited or not inhibited by either compound (Table 3), suggesting that perhaps the inhibitors target LasR in a rather specific fashion.

The inhibitors decrease production of virulence factors. To confirm the microarray results and to test whether V-06-018 and PD12 inhibit production of LasR-regulated virulence factors, we measured the levels of two extracellular virulence factors, pyocyanin and elastase, in *P. aeruginosa* PAO1 culture fluid. The transcript levels of the pyocyanin biosynthetic genes (*phz* genes) were reduced about 85% by V-06-018 and only marginally by PD12. Inhibition of pyocyanin production by V-06-018 was about 90%, and inhibition by PD12 was about 40% (Fig. 2). The product of the 3OC12-HSL-regulated *lasB* is an extracellular protease, elastase (1). The transcription of *lasB* was inhibited 58% in PAO1 by V-06-018 (Table 3) and inhibited weakly by PD12 (Table 3). We found that elastase production in PAO1 was reduced 60% by V-06-018 and 20% by PD12 (Fig. 3). Thus, the inhibitors not only affect the tran-

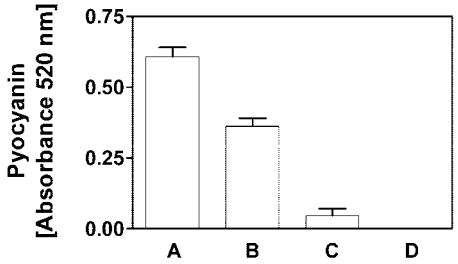


FIG. 2. The influence of V-06-018 and PD12 on pyocyanin production by *P. aeruginosa*. Pyocyanin was extracted from cultures and measured as absorbance at 520 nm. (A to C) Wild-type PAO1 was grown with no added inhibitors (A), 10 μM PD12 (B), or 100 μM V-06-018 (C). (D) A *lasR rhlR* mutant (PAO *lasR rhlR*) without inhibitors was included as a negative control. In all cases, DMSO was present (10 μM). The results shown are the means and standard deviations from two experiments, each performed in triplicate.

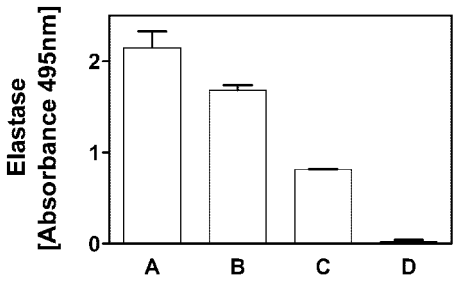


FIG. 3. The influence of V-06-018 and PD12 on elastase production by *P. aeruginosa*. Elastase activity in the culture supernatant fluid was measured with a colorimetric substrate (absorbance at 495 nm). Wild-type PAO1 was grown in the absence of added inhibitors (A) or in the presence of 10 μM PD12 (B) or 100 μM V-06-018 (C). (D) A *lasI rhlI* mutant (*P. aeruginosa* MW1) grown in the absence of acylated HSL was included as a negative control. In all cases, DMSO was present (10 μM). The results shown are the means and standard deviations from two experiments, each performed in triplicate.

scription of virulence genes but also reduce the production of virulence factors in a wild-type strain of *P. aeruginosa*.

Influence of the quorum-sensing inhibitors on LasR stability. Previously identified furanone inhibitors of quorum sensing are thought to stimulate degradation of acyl-HSL receptor proteins by cellular proteases (21). In a heterologous expression system, the half-life of LuxR (a *Vibrio fischeri* LasR homolog) was significantly reduced when cells were grown in the presence of a furanone. We assessed the effect of the quorum-sensing inhibitor V-06-018 on the stability of LasR in cells of *P. aeruginosa* MW1(pUM15) by Western blotting. V-06-018 did not affect LasR levels, nor could we detect any LasR degradation products (data not shown).

DISCUSSION

Quorum sensing controls the expression of more than 300 genes in *P. aeruginosa* (35), and it is important for virulence (6) and for normal biofilm development (4). Inhibitors of quorum sensing have been identified previously (26, 33). To identify new classes of inhibitors, we developed an ultra-high-throughput *P. aeruginosa* cell-based screen and tested a library of approximately 200,000 compounds. This is a stringent screen that requires the test compound to be capable of entering *P. aeruginosa* cells, to be able to interfere with 3OC12-HSL reception, and to do this in a specific way so that it does not interfere with the growth of *P. aeruginosa*. From this screen and secondary assays in dose-response mode, we selected two molecules for further study, PD12 and V-06-018.

We have no direct evidence that either compound binds to LasR. However, based on their structural similarity to the natural signal, 3OC12-HSL, we suggest binding to LasR to be a likely mode of inhibition. Of possible relevance, our examination of PD12 analogs (Table 2) showed that acyl chain length was a determinant for inhibitory activity; active compounds had acyl chains similar in length to 3OC12-HSL. This also suggests that the inhibitors may interact with the 3OC12-HSL binding region of LasR. Unlike previously described furanone inhibitors of quorum sensing, V-06-018 did not appear to stimulate receptor degradation.

Microarray experiments showed that PD12 and V-06-018 decreased the transcription of many 3OC12-HSL-regulated genes. Like the other inhibitors that have been studied with microarrays, these compounds did not inhibit every $3OC_{12}$-HSL-regulated gene and different genes were affected to various degrees (14, 32). With our criterion of 50% inhibition, about half of the genes activated by 3OC12-HSL in our experiments were inhibited by V-06-018. Many quorum-sensing genes are controlled by multiple factors, and these factors differ from one gene to another (for a recent review, see reference 35). Expression of very few non-quorum-controlled genes was inhibited by V-06-018 or by PD12, indicating that these compounds were specific effectors of 3OC12-HSL signaling. A similar specificity has been observed with a furanone inhibitor developed from a natural product screening approach (14) and appears to be better than the specificity of another recently identified inhibitor, 4-nitro-pyridine-*N*-oxide (32).

We also showed that PD12 and V-06-018 inhibited production of two quorum-controlled extracellular virulence factors by wild-type *P. aeruginosa* (Fig. 2 and 3). This confirms conclusions that these are general quorum-sensing inhibitors and provides support for the notion that these classes of molecules could be exploited for therapeutic applications. We consider the inhibition of virulence factors in a wild-type strain to be a more stringent evaluation of inhibitor efficacy than the inhibition of transcription in a signal generation mutant to which a limiting amount of 3OC12-HSL has been added.

Although the library we screened contained approximately 200,000 compounds selected to cover a wide range of chemical scaffolds, both inhibitors resemble the authentic signal molecule 3OC12-HSL (Fig. 1). Each has an extended tail of 12 carbons and either a tetrazole or a benzyl head. Presumably, these inhibitors compete with 3OC12-HSL for binding to LasR but do not provoke whatever conformation change is needed in LasR to activate transcription. While it was not surprising to find inhibitors that are structurally similar to the natural signal, our failure to identify other classes of inhibitors was not anticipated. Both V-06-018 and PD12 are stable for many months during storage in DMSO at −20°C, unlike many furanones but similar to the compounds described by Smith et al., who have synthesized both agonists and antagonists by replacing the homoserine lactone with various amines and alcohols (38, 39). We believe that V-06-018 and PD12 are useful tools for the inhibition of quorum sensing. We also believe that they present promising scaffolds for developing additional compounds for applications where inhibition of quorum signaling would be desired.

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