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Articles

Discovery of PqsE Thioesterase Inhibitors for *Pseudomonas aeruginosa* Using DNA-Encoded Small Molecule Library Screening

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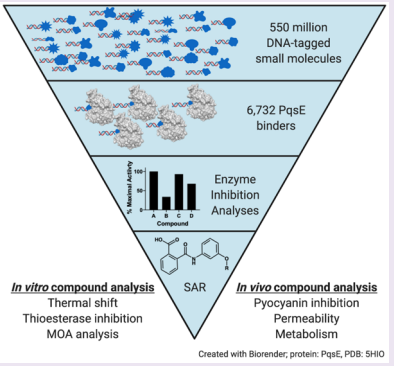
ABSTRACT: *Pseudomonas aeruginosa* is a leading cause of hospital-acquired infections in the United States. PqsE, a thioesterase enzyme, is vital for virulence of *P. aeruginosa*, making PqsE an attractive target for inhibition. Neither the substrate nor the product of PqsE catalysis has been identified. A library of 550 million DNA-encoded drug-like small molecules was screened for those that bind to the purified PqsE protein. The structures of the bound molecules were identified by high throughput sequencing of the attached DNA barcodes. Putative PqsE binders with the strongest affinity features were examined for inhibition of PqsE thioesterase activity *in vitro*. The most potent inhibitors were resynthesized off DNA and examined for the ability to alter PqsE thermal melting and for PqsE thioesterase inhibition. Here, we report the synthesis, biological activity, mechanism of action, and early structure-activity relationships of a series of 2-(phenylcarbamoyl)benzoic acids that noncompetitively inhibit PqsE. A small set of analogs designed to probe initial structure-activity relationships showed increases in potency relative to the original hits, the best of which has an IC_{50} = 5 μM. Compound refinement is required to assess their *in vivo* activities as the current compounds do not accumulate in the *P. aeruginosa* cytosol. Our strategy validates DNA-encoded compound library screening as a rapid and effective method to identify catalytic inhibitors of the PqsE protein, and more generally, for discovering binders to bacterial proteins revealed by genetic screening to have crucial *in vivo* activities but whose biological functions have not been well-defined.



Pseudomonas aeruginosa is an opportunistic Gram-negative bacterial pathogen that colonizes a variety of niches, including the skin, lungs, plants, soil, and abiotic surfaces.[1] *P. aeruginosa* is notorious for causing nosocomial infections, most notably of immunocompromised individuals, cystic fibrosis (CF) sufferers, burn victims, and patients with indwelling medical devices, creating a significant health burden.[2] *P. aeruginosa* is resistant to many commonly used antibiotics, hindering eradication of infections.[3,4] Antibiotic resistance is linked to the ability of *P. aeruginosa* to form sessile communities of cells adhered to surfaces, called biofilms, a trait that has propelled *P. aeruginosa* to become a leading cause of hospital-acquired infections in the United States.[5−8] New approaches are urgently needed to combat *P. aeruginosa* pathogenesis.

P. aeruginosa virulence and biofilm formation are controlled by the cell−cell communication process called quorum sensing that relies on the production, release, and group-wide detection of extracellular signal molecules called autoinducers.[9−11] Quorum sensing enables bacteria to orchestrate collective behaviors, and in the case of *P. aeruginosa*, these behaviors include biofilm formation and the production of the virulence factor pyocyanin, among other pathogenicity traits (recently reviewed in ref 12). Germane to the present work is the recent finding that the *P. aeruginosa* RhlR quorum-sensing receptor responds to two autoinducers: a canonical homoserine lactone (C4-homoserine lactone) that is synthesized by RhlI, and a second, alternative autoinducer of unknown structure that is produced by the PqsE thioesterase enzyme.[13−17] Thus, RhlR−RhlI and RhlR−PqsE form quorum-sensing receptor-autoinducer synthase pairs. What is crucial is that the RhlR−PqsE pair, and not the RhlR−RhlI pair, is essential for virulence in both a nematode and a mouse model of infection.[16] Specifically, both *rhlR* and *pqsE* mutants are highly attenuated for virulence.[16] Indeed, the *pqsE* mutant is even more defective for virulence than the *rhlR* mutant, making PqsE an interesting target for *in vivo* inhibition in efforts to develop new therapeutics.[16]

The conundrum is that the *in vivo* role of PqsE is unknown. In brief, the *pqsE* gene is a member of the *pqsABCDE* operon responsible for synthesis of the signaling molecule 2-heptyl-3-hydroxy-4-quinolone (PQS), the autoinducer for a different quorum-sensing pathway.[18] However, PqsE, the final gene in the operon, is not required for PQS synthesis.[15] PqsE is known

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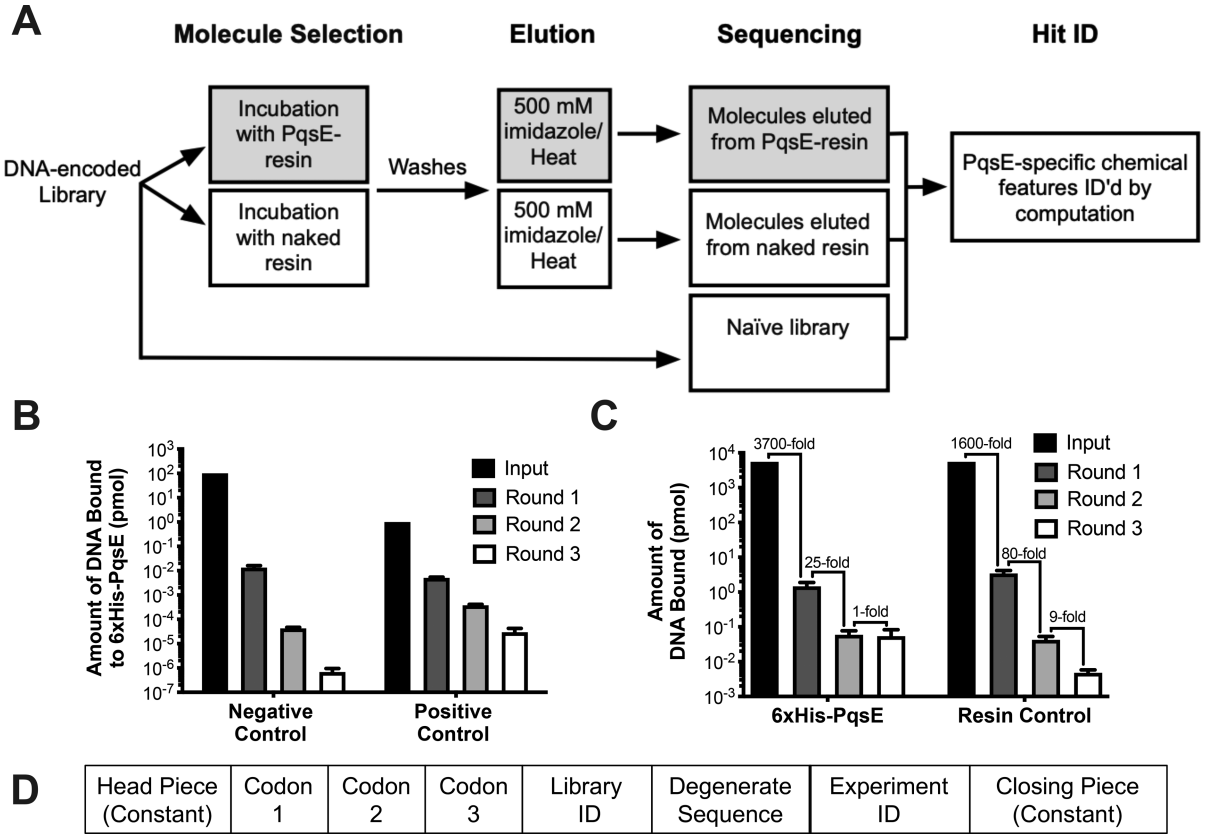

Figure 1. (a) Flowchart summarizing the screening method. (b) Quantitation of positive and negative control DNA barcodes following three rounds of selection with purified 6xHis-PqsE. Error bars represent standard deviation (SD) of technical replicates, $n = 4$. (c) Quantitation of the amount of library barcode DNA remaining following three rounds of selection with resin coated with 6xHis-PqsE or naked resin. The fold changes in remaining molecules are denoted. Error bars represent SD of technical replicates, $n = 5$. (d) Diagram showing the major motifs in the DNA-tag.

to be a thioesterase enzyme based on structural studies and *in vitro* enzyme activity assays.[19,20] However, neither the endogenous substrate(s) nor the product(s) of PqsE catalysis are known. It is also known that PqsE, *via* an undefined mechanism, is required for production of the virulence factor pyocyanin.[21,22] Specifically, a *P. aeruginosa* Δ*pqsE* mutant and *P. aeruginosa* harboring the catalysis-inactive PqsE S273A protein make no pyocyanin.[16] Curiously, however, a previously discovered small molecule, C1, that inhibits PqsE thioesterase activity *in vitro*, does not affect *in vivo* pyocyanin production levels.[23]

Given the critical role of PqsE in *P. aeruginosa* virulence coupled with the poor understanding of PqsE function, we reasoned that an unbiased method of screening together with a strategy for rapidly and efficiently testing very large numbers of compounds could deliver the best opportunity to identify structurally diverse small molecule PqsE inhibitors. High-diversity DNA-encoded libraries (DELs) can be used for unbiased, pooled screening to identify molecules that bind to a purified, functional protein target of interest.[24−26] Several hundred million molecules are screened rapidly and simultaneously (reviewed in refs 27, 28). The approach yields molecules that bind the protein target irrespective of the protein's biochemical activity, in our case, enabling us to subsequently interrogate their PqsE-related functions and correlate those functions with *in vivo* effects relevant to antimicrobial activity. Sorting through hits to identify those compounds with desired properties does demand robust, high-capacity, secondary screens that report on activities of interest.[29] A final attractive feature of DELs is that the size of these libraries generally provides sufficient numbers of structurally related hit compounds to support convincing structure-activity relationships (SAR) and minimizes the risk of prosecuting "singleton" screening hits in which structural analogs of the original hit molecule have no specific activity for the desired target.[30]

Here, we describe the affinity-based selection method we used to screen a DEL of drug-like compounds for binders to purified PqsE. Multiple clusters of hits were identified with shared chemical features, representing potential small molecule tools to study a range of catalytic and regulatory roles of PqsE. Following a two-step validation strategy, we identified the subset of binders that inhibit PqsE thioesterase activity. We report here the initial results from hit compounds of one chemotype, a series of 2-(phenylcarbamoyl)benzoic acids. We prepared a small set of analogs based on the structures of two hit molecules and performed an initial probe of SAR. This strategy enabled identification of compounds with increased potency. Mechanism of action studies indicate these inhibitors exhibit noncompetitive inhibition of PqsE-mediated hydrolysis of a synthetic substrate. The compounds failed to inhibit *in vivo* production of pyocyanin because they do not enter, are rapidly metabolized by, or are rapidly exported from *P. aeruginosa*. Additional structural classes revealed by this screen may provide future opportunities for further exploration of PqsE inhibition.

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RESULTS

Development of a DNA-Encoded Small Molecule Screen for PqsE Binders. To develop an affinity-based screen for DNA-encoded small molecules that bind to PqsE, we first examined the requirements for the protein target. A plasmid carrying the *P. aeruginosa pqsE* gene fused to the DNA encoding an N-terminal 6xHis tag was introduced into *E. coli* BL21.[16,19] Protein production was induced, and 6xHis-PqsE was purified to >90% on nickel resin using FPLC. Purified 6xHis-PqsE protein was assessed for the ability to bind to cobalt resin and to withstand the mandatory washing steps that would later be used in the screen (Figure S1A).[31] We selected cobalt resin on which to perform the screen to exploit resin binding by the 6xHis tag, with the notion that contaminating proteins that co-purified with 6xHis-PqsE on nickel would be eliminated (Figure S1A, designated FT).[31] The majority of the 6xHis-PqsE protein could be eluted from the cobalt resin with 500 mM imidazole (Figure S1A, Eluate 1), and a portion of the remaining protein could be eluted in buffer containing 1 M imidazole (Figure S1A, Eluate 2). Nonetheless, a fraction of the 6xHis-PqsE protein remained bound to the resin after these treatments. That fraction could be recovered by heating the resin to 60 °C for 5 min (Figure S1A Eluate 3). The rationale was that heat treatment would enable recovery of the remainder of the protein and simultaneously destabilize PqsE, resulting in release of the bound small molecules.

We next made two constructs as controls to verify that the covalently attached DNA barcodes do not interfere with compound binding to PqsE. First, as a positive control, we needed a small molecule PqsE binder that could be linked to DNA at a site of attachment on the molecule that would not interfere with compound binding. Hartmann[23] has published a set of small molecule PqsE inhibitors, three of which have been co-crystallized with PqsE, providing key structural information on potential points of attachment for a linker to DNA. We selected the highest affinity inhibitor, 2-(pyridin-3′-yl) benzoic acid (called C1), for modification. Inspection of the X-ray co-crystal structure of C1 complexed with PqsE suggested that a linker attached at the 4′ position (Figure S1B) would likely project out of PqsE into a solvent-exposed region. We therefore synthesized probe molecule P1 (Figure S1B, synthesis details in Supporting Information) and coupled the molecule to an azide-based eight-base-pair DNA headpiece using click chemistry.[32] A full-length DNA barcode (~120 bp) was subsequently added by ligation (Figure S1B, called positive control). As a negative control, we tested a ~120 bp DNA barcode without an attached small molecule.

Prior to examining binding of the two controls to PqsE, we validated our ability to quantify each control by RT-PCR of the respective barcode, including in mixtures (Table S1 and Supplemental Methods). The PCR primers used to amplify the positive control DNA barcode did not amplify the unmodified negative control DNA barcode (Figure S1C, left). However, the PCR primers used to amplify the negative control barcode DNA exhibited modest amplification of the positive P1-attached barcode DNA (Figure S1C, right). Thus, we could expect a false elevation in the PCR value from the negative control in mixtures quantified by PCR. Our linear range for PCR amplification spans seven logs, so we reasoned that so long as we could discern a significant increase in amplification of the P1-attached positive control DNA barcode relative to that of the unmodified negative control DNA barcode, we could accept some background noise in the readout. Therefore, we went forward with the binding analysis.

A schematic of the overall screening process is shown in Figure 1A. In brief, to test if the PqsE protein could selectively bind and retain a DNA-encoded molecule, we combined 100 pmol of the unmodified negative control DNA barcode with 1 pmol of the positive control P1-DNA-encoded compound and assessed binding to 6xHis-PqsE-bound cobalt resin. This compound mixture is referred to as the input. First, the input mixture was preincubated with the 6xHis-PqsE purified protein. Following incubation, the 6xHis-PqsE-input mixture was loaded onto cobalt resin. The resin was washed five times to remove all unbound molecules, then 6xHis-PqsE and bound molecules were eluted by heating in 500 mM imidazole. We call this the round 1 selection. The molecules isolated from the 6xHis-PqsE in round 1 were incubated with DNA-binding magnetic beads to remove imidazole. This process was repeated two more times, each time with fresh 6xHis-PqsE protein (designated round 2 and round 3 selection). After each round of selection, the amounts of bound P1-encoded and unmodified barcode DNAs were quantified by RT-PCR (Figure 1B). Starting with a 100-fold excess of negative control DNA, three rounds of selection enabled us to recover a ~50-fold excess of positive control DNA, representing a 5000-fold enrichment, suggesting the potential for identification of specific PqsE binders using this protocol.

Production of the Compound Library. The Macroceutics DEL that was screened against PqsE contained approximately 550 million small molecules. This compound set was generated from five different library builds (DEL01-DEL05), and all of the libraries were synthesized using traditional split-pool-mix techniques outlined previously.[26,33] The various chemistries used to produce these libraries included acylation, reductive amination, sulfonylation, and S_N2-mediated alkylation.[34] Libraries were made in both branched and linear geometries. This Macroceutics DEL was designed to maximize diversity, while maintaining compound physical properties suitable for lead optimization in follow-up medicinal chemistry efforts. An example schematic of DEL02, a library produced as a branched dimer, is shown in Figure S1D. Cycle 1 (position c_1) consisted of approximately 65 trifunctional cores, each of which had orthogonal protection moieties on two nitrogen atoms as depicted in Figure S1D. These 65 cores were loaded on DNA and, using the subsequent split-pool-mix methodology,[26,33] reacted with ~1000 building blocks each in cycle 2 (position c_2) and cycle 3 (position c_3), to provide a branched design with a final library size of 65 million compounds in DEL02.

Screening the DNA-Encoded Compound Library for PqsE Binders. We used the above protocol to screen the DNA-encoded small molecule library for compounds that bind to PqsE (Figure 1A). We preincubated 5000 pmol of the library, representing a pool of 550 million unique molecules, with 6xHis-PqsE protein and performed the three rounds of selection. Following each round of selection, an aliquot was removed, and total DNA bound to PqsE was quantified by RT-PCR to assess how many encoded molecules remained (Figure 1C). We performed the identical procedure with cobalt resin to which no 6xHis-PqsE had been added to assess the background level of encoded molecules binding to the resin. Following three rounds of selection, 10-fold more DNA-encoded molecules remained bound to the resin coated with 6xHis-PqsE than to the resin itself, suggesting that, while there

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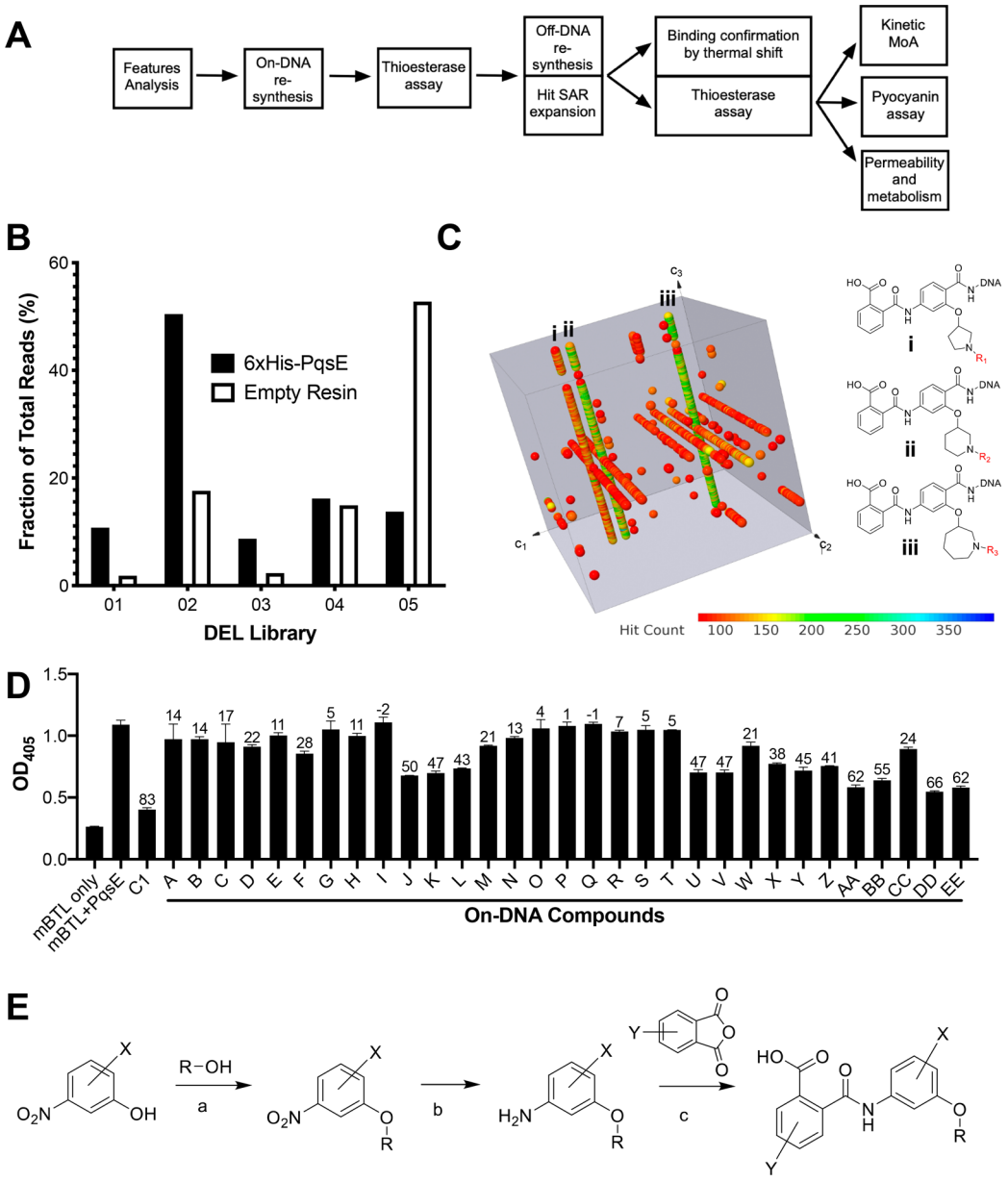

Figure 2. (a) Flowchart summarizing the pipeline for assessment of screening hits. (b) Fraction of total reads from each DEL based on sequencing of DNA tags for compounds bound by 6xHis-PqsE versus those bound to the empty resin. (c) Data cube showing the screening hits from DEL02 that possessed codon combinations with hit counts of 75 or greater. The *x*, *y*, and *z* axes represent, respectively, compound building blocks in the three codons representing synthetic positions c_1, c_2, and c_3 as shown in Figure S1D. Codon combinations that yielded hits in the resin binding or in the naïve library controls have been removed from the view. Three related molecules i, ii, and iii corresponding to strong features in the data cube are shown. The moieties designated R_1, R_2, and R_3 represent the various cycle 3 capping moieties from the library. (d) Thioesterase inhibition by 10 μM of the on-DNA candidate compounds in the Ellman's assay. We note that the positive control inhibitor C1 was tested at 100 μM explaining its higher potency in the graph. The % inhibition caused by each compound is shown above its corresponding bar. Error bars represent the SD of technical replicates, n = 2. (e) General synthesis of compounds **1**−**16**. Reagents and conditions: (a) diisopropyl azodicarboxylate (DIAD), triphenylphosphine (Ph_3P), tetrahydrofuran (THF), 0 °C to RT, 12 h; (b) iron (Fe), ammonium chloride (NH_4Cl), ethanol (EtOH), 80 °C, 12 h; (c) dichloromethane (CH_2Cl_2), RT, 6 h.

may be overlap, the resin containing 6xHis-PqsE protein shows high selectivity to bind a subset of our DNA barcoded compounds. The library molecules remaining on both the 6xHis-PqsE-coated and naked resins were collected following heat treatment of the resins, and the DNA barcodes were sequenced (Figure 1A). Additionally, an aliquot of the naïve library that had not been subjected to any selection was sequenced to eliminate any sequencing bias arising from the DNA tags (Figure 1A).

Analysis of Small Molecule Hits. The DNA from our selection experiments was assessed by next-generation sequencing.[25] Using Macroceutics' in-house proprietary software, the sequence data were analyzed and compared to the naïve library and resin-binding control data. Important for this analysis is that each DNA tag contained multiple motifs for decoding the screening results (Figure 1D). First, the terminal DNA sequences, called the head and closing pieces, were constant. Primers were designed to hybridize with these two

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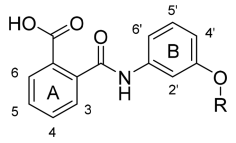
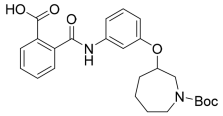
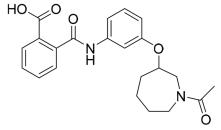
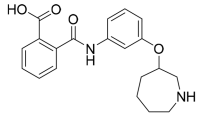
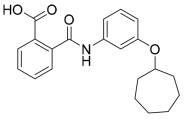
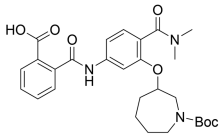
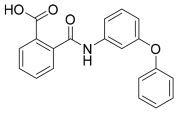
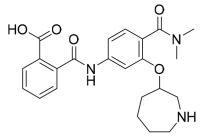
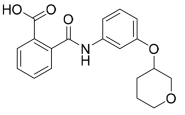
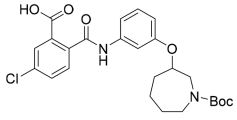
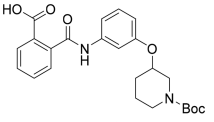
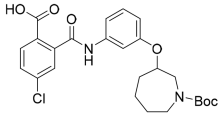
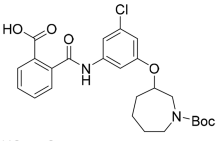
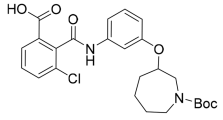
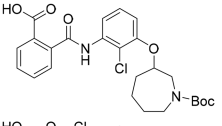
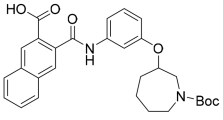
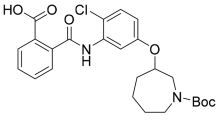
Table 1. PqsE Enzyme Inhibition Activity of Compounds 1−16



Compound	Structure	PqsE Thioesterase IC_{50} (μM)	Compound	Structure	PqsE Thioesterase IC_{50} (μM)
1		18.1 ± 1.7	9		18.9 ± 4.8
2		>100	10		11.5 ± 3.8
3		19.1 ± 3.2	11		10.3 ± 1.5
4		>100	12		24.1 ± 8.9
5		>100	13		32.1 ± 15.6
6		>100	14		5.6 ± 1.0
7		>100	15		9.0 ± 2.0
8		>100	16		>100

regions to enable PCR amplification of the tags. Second, each DNA sequence contained a barcode identifying the DEL from which the compound came (Library ID), allowing multiple libraries to be screened simultaneously. In our screen, five DELs were pooled and screened, so there were five different library ID sequences. Third, the tags contained an experiment ID DNA sequence, unique to each screen. Fourth, individual chemical building blocks incorporated into the compounds were encoded by unique DNA sequences called codons. Since there were three chemical cycles used to produce each compound in each library, the DNA tag for each compound contained all three of its corresponding codons. This feature enables compound structure identification. Finally, a degenerate DNA sequence was included as a unique ID for each individual compound. Since the libraries were amplified by PCR prior to sequencing, the degenerate sequence allowed differentiation of unique hits from duplications arising from PCR amplification. Our strategy for hit analysis is shown in Figure 2A.

Regarding the success of our screen, the library ID sequences showed that different fractions of total reads came from each of the five DELs and each DEL had a distinct background profile (Figure 2B) demonstrating that the libraries behave differently with respect to binding to PqsE and to the control resin. Each codon combination encodes a unique compound and the number of times that combination appears (hit count) can be determined using the unique sequences of the codons and the corresponding degenerate sequence. A high hit count is assumed to represent an enhancement of that compound during selection. Since each library has a three building-block design, with three synthetic codons, it is convenient to represent the screening data as a cube, with each axis composed of building blocks for one of the cycles (Figure 2C shows the cube for DEL02). Patterns, or "features," in the cube can be interpreted as SAR. DEL02 contains a total of 65 million compounds. Assessment of the sequencing data from DEL02 is summarized in Table S2 and Figure S2. Even with a comparable number of DNA sequences from the resin control and a larger number of naïve DNA

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sequences, the PqsE-selected DNA sequences yielded a much larger number of high hit count DNA sequences. After removing sequences that appeared in the resin-binding or naïve sets, there were 6732 PqsE-selected sequences that exhibited a hit count of 75 or greater, which was about 0.1% of the unique codon combinations recovered or about 0.01% of the compounds applied.

A representative compound cube from DEL02 is shown in Figure 2C. Three extremely strong features can be observed representing three closely-related core structures (out of 65 cores) in this library (Figure 2C). In the figure, the three features are the vertical lines representing the three chemical structures: cycle 1 is denoted as "c_1," cycle 2 is "c_2," and cycle 3 is "c_3." All three core structures featured a central *p*-aminobenzoic acid with a pendant five-, six-, or seven-membered nitrogen heterocycle. Note that the benzoic acid depicted in Figure 2C is the cycle 2 moiety coded for in those features. The heavy vertical patterns in Figure 2C show that strong binding to PqsE is independent of the identity of the cycle 3 building block. Cycle 3 included acylations, reductive aminations, and sulfonylations, and all three reactions led to hits. This result corresponds to an SAR "dead-spot" that does not influence binding to PqsE. On the basis of these results, we resynthesized hit compounds from these core structures using on-DNA resynthesis.

For our initial retest, we focused on the compounds with the strongest and/or most frequent hit features from the initial screening campaign. Thirty-one exemplar molecules attached to their DNA barcodes were selected for assessment of inhibition of PqsE catalytic activity. Although the *in vivo* substrate and product for PqsE are not known, PqsE can catalyze thioester bond cleavage. We monitor this catalytic activity using a surrogate substrate called mBTL (*meta*-bromothiolactone) that we previously identified as an agonist of the *P. aeruginosa* RhlR quorum-sensing receptor.[35] The molecular weight (MW) of mBTL is 357 g/mol, and PqsE-mediated hydrolysis of the thiolactone yields a product with a mass of 375.01 g/mol (Figure S3A) that can be followed by mass spectrometry. Indeed, Figure S3B shows that mBTL, when incubated with purified PqsE protein, but not in its absence, results in the appearance of the 375.01 m/z peak. This result establishes that we can use mBTL as a convenient and inexpensive artificial substrate for PqsE, enabling efficient assessment of screening hits for the ability to inhibit PqsE thioesterase activity.

After confirming PqsE thioesterase activity with mass spectrometry, we developed an efficient, inexpensive, high-throughput plate-based method using Ellman's reagent (5,5′-dithio-bis-[2-nitrobenzoic acid]) for routine assessment of enzyme inhibition.[19] First, we monitored inhibition of PqsE-driven cleavage of mBTL by compounds from the screen, still containing their DNA tags (Figure 2D). Seventeen of the 31 test compounds, when assayed at 10 μM with 500 nM 6xHis-PqsE, showed at least 20% inhibition of PqsE thioesterase activity, with five compounds (on-DNA compounds J, AA, BB, DD, and EE, Figure 2D) showing at least 50% inhibition. We chose to focus on the structural class represented by the on-DNA hit BB (Figure 2D). BB is in the family of compounds depicted in Figure 2C. It contains an azepane ring (Figure 2C, compound iii) with a *tert*-butoxycarbonyl (Boc) group in the R_3 position. We selected this chemotype based on consistency of hit features within the primary screen, synthetic tractability, and calculated physicochemical properties such as MW, polar surface area, and cLogP values of the corresponding off-DNA analog of compound BB. Interestingly, on-DNA hit BB possesses structural features similar to the known C1 inhibitor.

To confirm off-DNA activity of this chemotype, we synthesized an initial set of four analogs of the hit designated BB in Figure 2D off of their DNA barcodes, which correspond to compounds **1**−**4** in Table 1. The point of attachment to DNA is at the 4′-position, as shown for the three compounds in Figure 2C. We synthesized the corresponding analog in which the attachment point is removed altogether by substitution with a hydrogen atom (compound **1**, Table 1) and the analog with that attachment point capped by a simple N,N-dimethyl amide (compound **3**, Table 1). Compound **3** is the closest structural analog to on-DNA hit BB in our set. Because the Boc group is easily removed under acidic conditions,[36] we sought to understand whether the presence of that group was important to PqsE activity. Thus, we also prepared the corresponding compounds **2** and **4** (Table 1) in which the Boc group was removed.

The general synthetic scheme by which compounds **1**−**4** and subsequent analogs **5**−**16** were prepared is shown in Figure 2E. In brief, 3-nitrophenol and the appropriate alcohol were subjected to typical Mitsunobu reaction conditions[37] using diisopropylazodicarboxylate to afford the desired phenyl ether. The nitro group was reduced using elemental iron and ammonium chloride,[38] and the corresponding aniline product was treated with the appropriate phthalic anhydride to afford the desired targets.

Assessment of Activity of Compounds 1−16. Compounds **1**−**4** (Table 1) were tested both for the ability to bind to PqsE via a thermal shift assay (Figure 3A) and for the ability to inhibit PqsE thioesterase activity (Table 1). All four compounds were soluble under both assay conditions (Figure S4A,B). Compounds **1** and **3** significantly shifted the melting temperature of PqsE in a dose-dependent manner (Figure 3A), indicating these compounds bind to and stabilize PqsE. Specifically, at 500 μM, compounds **1** and **3** shift the PqsE melting temperature by >3 °C. Compounds **2** and **4**, in which the Boc group has been removed, do not cause a melting temperature shift at concentrations below 500 μM, indicating a considerable loss in affinity for PqsE. This result raised the question of whether the decrease in binding is a consequence of the strongly basic amine moiety that is unmasked by Boc removal or the loss of important binding interactions between PqsE and the Boc group. Since the DEL02 screening results indicated that PqsE binding was largely independent of the composition of the Cycle 3 building block, we surmised that the loss of activity was due to the presence of a strongly basic amine in compounds **2** and **4**.

To more rigorously examine compounds for inhibition of PqsE, we developed a thioesterase assay with a >5-fold larger dynamic range than that provided by the Ellman's reagent used in our initial enzyme inhibition test. We employed 7-diethylamino-3-(4′-maleimidyl-phenyl)-4-methylcoumarin (CPM), a reagent that fluoresces following reaction with free sulfhydryl groups.[39] We incubated 100 μM mBTL with 250 nM PqsE at RT for 20 min with a range of concentrations of test compounds to determine their IC_{50} values. Compounds **1** and **3** profiled as modestly potent thioesterase inhibitors in this assay with IC_{50} values of 18 and 19 μM, respectively (Table 1 and Figure 3B,C). Compounds **2** and **4** did not yield IC_{50} values even when tested at 100 μM (Table 1). These data parallel those from the thermal shift assay. Verifying the results

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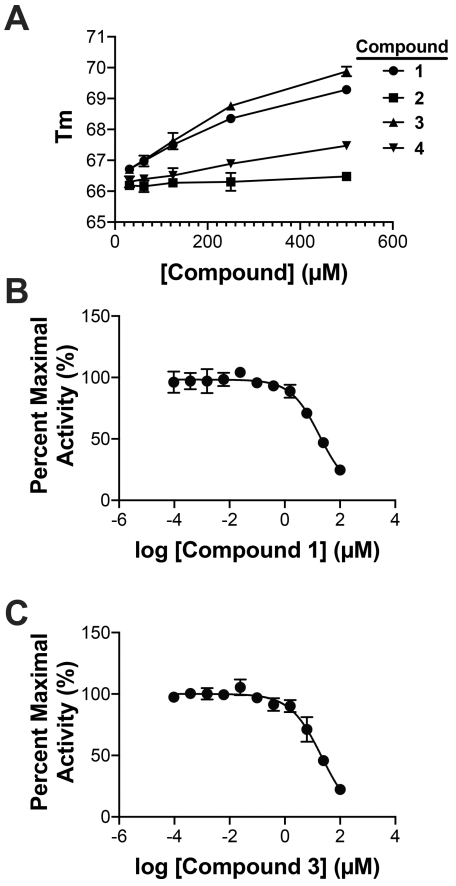

Figure 3. (a) Melting temperatures for 6xHis-PqsE in the presence of the designated compounds. Error bars are shown as SD of biological replicates, $n = 3$. (b) PqsE thioesterase inhibition curve for compound **1**. Percent maximal activity is defined as the fluorescence value in the presence of the inhibitor divided by the fluorescence value in the absence of inhibitor. A curve was fit to these data points to determine the IC_{50} value, as reported in Table 1. Error bars represent SD of biological replicates, $n = 3$. (c) Inhibition curve as in panel B for compound **3**.

of the CPM-based assay, analysis by mass spectrometry showed that PqsE-mediated cleavage of mBTL was reduced in the presence of compounds **1** and **3** relative to controls lacking inhibitor compounds (Figure S3B).

We next prepared a small set of additional compounds in this chemotype to probe initial SAR (compounds **5**−**16**, Table 1) and evaluated them for inhibition of PqsE thioesterase activity (Table 1 provides all of the IC_{50} values, and Figure 4 shows the dose response curves for all compounds exhibiting activity (compounds **9**−**15**)). All compounds were soluble under the thioesterase assay conditions (Figure S4B). On the basis of the results from compounds **1**−**4**, the Boc groups were retained in compounds **5**−**8** and **13**−**16**. A chlorine atom was used as a "probe" moiety to evaluate the tolerance to substitution at each open position of the two aromatic rings contained in the structure (compounds **5**−**7** and **14**−**16**). Compounds **9**−**13** were designed to provide a cursory examination of the importance of the *t*-butyl-3-hydroxyazepanecarboxylate group.

The screening results from this set of analogs reveal three key pieces of information. First, it appears that phenyl ring A is quite intolerant of substitution, as compounds **5**−**7**, in which a chloride is placed in positions 3−5, (for compound position numbering see the structure at the top of Table 1), are all inactive at the highest concentration tested. Additionally, compound **8**, in which the phenyl group has been replaced by the sterically larger naphthyl group, is also inactive. These data suggest that positions 4 and 5 of the aromatic ring pack tightly against the PqsE protein. This same case can be made for position 3; however, loss of activity in analog **7** could also be due to a shift in the preferred relative conformation of the carboxylic acid and amide moieties. Substitution of hydrogen with the sterically larger chlorine atom will affect the conformation of those functional groups, and it is likely that the relative spatial positions of those groups are important aspects of compound binding to PqsE. We were unable to prepare the analog with a chlorine in the 6 position, thus effects of substitution at that position remain unknown.

Second, the data suggest that the 2′ and 5′ positions of phenyl ring B are tolerant to substitution (see compounds **14** and **15**). In fact, compounds **14** and **15** both show a modest increase in potency relative to the hit compounds **1** and **3**, suggesting substitution at either of these two positions may be beneficial. By contrast, substitution at the 6′ position is detrimental to activity (compound **16**). Compound **14** is the most potent inhibitor we have discovered to date in this chemical series. We did not prepare the analog with a chlorine atom in the 4′ position because compound **3** had already shown us that substitution in this position was tolerated.

Finally, it appears that the substituent on the phenol is also tolerant to substitution. Compound **9**, in which the *t*-butylcarbamate moiety in compound **1** has been altered to an acetamide, is equipotent to compound **1**. Replacement of the azepane ring with a cycloheptyl ring (compound **10**) or a phenyl ring (compound **11**) provided a slight increase in inhibitory potency relative to hit compound **1**, while replacement with tetrahydropyranyl (compound **12**) or piperidinyl (compound **13**) moieties led to no change in potency relative to compound **1**. These results suggest that nonpolar groups are slightly more preferred than polar groups in this region, although the differences in potency between these compounds are minor.

Overall, the data from this small set of analogs provide proof of SAR with respect to PqsE thioesterase inhibition with this chemotype and suggest that the 2′, 4′, and 5′ positions in ring B, along with the substituent on the ring B phenol are the most productive positions on the molecule for additional structure-activity work. The importance of the carboxylic acid and the amide functional groups to biological activity still need to be determined. While these compounds are somewhat similar in structure to the small heterocyclic ligands reported by Hartmann[23] to be active site inhibitors, we do not know where on the PqsE protein these compounds bind, and it would be premature to assume they bind analogously to the Hartmann inhibitors.

Assessment of Activities *in Vivo* in *P. aeruginosa*. To discover whether the active thioesterase inhibitors (compounds **1**, **3**, **9**−**15**) have effects in *P. aeruginosa*, we examined them for their influence on PqsE-dependent pyocyanin production by *P. aeruginosa*. On the assumption that catalytic activity is required for pyocyanin production,[16] reduction of pyocyanin would be an indication that our PqsE thioesterase inhibitors are soluble, stable overnight at 37 °C, permeable to the bacterial membrane, and actively inhibiting cytoplasmic PqsE enzyme activity in the critical period when pyocyanin is produced as a consequence of quorum sensing. *P. aeruginosa*

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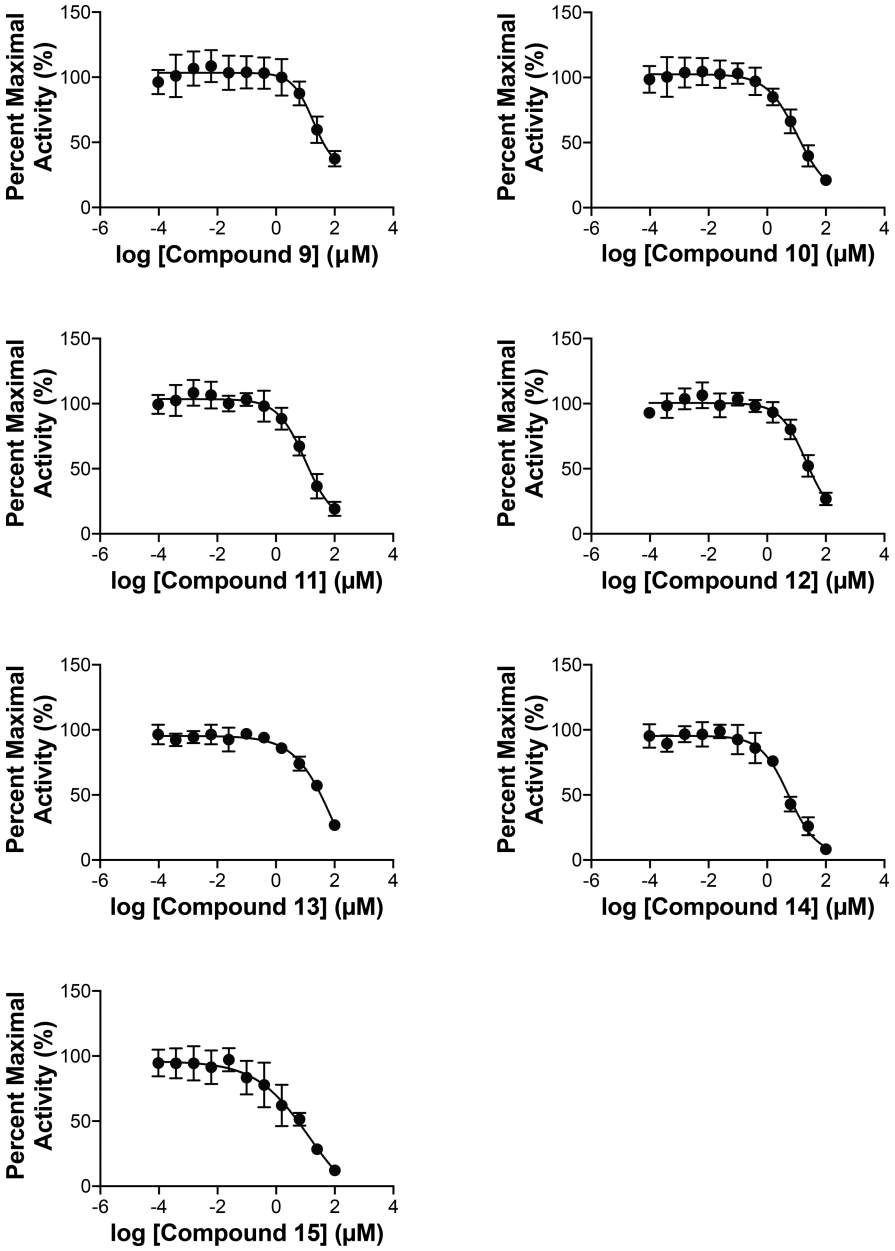

Figure 4. Thioesterase dose−response inhibition analyses for compounds **9**−**15** from Table 1, which reports the IC_{50} values. Curve fits to these data were used to determine the IC_{50} values for Table 1. Error bars represent SD of biological replicates, $n = 3$.

cells were back-diluted 1:1000 from a saturated, overnight culture, and compounds were added at 100 μM. All compounds are soluble in these conditions (Figure S4C). The cultures containing the test compounds were incubated overnight at 37 °C with aeration to allow the cells to regrow. Pyocyanin production was monitored by OD_{695}. Unlike mBTL, a known inhibitor of pyocyanin production, none of these new compounds exhibit *in vivo* activity (Figure 5A).

Analysis of Compound Stability and Metabolism. Compounds **1** and **3** are micromolar inhibitors of PqsE thioesterase activity. However, given that they do not reduce pyocyanin production in *P. aeruginosa*, which requires *in vivo* PqsE activity, we suspected that the compounds may not reach the target at concentrations sufficient to elicit a response. Moreover, the overnight incubations necessary for laboratory measurements of pyocyanin production provide a high bar for metabolic stability of PqsE inhibitors *in vivo*. To investigate the fates of compounds **1** and **3** in our assays, we used mass spectrometry to quantify their levels over time following administration to *P. aeruginosa*. mBTL, which is known to be internalized and maintained in *P. aeruginosa*, was used as the positive control. Indeed, as expected, over time, the concentration of mBTL progressively decreased in the cell-free culture fluid (Figure 5B) and increased in the cell lysate (Figure 5C). By contrast, for the first 7 h of incubation, the amounts of compounds **1** and **3** in the cell-free culture fluids remained steady (Figure 5B), and neither compound could be detected in the cell lysate (Figure 5C). Following overnight incubation, we could not detect compound **1** or **3** in the cell-free culture fluid or the cell lysate (Figure 5B,C, respectively). We conclude that compounds **1** and **3** are unable to enter *P. aeruginosa*, or if they are internalized, they are subsequently metabolized or effluxed. Neither compound has sufficient stability to withstand overnight incubation under our assay

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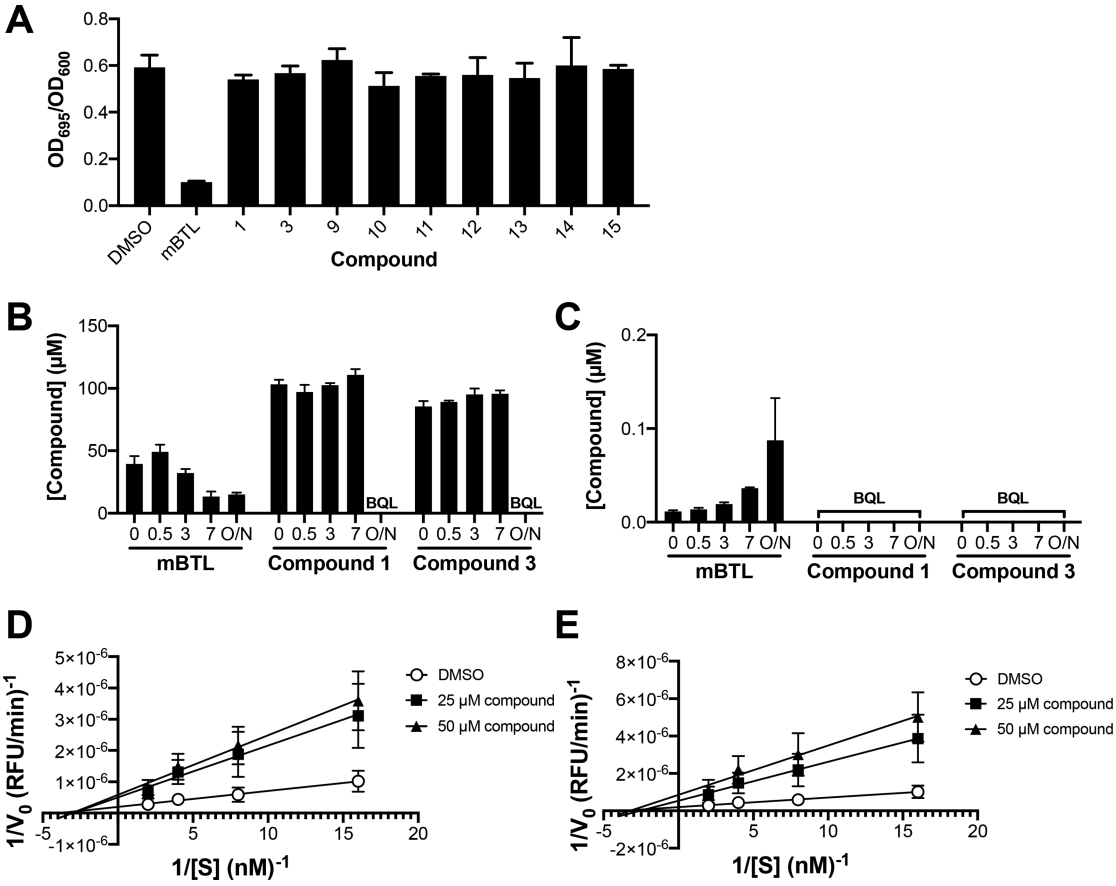

F

	DMSO	Compound 1 25 μM	Compound 1 50 μM	Compound 3 25 μM	Compound 3 50 μM
V_{max} (RFU/min)	$5.1x10^6$	$2.0x10^6$	$1.7x10^6$	$1.9x10^6$	$1.2x10^6$
K_m (μM)	261	331	328	390	310

Figure 5. (A) Pyocyanin levels as judged by OD_{695} per OD_{600} (growth) following treatment of *P. aeruginosa* with the designated compounds at 100 μM. Error bars represent SD of biological replicates, $n = 3$. (B) Concentrations of mBTL, compound **1**, and compound **3** in cell-free culture fluids following incubation with *P. aeruginosa* for the designated times. O/N designates 16 h of treatment. BQL = below the quantitation limit. Error bars represent SD of biological replicates, $n = 4$. (C) As in panel B for the concentrations of mBTL, compound **1**, and compound **3** in cell lysates. (D) Lineweaver–Burk plots of thioesterase inhibition by compound **1** at a range of concentrations of the mBTL substrate. The inhibitor was tested at 25 μM and 50 μM. Error bars represent SD of biological replicates, $n = 3$. (E) Lineweaver–Burk plots as in panel D for compound **3**. (F) Calculated V_{max} and K_m for each condition from the data in panels D and E.

conditions. These results apparently explain why compounds **1** and **3** do not inhibit *in vivo* pyocyanin production.

Mechanism of Action (MoA) of Compounds 1 and 3. We assessed the MoA of compounds **1** and **3** by quantifying initial rates of mBTL cleavage over a range of concentrations (Figure 5D,E). In the absence of inhibitor, the V_{max} of the reaction is 5.1×10^6 RFU/min, and the K_m is 261 μM. Modest changes in K_m occurred in the presence of the inhibitors, but the changes did not depend on inhibitor concentration (Figure 5F). In contrast, the V_{max} for PqsE catalysis decreased in the presence of each inhibitor and in a dose-dependent manner (Figure 5D–F). We therefore conclude that compounds **1** and **3** act noncompetitively to inhibit the ability of PqsE to cleave mBTL.

Hartmann *et al.* have previously reported on a set of PqsE thioesterase inhibitors which coincidentally have some structural similarity to our series.[23] X-ray co-crystal structures of those compounds revealed they bound in the active site of PqsE. The authors posited a competitive inhibition mechanism for their inhibitors; however, no formal MoA studies were reported. Our MoA studies with compounds **1** and **3** indicate that these inhibitors are noncompetitive. However, we do not know whether our compounds are orthosteric or allosteric inhibitors. Either our inhibitory compounds bind at a site that is distinct from the previously reported compounds, or both chemotypes bind in the active site but our compounds do not block substrate binding. We have begun structural analyses to further characterize the compounds we identified here, and we are also investigating additional hit chemotypes from the DEL screening campaign to identify compounds that exhibit other mechanisms of interference with PqsE function.

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CONCLUSIONS

P. aeruginosa infection is a significant health burden, especially to the immunocompromised population.[40] PqsE is now established as a therapeutic target as it is essential for *P. aeruginosa* pathogenicity, and it has a binding pocket that should be susceptible to small molecule inhibition.[19] In this initial work, we, like others in the field, have encountered long-standing challenges regarding cell penetration, metabolism, and efflux of interesting anti-virulence compounds.[41−43] Future SAR expansion of the hits from this screen as well as characterization of hits from the other DELs screened as part of the current study could reveal potent PqsE inhibitors that are also internalized by and exhibit *in vivo* PqsE inhibition in *P. aeruginosa*, either through inhibition of thioesterase activity or other potential activities of the PqsE protein.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.9b00905.

Four supplemental figures, two supplemental tables, methods, compound syntheses, and analytical chemistry data (PDF)

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Notes

The authors declare no competing financial interest.

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