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Chemical Inhibition of Kynureninase Reduces *Pseudomonas aeruginosa* Quorum Sensing and Virulence Factor Expression

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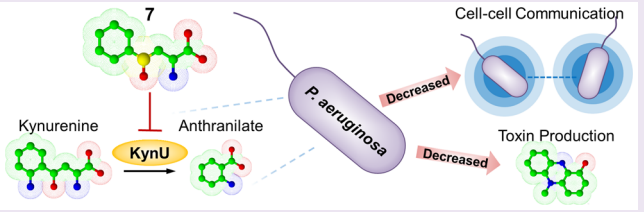
Supporting Information

ABSTRACT: The opportunistic pathogen *Pseudomonas aeruginosa* utilizes multiple quorum sensing (QS) pathways to coordinate an arsenal of virulence factors. We previously identified several cysteine-based compounds inspired by natural products from the plant *Petiveria alliacea* which are capable of antagonizing multiple QS circuits as well as reducing *P. aeruginosa* biofilm formation. To understand the global effects of such compounds on virulence factor production and elucidate their mechanism of action, RNA-seq transcriptomic analysis was performed on *P. aeruginosa* PAO1 exposed to *S*-phenyl-L-cysteine sulfoxide, the most potent inhibitor from the prior study. Exposure to this inhibitor down-regulated expression of several QS-regulated virulence operons (e.g., phenazine biosynthesis, type VI secretion systems). Interestingly, many genes that were differentially regulated pertain to the related metabolic pathways that yield precursors of pyochelin, tricarboxylic acid cycle intermediates, phenazines, and *Pseudomonas* quinolone signal (PQS). Activation of the MexT-regulon was also indicated, including the multidrug efflux pump encoded by *mexEF-oprN*, which has previously been shown to inhibit QS and pathogenicity. Deeper investigation of the metabolites involved in these systems revealed that *S*-phenyl-L-cysteine sulfoxide has structural similarity to kynurenine, a precursor of anthranilate, which is critical for *P. aeruginosa* virulence. By supplementing exogenous anthranilate, the QS-inhibitory effect was reversed. Finally, it was shown that *S*-phenyl-L-cysteine sulfoxide competitively inhibits *P. aeruginosa* kynureninase (KynU) activity *in vitro* and reduces PQS production *in vivo*. The kynurenine pathway has been implicated in *P. aeruginosa* QS and virulence factor expression; however, this is the first study to show that targeted inhibition of KynU affects *P. aeruginosa* gene expression and QS, suggesting a potential antivirulence strategy.



Pseudomonas aeruginosa is a Gram negative bacterium that poses a significant threat to susceptible immunocompromised individuals, such as those with cystic fibrosis and those with severe and/or chronic wounds. Contributing to its pathogenicity is a wide-ranging battery of virulence traits, many of which are regulated by a process known as quorum sensing (QS). QS is a complex system that coordinates multicellular behaviors based on the bacterial population present.[1] In the most simplistic model, bacteria synthesize, secrete, and detect molecules known as autoinducers (AI). As the bacterial population increases, so does the concentration of AI in the medium. When a threshold concentration has accumulated, AIs can bind to their cognate receptor and induce the expression of genes in the entire population simultaneously. These genes often encode products involved in biofilm formation, virulence factors, or other proteins that would be less efficient or effective if expressed by individual bacteria versus coordinated expression.[2]

There are three well-characterized QS networks that have been identified in *P. aeruginosa*: the *las*-pathway, the *rhl*-pathway, and the *pqs*-pathway. The *las*, *rhl*, and *pqs*-pathways utlize the corresponding AIs, respectively *N*-3-oxo-dodecanoyl homoserine-lactone ($3OC_{12}$-HSL), *N*-butanoyl homoserine lactone (C_4-HSL), and 2-heptyl-3-hydroxyl-4-quinolone (*Pseudomonas* quinolone signal, or PQS).[3−5] These communication pathways have become targets for the development of new drugs that abrogate bacterial virulence and have the potential to mitigate drug resistance.[6,7]

QS inhibition can be achieved through a variety of approaches, including interfering with AI-receptor binding, degrading, or inactivating AIs, or by inhibiting the metabolic processes through which AIs are synthesized.[8,9] Acyl-homoserine lactone (acyl-HSL) analogues have been designed to competitively bind to LuxR-type acyl-HSL receptors and inhibit quorum sensing and virulence.[10−12] Acyl-HSL deactivation has been shown by both chemical (e.g., alkaline-induced

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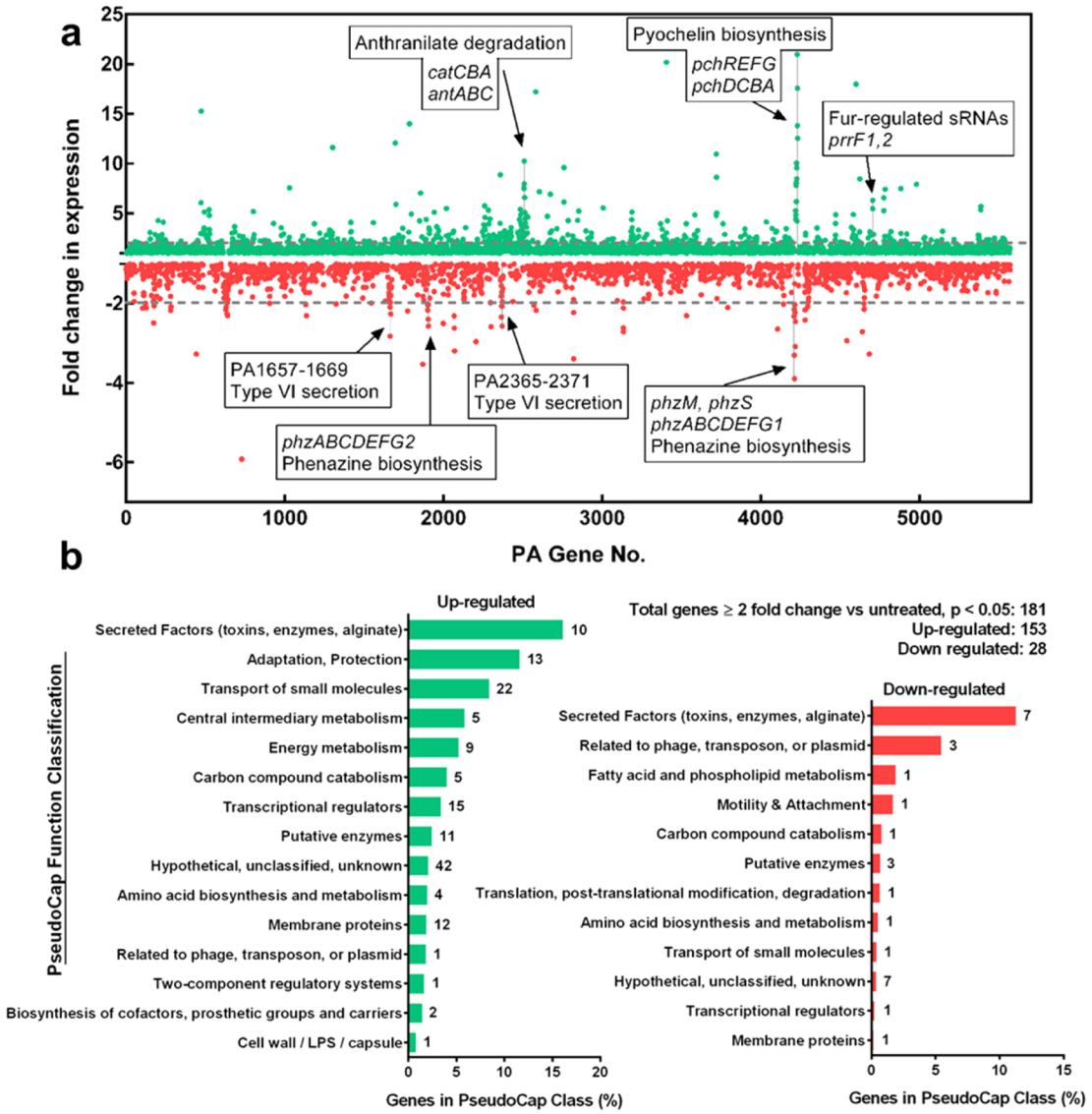

Figure 1. (a) The genome wide transcriptomic profile of *P. aeruginosa* PAO1 in response to compound **7**. Green dots represent genes induced in response to compound **7**, and red dots indicate repressed genes. The gray dashed lines represent 2-fold changes in expression. Several related operons that stand out are indicated. (b) Functional classes of genes that were significantly up-regulated (green, left) or down-regulated (red, right) in response to compound **7**. Functional classes are plotted based on the percentage of the total number of genes in their respective PseudoCap class that were significantly affected. The actual number of genes in each class that were affected is indicated next to the corresponding bar.

lactonolysis)[13] and enzymatic degradation (e.g., acylase and lactonase activities).[14,15] Small molecule inhibition of acyl-HSL synthases has been explored for QS disruption.[16] Methyl anthranilate, an analogue of the PQS precursor anthranilate, has also been shown to obstruct QS signal generation and associated virulence.[17]

P. aeruginosa can leverage the kynurenine pathway to generate anthranilate from tryptophan. The importance of this pathway in PQS signaling was first suggested by D'Argenio et al.[18] Although *P. aeruginosa* possesses two other anthranilate synthases (i.e., PhnAB and TrpEG) that utilize chorismate as a precursor,[19] it has previously been shown that the kynurenine pathway is the preferred source of anthranilate for PQS when cells are growing in rich media.[20,21] While it has been speculated that exploitation of the kynurenine pathway could serve as a novel therapeutic target,[20,21] there have been no reports to date of small molecule effectors that disrupt the pathway in this pathogen. It should be noted that inhibition of human kynureninase has been explored and multiple inhibitor compounds have been reported. Kynureninase is highly conserved between humans and bacteria, including *Pseudomonas fluorescens*.[22−25] To study kynureninase activity and inhibition for humans, KynU has been extracted from *P. fluorescens*. However, there have been no reports or detailed studies of kynureninase inhibitors in *P. fluorescens* or *P. aeruginosa*. Further, there have been no reports of QS modulatory properties by such inhibitors. Thus, exploration of kynureninase inhibition in *P. aeruginosa* is both novel and significant.

Previously, in a high-throughput phenotypic screening effort, we identified several compounds, inspired by naturally occurring small molecules from the tropical plant *Petiveria alliacea*, that inhibit QS in *las*- and *rhl*-based QS reporters systems.[26] Our lead compound from this study, *S*-phenyl-L-cysteine sulfoxide (compound **7**), was shown to inhibit biofilm formation in multiple species, including *P. aeruginosa*, and to

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reduce bacterial load in an *in vivo Drosophila melanogaster* infection model.[26,27] However, these compounds lack structural similarity to previously reported *P. aeruginosa* autoinducers, which led us to conduct further mechanistic studies to determine potential novel targets.

In this work, we performed a global transcriptomic analysis of *P. aeruginosa* in response to *S*-phenyl-L-cysteine sulfoxide (compound 7) using RNA-seq. Compound 7 was found to affect the expression of a number of genes in *P. aeruginosa*, including those associated with iron acquisition, virulence factor production, and efflux pumps. In particular, we observed altered expression of genes in the pyochelin, phenazine, and anthranilate biosynthetic pathways in response to treatment with compound 7. This led us to hypothesize that compound 7 functions by inhibiting kynureninase, an enzyme that converts kynurenine to anthranilate. This would in turn inhibit QS since anthranilate is a precursor of PQS. Consistent with this hypothesis, we show that the addition of exogenous anthranilate, but not kynurenine, can reverse the QS-inhibitory effect of compound 7 and that the molecule competitively inhibits enzymatic activity of a recombinant *P. aeruginosa* KynU. Furthermore, we show that PQS, and its precursor HHQ, are significantly less abundant when *P. aeruginosa* is exposed to compound 7. We conclude that targeting kynureninase is a viable strategy for inhibiting QS in *P. aeruginosa*.

RESULTS

To interrogate the *P. aeruginosa* transcriptomic response to compound 7, we prepared RNA samples from two independent replicates of both untreated and compound-7-treated *P. aeruginosa* cultures grown for 24 h. Illumina-based RNA-seq was performed. Transcript reads were subsequently mapped to the *P. aeruginosa* PAO1 genome, and the expression level for each gene was determined. Each independent treatment replicate showed extremely high correlation to its respective counterpart (untreated R^2 = 0.9861; compound-7-treated R^2 = 0.9960, Supporting Information Figure 1), which indicated high reproducibility. The fold change for each gene was plotted against its chromosomal location (Figure 1a), enabling the identification of differentially regulated operons. Of the ~5570 genes contained on the PAO1 chromosome, 181 (153 up-regulated, 28 down-regulated) were differentially regulated by 2-fold or greater ($p < 0.05$), in comparison to the untreated control (Figure 1a, Supporting Information Table 1). Interestingly, of the down-regulated genes, 17 (61%) have been reported as QS-controlled (Supporting Information Table 1).[28−30] This is a significant over-representation of QS-controlled genes compared to what would be expected in a randomized set of genes (cumulative binomial distribution, p = 1.4e^{-10}, using the QS regulon reported by Schuster *et al.*[28]). Affected genes were grouped according to their annotated PseudoCAP (*Pseudomonas* community annotation project) function[31,32] and plotted relative to their PseudoCAP class (Figure 1b). The most frequently up-regulated classes were those transcripts involved in expression of secreted factors (16%), adaptation/protection (12%), and transport of small molecules (8%; Figure 1b). Secreted factors were also the most abundant class of down-regulated genes (11%; Figure 1b).

Compound 7 Inhibits Pyocyanin Production. *P. aeruginosa* uses diverse secreted factors to establish infection and to increase pathogenicity.[33−35] We focused on the effects of compound 7 on different virulence systems.[36] Phenazine biosynthesis genes, which ultimately lead to the production of the secreted redox-active toxin pyocyanin, were the most highly down-regulated virulence traits (Figure 2, green circles) and

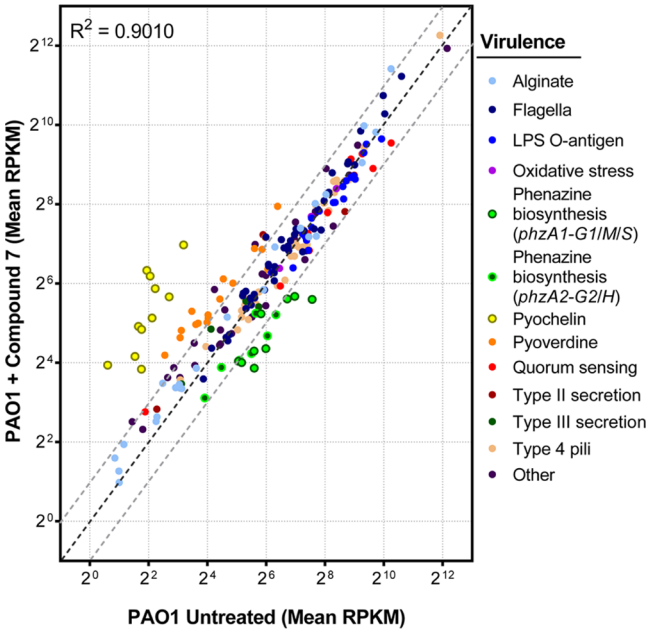

Figure 2. (a) Expression levels of *P. aeruginosa* genes that are known to be virulence-related[36] were plotted for comparison of untreated and compound-7-treated samples. The relative expression level is denoted by the mean normalized reads per kilobase of gene model per million mapped reads (RPKM) values[63] of the duplicate samples. Individual genes are represented by single points and are color-coded to represent their virulence function. The gray dashed lines represent a 2-fold change. Genes above the upper gray dashed line are up-regulated ≥2-fold when exposed to compound 7. Genes below the lower line indicate ≥2-fold reduction when exposed to compound 7.

among the most highly down-regulated transcripts overall. The expression of these operons is regulated by the *las*, *rhl*, and *pqs* QS systems.[28−30] Inhibitors of pyocyanin production are of significant interest as potential novel anti-infective agents.[12,37] We quantified the relative pyocyanin concentration in supernatants of *P. aeruginosa* compared to levels achieved in compound-7-treated cultures. Although compound 7 does not have a considerable effect on *P. aeruginosa* growth rate (Figure 3a), the relative pyocyanin concentration was significantly reduced in compound-7-treated cultures (unpaired t test, $p < 0.0001$) when compared to that observed in the untreated control (Figure 3b), consistent with the transcriptomic data.

Compound 7 Induces Expression of Iron Acquisition Systems. Genes that code for the biosynthesis of iron siderophores pyochelin and pyoverdine were among the most highly up-regulated virulence factors, indicating an iron starvation response (Figure 2, yellow circles (pyochelin) and orange circles (pyoverdine)). In fact, 38 (25%) of the observed up-regulated genes were previously identified to be induced under iron-limiting conditions (Supporting Information Table 1).[38] One protein involved in pyoverdine biosynthesis, PvdQ, has also been shown to function as a quorum quenching enzyme, as it has the ability to cleave 3OC$_{12}$−HSL, and subsequently inhibit *las*-based QS as well as the production of related virulence factors.[14] The encoding gene, *pvdQ*, was up-

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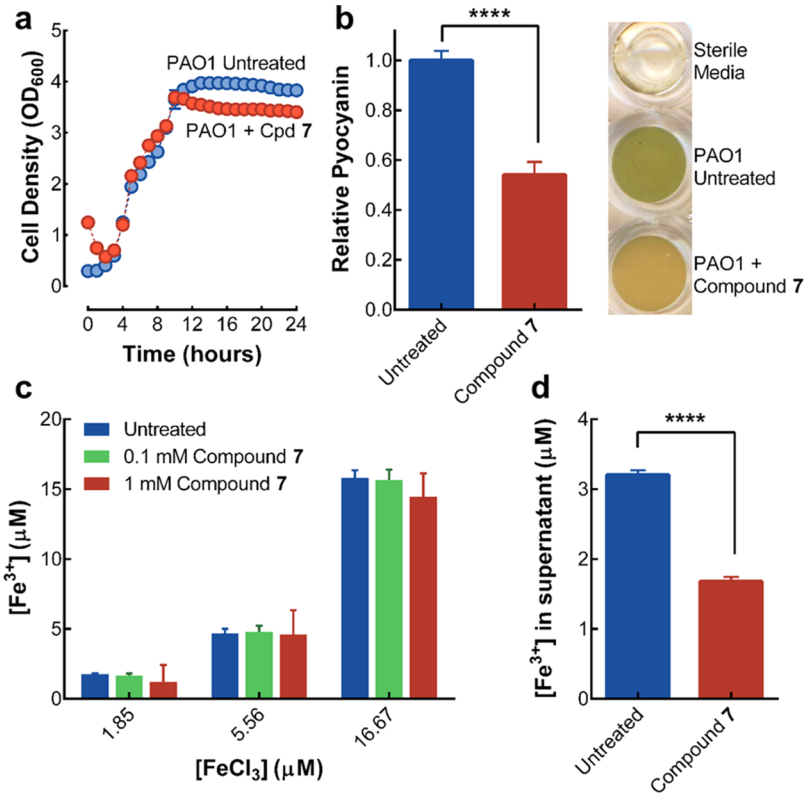

Figure 3. (a) Growth curves of *P. aeruginosa* when untreated and treated with compound 7. The initial higher absorbance and subsequent decrease in absorbance is due to the addition of compound 7, which takes approximately 2 h to completely dissolve at 37 °C. (b) The relative quantification of pyocyanin in the supernatants of untreated and compound-7-treated *P. aeruginosa* cultures (****, $p < 0.0001$). Image shows visual appearance of cultures grown on 96-well plate. Blue-green pigmentation is characteristic of pyocyanin production.[42] (c) Known concentrations of iron chloride were mixed with 0.1 mM compound 7 or 1 mM compound 7 or were untreated, and available iron concentration was measured. (d) Cell-free supernatants of untreated *P. aeruginosa* and compound-7-treated samples were measured to determine the concentration of available iron (****, $p < 0.0001$). Error bars represent the standard deviation of at least three replicates.

regulated in compound 7 treated cells (Supporting Information Table 1). We postulated either that compound 7 has iron chelating ability or that *P. aeruginosa* treated with compound 7 actively recruits more iron for intracellular processes. Using a colorimetric assay with the iron indicator 1,10-phenanthroline, which forms a deep red-colored complex with free iron, we found that compound 7 does not appear to chelate iron (Figure 3c). However, in a comparison of the free iron concentration in 24 h cell-free spent supernatants of compound-7-treated vs untreated *P. aeruginosa* cultures, the former contained significantly less iron than the latter (unpaired *t* test, $p < 0.0001$; Figure 3d). This result indicated that compound-7-treated cells exhibited increased iron uptake, which agreed well with the gene expression data that demonstrated an increase in iron siderophore biosynthesis. We next sought to investigate the cause of the increased demand for intracellular iron.

Expression of Genes Encoding Enzymes Involved in the Secondary Metabolism of Aromatic Metabolites Is Significantly Affected by Compound 7. Both pyochelin and phenazines are synthesized from a common precursor, chorismate, by way of the enzymes coded by the *pch* and *phz* genes, respectively[39] (Figure 4a). Chorismate can also be converted to anthranilate by TrpEG or PhnAB[40] or from tryptophan, via kynurenine and the *kyn* enzymes.[20,21] Anthranilate can then be further processed to catechol via the *ant* enzymes or used by the *pqs* enzymes for the biosynthesis of 2-heptyl-4-quinolone (HHQ) and ultimately PQS (Figure 4a). Our RNA-seq analysis indicated overexpression of the *ant* operon in response to compound 7 (Supporting Information Table 1). Interestingly, two of the enzymes encoded by this operon, AntA and AntC, require iron as cofactors,[31,41] which could potentially contribute to the iron stress response. Because the biosynthetic pathways involving chorismate and anthranilate have well-established effects on iron starvation, iron scavenging, phenazine synthesis, and QS, we examined these pathways and their branches.[39,42−45]

Compound 7 Is Structurally Similar to Both Kynurenine and a Known Kynureninase Inhibitor. The kynurenine pathway is critical for PQS and virulence factor production.[20] In this pathway, tryptophan is converted to kynurenine via kynurenine formamidase (KynB), which in turn is converted to anthranilate via kynureninase (KynU; Figure 4a). When examining this pathway, we found that compound 7 has structural similarity to kynurenine as well as a previously reported kynureninase inhibitor, *S*-phenyl-L-cysteine *S*,*S*-dioxide (Figure 4b).[23] To quantify the similarity among these compounds, we determined the Tanimoto coefficients for each pair of compounds. The Tanimoto coefficient represents a ratio of the number of similar chemical features in both molecules to the total number of chemical features in either. The numerical range of this value is between 0 and 1, with a value of 1 representing identical structures. Compound 7 shares Tanimoto coefficients of 0.66 and 0.52 with *S*-phenyl-L-cysteine *S*,*S*-dioxide and kynurenine, respectively. *S*-phenyl-L-cysteine *S*,*S*-dioxide and kynurenine share a Tanimoto coefficient of 0.49. Because compound 7 is more similar to kynurenine than *S*-

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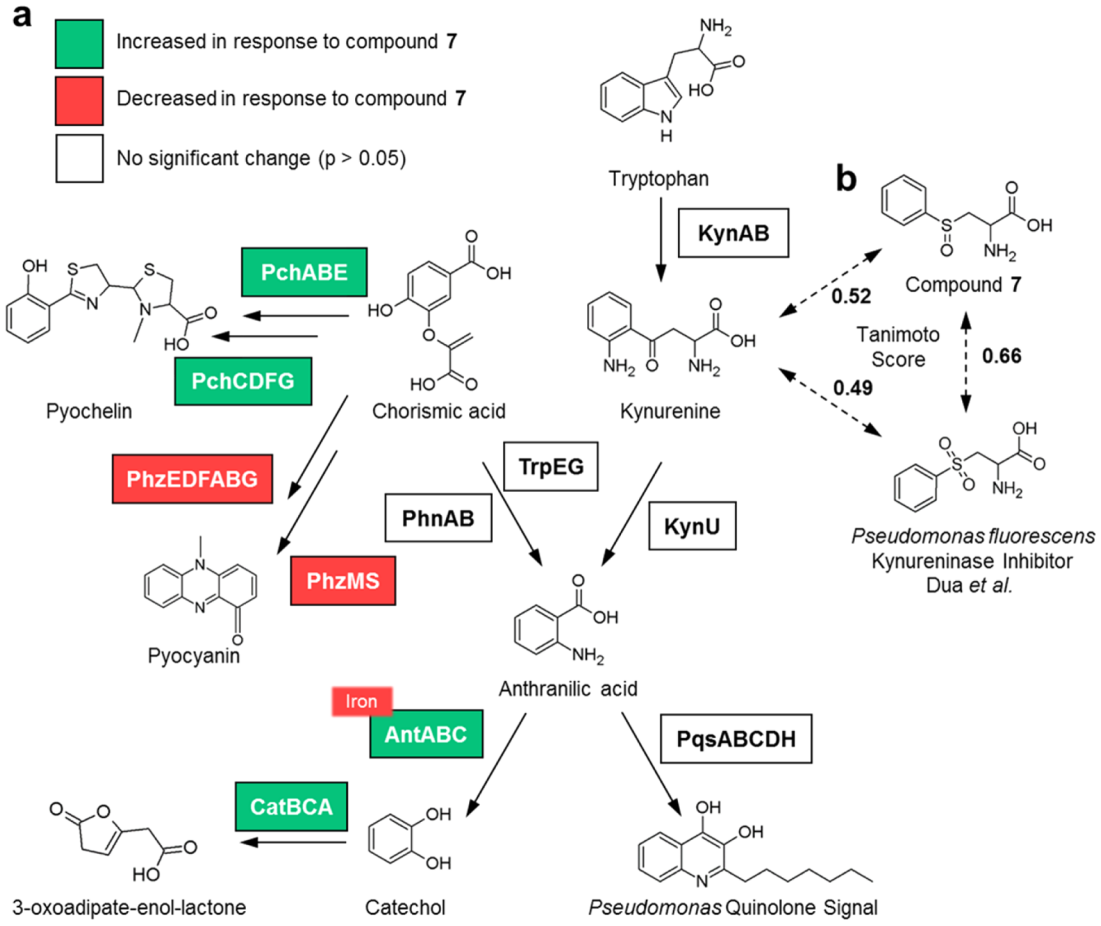

Figure 4. (a) The effect of compound 7 on *P. aeruginosa* metabolic pathways that yield pyocyanin, pyochelin, PQS, and tricarboxylic acid cycle intermediates from chorismate and anthranilate. Proteins whose transcripts were upregulated are represented in green, and those of which were repressed are represented in red. (b) Similarity comparisons of kynurenine, compound 7, and *S*-phenyl-L-cysteine *S*,*S*-dioxide. Higher Tanimoto scores indicate a higher degree of similarity.

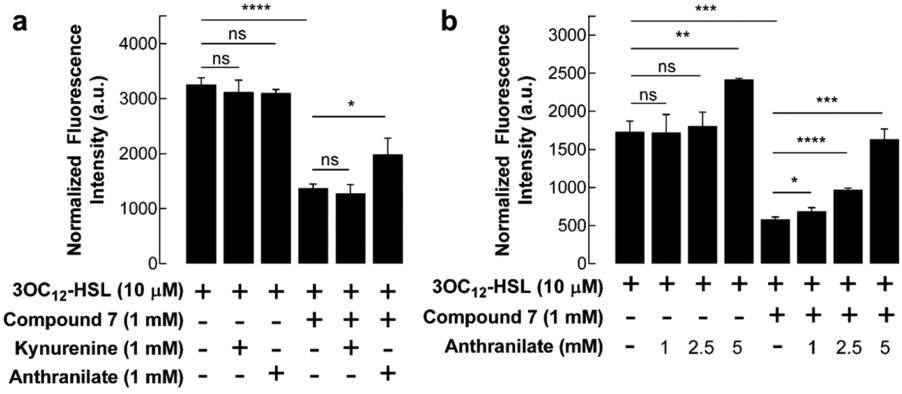

Figure 5. (a,b) Fluorescence normalized to cell growth (OD_{600}) of the *P. aeruginosa* MW1 pUM15 reporter strain containing the *rsaL* promoter fused to *yfp* after 8 h. (a) Cultures were supplemented with different combinations of 10 μM $3OC_{12}$–HSL, 1 mM kynurenine (KYN), or 1 mM anthranilate (ANT), with and without 1 mM compound 7. (b) Dose–response of MW1 pUM15 reporter strain to increasing concentrations of ANT. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns = not significant. Error bars represent the standard deviation of at least three replicates.

phenyl-L-cysteine *S*,*S*-dioxide, and kynureninases from both species show considerable homology (Supporting Information Figure 2), we hypothesized that compound 7 may be functioning as a kynurenine analogue, leading to the observed expression changes in these metabolic pathways. Previously, Calfee *et al.* demonstrated that methyl anthranilate, a congener of anthranilate, could attenuate PQS production and related virulence,[17] leading to the hypothesis that an analogue of a precursor of anthranilate (e.g., kynurenine) could produce similar effects.

Exogenous Anthranilate Reverses the QS-Inhibitory Effect of Compound 7. In previous work, we identified

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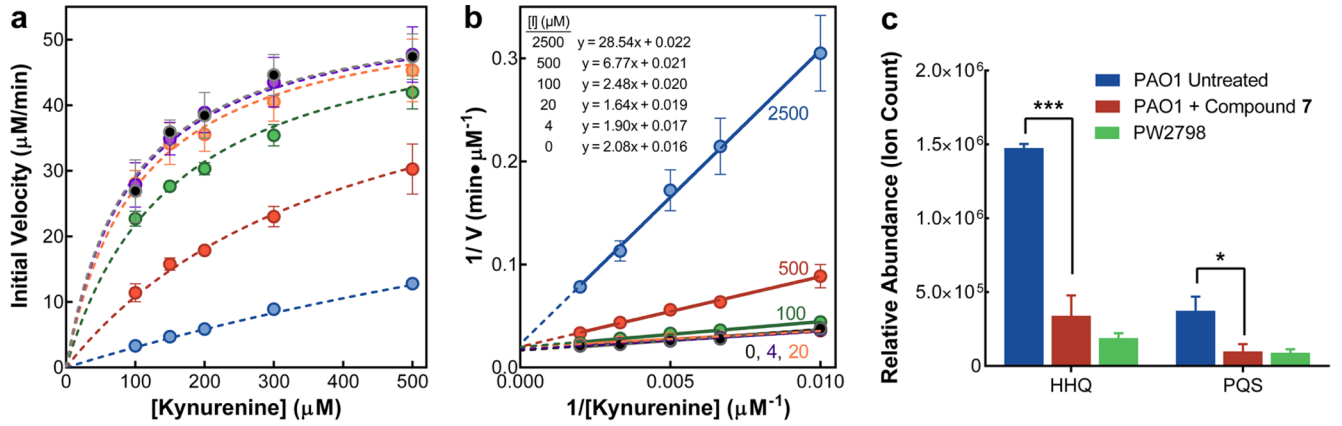

Figure 6. (a) Michaelis–Menten and (b) Lineweaver–Burk plots of rKynU kinetics with increasing concentrations of compound 7. Black circles, 0 μM compound 7; purple circles, 4 μM; orange circles, 20 μM; green circles, 100 μM; red circles, 500 μM; blue circles, 2500 μM. (a) Dashed lines represent best fit curves for a competitive inhibition model. (b) Solid lines represent the line of best fit for each set of data points, and dashed lines represent a traced extrapolation of best fit lines to visualize the point of intersection. (c) Differences in ion counts for masses representing HHQ and PQS determined by DART-HRMS in positive ion mode. The data represent the average of three replicates of the actual DART-HRMS derived ion counts at nominal m/z 244 and 260 representing M + H^+ for HHQ and PQS, respectively. The ion counts reflect actual amounts of the observed ions and illustrate the relative production of AHQs. *P. aeruginosa* PW2798 is a transposon mutant strain incapable of producing AHQs. *$p < 0.05$; ***$p < 0.001$. Error bars represent the standard deviation of three replicates.

compound 7 as a QS inhibitor using the *P. aeruginosa* MW1 pUM15 reporter strain.[26,46] The MW1 strain is a *lasI*, *rhlI* double insertional mutant, deficient in synthesis of $3OC_{12}$–HSL and C_4–HSL, respectively.[47] The pUM15 plasmid contains a yellow fluorescent protein (*yfp*) reporter gene driven by the *rsaL* promoter, which is induced when bound by LasR in response to the autoinducer $3OC_{12}$–HSL. It has previously been shown that the transcriptional regulator MvfR, the cognate receptor to PQS, can regulate a number of LasR- and RhlR-controlled genes, including *rsaL*[30] whose promoter drives *yfp* in pUM15 (as described above). Thus, changes in PQS concentration could directly affect *yfp* expression in this reporter strain. Because MW1 pUM15 can still synthesize and respond to PQS, we hypothesized that the inhibitory effects observed in this strain were actually a result of inhibition of the PQS pathway via anthranilate depletion and might therefore be reversed upon anthranilate supplementation. We examined the expression of *yfp* in MW1 pUM15 over 24 h when supplemented with 1 mM kynurenine (KYN) or anthranilate (ANT) in addition to 10 μM $3OC_{12}$–HSL, in the presence or absence of 1 mM compound 7 (Figure 5a). When compound 7 and $3OC_{12}$–HSL are added to this reporter, *yfp* expression is significantly lower than when $3OC_{12}$–HSL is added alone ($p < 0.0001$; Figure 5a). The addition of KYN or ANT to $3OC_{12}$–HSL did not exhibit significant activity toward *yfp* expression when compound 7 was not present (Figure 5a). When compound 7 was present, the addition of KYN had no effect (Figure 5a). However, upon the addition of ANT in the presence of $3OC_{12}$–HSL and compound 7, there was some restoration of *yfp* expression (unpaired t test, $p < 0.05$; Figure 5a).

This restoration of *yfp* expression was further demonstrated by varying the concentration of ANT (Figure 5b). Increasing the concentration of ANT in the presence of $3OC_{12}$–HSL and compound 7 displayed a dose-dependent response and *yfp* expression was completely recovered upon the addition of 5 mM ANT (Figure 5b). The addition of 2.5 mM ANT to $3OC_{12}$–HSL (without inhibitor) had no effect on *yfp* expression. The addition of 5 mM ANT to $3OC_{12}$–HSL did result in increased *yfp* expression. However, this occurred to a much lower extent than was observed when the inhibitor was present (Figure 5b). These data indicate that compound 7 inhibits the QS response in reporter MW1 pUM15 and that this inhibition can be reversed upon addition of anthranilate, the downstream product of kynurenine and upstream precursor of PQS.

Compound 7 Is a Competitive Inhibitor of *P. aeruginosa* Kynureninase. Because of the structural similarity of compound 7 with kynurenine, as well as its structural similarity to the previously reported KynU inhibitor *S*-phenyl-L-cysteine *S*,*S*-dioxide (Figure 4b),[23] we sought to investigate if compound 7 could inhibit *P. aeruginosa* KynU. Therefore, we cloned, expressed, and purified recombinant, His-tagged *P. aeruginosa* KynU (rKynU). The KynU-mediated conversion of kynurenine to anthranilate can be monitored by UV–vis absorbance measurements, as kynurenine has a strong absorbance near 350 nm (Supporting Information Figure 3a).[23] Compound 7 alone contributed little to the UV absorbance profile (Supporting Information Figure 3a).

To test the inhibitory effect of compound 7 on kynureninase, we performed several enzyme kinetics experiments to characterize the enzyme inhibition model and potentially define an inhibition constant (K_i). The initial velocity of the kynureninase reaction was followed by monitoring the initial decrease in the 350 nm absorbance with increasing concentrations of compound 7 (Figure 6a). The best-fit inhibition model for the resulting data is competitive inhibition, yielding V_{max}, K_m, and K_i values of 55.93 (±1.31) μM/min, 91.32 (±7.35) μM, and 140.4 (±11.54) μM, respectively (Figure 6a). The hypothesis of competitive inhibition was further supported by the results of a Lineweaver–Burk plot of the reciprocal values and observing the intersection of best-fit lines at the y axis (Figure 6b). The calculated y intercepts range from 0.016 (±0.002) to 0.022 (±0.011) min·μM^{-1} (Figure 6b), agreeing with the calculated inverse V_{max} of 0.018 (±0.0004) min·μM^{-1}. To investigate whether compound 7 could permanently inactivate the enzyme, we performed several experiments of pre-exposing the enzyme to molar excess concentrations of

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compound 7 before introducing kynurenine. Even after 90 min of pre-exposure, and a subsequent 6 h of monitoring the reaction, there was still observable enzyme activity, illustrating that compound 7 does not inactivate rKynU (Supporting Information Figure 4).

Production of HHQ and PQS Is Inhibited when *P. aeruginosa* Is Exposed to Compound 7. The aforementioned observations suggested that production of alkyl quinolone (AHQ) signaling molecules (e.g., HHQ and PQS) was attenuated when *P. aeruginosa* was exposed to compound 7. Therefore, we investigated the possible differential production of these signaling molecules in PAO1 vs compound-7-treated PAO1 cultures using high resolution direct analysis in real time mass spectrometry (DART-HRMS). In these experiments, the actual relative amounts of HHQ and PQS for each of the cultures was determined by ambient ionization mass spectrometry. The results, which are presented in Figure 6c, show that compared with the untreated PAO1 control, the level of HHQ and PQS production fell by 73.4% and 76.9%, respectively, for compound-7-treated samples. Thus, when compound 7 was present, *P. aeruginosa* produced significantly less PQS (unpaired *t* test, $p < 0.05$) and HHQ (unpaired *t* test, $p < 0.001$) than when untreated. As expected, the extract from *P. aeruginosa* strain PW2798 (which has a transposon inserted in the *pqsA* coding region and is therefore deficient in HHQ and PQS production[48]) showed the lowest AHQ levels, with the observed ion count reflecting background. In a comparison of the entire DART-HRMS profile of the organic culture extracts, the compound-7-treated samples were observed to be more similar to those of strain PW2798, than to those of the untreated PAO1 control (Supporting Information Figure 5a). Furthermore, the visual appearance of compound-7-treated cultures more closely resembled that of PW2798 as opposed to the untreated wild-type culture (Supporting Information Figure 5b).

Since it has been observed that AHQs can inhibit growth of Gram positive bacteria,[49] we examined the effect of culture supernatants on *Staphylococcus epidermidis* growth. When *P. aeruginosa* supernatants were applied to an agar plate with *S. epidermidis* spread throughout, there is a noticeable reduction in the zone of inhibition for the compound-7-treated supernatant (Supporting Information Figure 5c). This indicates that, in addition to our previously reported data on the ability of compound 7 to reduce *P. aeruginosa* infection in a *Drosophila* model,[26] compound 7 also diminishes the Staphylococcal killing ability of *P. aeruginosa*.

DISCUSSION

Originally identified from a library of compounds that are substrates of the *P. alliacea* alliinase, or the products of *P. alliacea* alliinase mediated S-substituted cysteine sulfoxide breakdown, *S*-phenyl-L-cysteine sulfoxide (compound 7) adds to the growing list of natural-product-inspired QS inhibitors, which includes compounds such as the halogenated furanones and protoanemonin (comparisons shown in Supporting Information Table 1).[50,51] We also showed previously that compound 7 affects biofilm architecture and biofilm formation,[26] similar to a previously reported halogenated furanone derivative.[52] There are also interesting parallels between the gene expression measured in compound-7-exposed cultures and expression levels previously reported for protoanemonin exposed cultures (Supporting Information Table 1).[51] Both small molecules induced a number of iron starvation genes.[51] There were also four hypothetical proteins up-regulated by both compound 7 and protoanemonin that appear to be independent of iron availability (PA2486, PA2759, PA4623, and PA4881, Supporting Information Table 1). These genes are preceded by the consensus motif ATCA-N5-GTCGAT-N4-ACYAT, which serves as a binding site for the transcriptional activator MexT, a LysR-like regulator.[53] Compound 7 up-regulated the *mexEF-oprN* operon, another member of the MexT regulon, which encodes a multidrug efflux pump and an associated outer membrane porin. Multidrug efflux pumps can have a broad range of substrates including AI-precursors and metabolic byproducts, which have the potential to affect QS.[54−56] Although the molecular target of protoanemonin in *P. aeruginosa* is unknown, it has been suggested that the QS-inhibitory effect may be a result of fluctuations in iron homeostasis and carbon compound catabolism (i.e., anthranilate and catechol), and the resulting effect on the levels of one of the *Pseudomonas aeruginosa* QS autoinducers, PQS.[51] Thus, compound 7 and protoanemonin may function through the same pathway to inhibit QS.

Overexpression of *mexEF-oprN* has been shown to reduce cell−cell signaling and related virulence, potentially by extruding AI precursors, including kynurenine, and subsequently affecting intracellular AI concentration.[54,55,57] Therefore, compound 7 induced expression of the MexT regulon may further affect cellular processing of kynurenine, beyond direct KynU inhibition, and may also contribute to the observed QS-inhibitory effects. Furthermore, Olivares *et al.* showed that MexEF-OprN can extrude kynurenine, thereby decreasing PQS production and the expression of multiple QS-regulated genes.[55] These reductions were then shown to be reversed upon the addition of exogenous anthranilate, similar to what we observed in this study.

In the present study, RNA-seq based expression analysis showed that exposure to compound 7 caused changes to gene expression in metabolic pathways that yield aromatic precursors of pyochelin, tricarboxylic acid cycle intermediates, phenazines, and PQS. One of the central compounds in these pathways is the small molecule anthranilate. Interestingly, we found that compound 7 has significant structural similarities to kynurenine, a precursor of anthranilate. Compound 7 also displays structural similarity to previously reported inhibitors of *P. fluorescens* KynU,[23] the enzyme responsible for the conversion of kynurenine to anthranilate (Figure 4b). Because of these observations, we tested the inhibitory effect of compound 7 toward recombinant *P. aeruginosa* KynU and, as a result, found that compound 7 competitively inhibits rKynU activity.

The KynU enzyme is a dimer of identical subunits whose chemistry has been of great interest for nearly a century.[22,58,59] It is also highly conserved across multiple kingdoms and has been implicated in a number of diseases of the central nervous system.[60] We showed that compound 7 has a K_i value of 140.4 μM against *P. aeruginosa* rKynU, which is of the same order of magnitude as was reported for *S*-phenyl-L-cysteine (700 μM) and an order of magnitude higher than *S*-phenyl-L-cysteine *S*,*S*-dioxide (3.9 μM), both reported by Dua *et al.*[23] These compounds are structurally related to compound 7, in that compound 7 is the sulfoxide form of *S*-phenyl-L-cysteine, and *S*-phenyl-L-cysteine *S*,*S*-dioxide is the sulfone form. Future studies will investigate the effect of these various inhibitors on *P. aeruginosa* virulence. However, it has been shown that more potent QS inhibitors do not necessarily translate to greater virulence inhibition in *P. aeruginosa*.[12]

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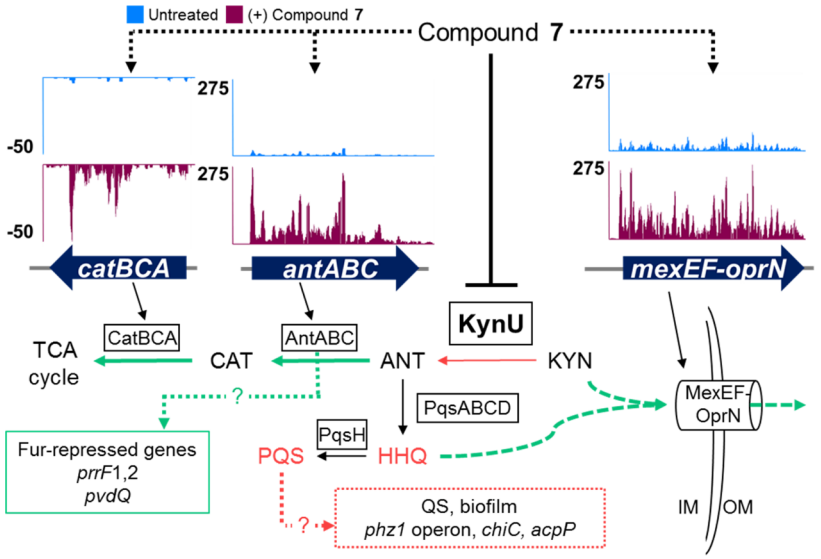

Figure 7. A proposed model of the intracellular response of *P. aeruginosa* to compound 7. Compound 7 competitively inhibits KynU and induces expression of the *antABC*, *catBCA*, and *mexEF-oprN* operons (this study). Up-regulation of the *ant* operon has been shown to induce expression of Fur-repressed genes, in order to increase intracellular iron.[41] The expression of sRNAs *prrF1* and *prrF2* are also Fur-regulated, and they play a role in destabilizing *antA* and *antR* transcripts to balance *antA* expression under varying iron conditions.[41] They were observed to be up-regulated in this study, along with the *ant* operon, illustrating high pressure on induction of the *ant* operon. *pvdQ* is also Fur-repressed and has potential to affect *P. aeruginosa* QS, as it has $3OC_{12}$–HSL acylase activity. Overexpression of *mexEF-oprN* has been linked with extrusion of the PQS precursors kynurenine and HHQ and subsequently affect QS.[54,55] We hypothesize that a combination of these events contributes to the observed QS inhibition and repression of QS-regulated genes (e.g., *phz* genes, *chiC*, *acpP*) in this study, as well as biofilm inhibition observed in our previous report.[26] The observed up-regulation of these operons is shown by their RNA-seq reads (blue, untreated; purple, compound-7-treated). Enzymes involved in these pathways are boxed by rectangles. TCA = tricarboxylic acid; CAT = catechol; ANT = anthranilate; KYN = kynurenine; HHQ = 4-hydroxy-2-heptylquinolone; PQS = *Pseudomonas* quinolone signal; IM = inner membrane; OM = outer membrane.

Although inhibitors of KynU expressed in *P. fluorescens* have been reported,[22−25] to the best of our knowledge this is the first report on the effects of small-molecule based KynU inhibition *in vivo*, and more specifically, for the pathogen *P. aeruginosa*. Using gene knock outs, it was previously demonstrated that *P. aeruginosa* uses the *kyn* pathway to supply PQS precursors and that mutants defective in this pathway (deletion mutants of *kynA*, *kynB*, and *kynU*) have reduced ability to kill *S. aureus*,[20] which demonstrates the importance of *P. aeruginosa* leveraging this pathway for competition with other organisms. Indeed, we found that treatment of *P. aeruginosa* with compound 7 reduces its ability to kill *S. epidermidis* (Supporting Information Figure 5c) which may be linked to attenuated expression of virulence factors in cultures treated with compound 7, particularly the decreased production of the redox-active toxin pyocyanin (Figure 3b) and AHQs (Figure 6c). These observations strongly support the possibility that KynU inhibitors have anti-infective potential against *P. aeruginosa* infections. Additional evidence is provided by the observation that methyl anthranilate, an analogue of the KynU product anthranilate, interferes with PQS synthesis and expression of related virulence factors.[17]

Based on our observations of how compound 7 affects *P. aeruginosa* gene expression, and more directly KynU activity, we propose a potential model for its mechanism of action (Figure 7). From our study, we found that compound 7 directly inhibits KynU activity while also inducing overexpression of the *cat*, *ant*, and *mexEF-oprN* operons. We propose that inhibition of KynU and the increase in *antABC* expression leads to a depletion of the available anthranilate pool for synthesis of PQS, a QS autoinducer. An increase in *antABC* expression and subsequent increase in AntABC concentration further triggers a need for iron, inducing an iron starvation response. This is due to the strict iron requirements for proper function of both AntA and AntC.[31,41] Interestingly, part of the iron starvation response is the increased expression of the PvdQ enzyme, which plays a role in pyoverdine synthesis and acts as an acylase to degrade the QS autoinducer $3OC_{12}$–HSL.[14] This would provide another potential mechanism for QS inhibition, beyond interference with PQS-based signaling. To further contribute to the depletion of PQS precursors, *mexEF-oprN* transcripts are also up-regulated. This system has previously been shown to extrude kynurenine and 4-hydroxy-2-heptylquinolone (HHQ), the direct precursor of the PQS signaling molecule, and reduce virulence factor expression.[54,55] All of these events would suggest a decrease in production of PQS and HHQ, which is consistent with what we observed in this study.

In summary, we used RNA-seq to investigate the mechanism of action of a natural products-inspired *P. aeruginosa* QS inhibitor, compound 7. Exposure to this compound resulted in significant fluctuations in the expression of 181 genes, a subset of which are involved in QS, virulence, and various secondary metabolism pathways. Because of the structural similarity of compound 7 to kynurenine, the reversal of its QS-inhibitory effects on the addition of the kynurenine derivative anthranilate, and its structural similarity to a previously reported KynU inhibitor, we studied the interaction of compound 7 with *P. aeruginosa* KynU. The results showed that compound 7 competitively inhibits KynU activity. Furthermore, we demonstrated that compound 7 can attenuate the production of PQS and HHQ by *P. aeruginosa*, as well as reduce its Staphylococcal killing ability. These findings provide a foundation upon which structure/activity relationship studies

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Table 1. Bacterial Strains and Plasmids Used in This Study

strains	description	source or reference
P. aeruginosa		
PAO1	wild-type	ref 62
MW1	PAO1 *rhlI*::Tn*501 lasI*::*tetA*	ref 47
PW2798	*pqsA*-H05::ISlacZ/hah	ref 48
E. coli		
OneShot Top10	F-*mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lacX*74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G; host for high copy plasmid replication	Invitrogen
BL21	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEco*RI-*B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*; T7 expression strain	New England Biolabs
S. epidermidis ATCC 14990	wild-type	ATCC
plasmids		
pUM15	*rsaL*::*yfp* transcriptional fusion	ref 46
pEXP5-NT/TOPO	T7-based expression vector, N-terminal 6xHis, Amp^R, TOPO cloning site	Invitrogen
pEXP5-NT/*kynU*	pEXP5-NT/TOPO with PAO1 *kynU* insertion	this study

of compound 7 derivatives with KynU can be conducted. This is the subject of further investigations in our laboratories.

METHODS

Bacterial Strains and Culture Conditions. Bacterial strains used in this study are listed in Table 1. *P. aeruginosa* PAO1 was propagated on tryptic soy agar (TSA) and cultured in tryptic soy broth (TSB). *P. aeruginosa* MW1 pUM15 was propagated on LB agar and cultured in LB broth with 150 μg mL^{-1} carbenicillin supplementation. *E. coli* strains harboring the pEXP5-NT/TOPO vectors were propagated on LB agar and cultured in LB broth containing 100 μg mL^{-1} ampicillin. All strains were grown at 37 °C.

***S*-phenyl-L-cysteine Sulfoxide (Compound 7) Synthesis and Small Molecule Structural Analysis.** *S*-phenyl-L-cysteine was purchased from Sigma-Aldrich and was subjected to hydrogen peroxide-mediated oxidation to yield *S*-phenyl-L-cysteine sulfoxide, as we have previously described.[26,27] Structural relationships among kynurenine and kynureninase inhibitors were analyzed by Tanimoto similarity coefficient using the ChemMine Tools service.[61]

RNA Harvesting, RNA-seq Library Preparation, and Sequencing. Two biological replicates of *P. aeruginosa* PAO1 were grown in 5 mL of TSB to the stationary phase (24 h) at 37 °C with shaking at 200 rpm in the presence of 1 mM compound 7 (in DMSO, 1% v/v final concentration) or DMSO alone (no compound control). Total RNA was harvested using the RNeasy kit (Qiagen). An aliquot of 0.5 mL was removed and added to 1 mL of RNAprotect Bacteria Reagent (Qiagen). Cells were pelleted and resuspended in 200 μL of TE buffer (Ambion, Life Technologies) containing 15 mg mL^{-1} lysozyme (EMD Millipore) and 6 mAU Proteinase K (Qiagen). Samples were left shaking at RT for 10 min before continuing the rest of the extraction in line with the manufacturer's protocol. The sample was resuspended in RNase-free water. RNA-seq libraries were generated by first removing rRNA using the RiboZero kit (Epicenter) followed by preparation of strand-specific cDNA libraries for Illumina sequencing using the ScriptSeq 2.0 kit (Epicenter). Libraries were sequenced using a HiSeq 2000 sequencer (Illumina; University at Buffalo Next Generation Sequencing Core Facility).

Read Mapping, Visualization, and Sequencing Analysis. Data were analyzed using the Partek Genomics Suite 6.6. Sequencing reads were mapped to the *P. aeruginosa* PAO1 reference genome annotation.[31,62] The number of overlapping reads per ORF were normalized as reads per kilobase of gene per million mapped reads (RPKM) as described by Mortazavi *et al.*[63] The average RPKM of the treated samples was compared to the average RPKM of the control to generate a fold change value. Gene expression quantification and statistical analysis were performed using the web-based genome analysis platform Galaxy.[64−66] Sequences were aligned to the PAO1 genome (NC_002516.2) using CLC Workbench, converted to the gff file format using in house Python scripts, and visualized with SignalMap (Nimblegen). The RNA-seq images displayed are exported screenshots from SignalMap.

Pyocyanin Assay. Pyocyanin quantification was performed as described be Lee *et al.*[67] *P. aeruginosa* was grown at 37 °C in TSB with and without compound 7 as described above. After 24 h, cells were centrifuged for 3 min at 10 000*g*, and the supernatant was removed and filtered through a 0.2 μm-pore filter. A total of 1 mL of the filtered supernatant was added to 0.6 mL of chloroform and shaken vigorously. The organic phase was extracted and added to 0.2 mL of 0.2 N HCl and shaken vigorously again. The aqueous phase was then removed, and the absorbance was measured at 520 nm. This was normalized against cell density (absorbance at 600 nm of original culture).

Iron Assay. Free iron concentration was measured using a phenanthroline-based iron test kit (0−1 mg mL^{-1}, Hach Company) with a modified protocol for microplate-based spectrometry. Absorbance of varying concentrations of $FeCl_3$ and indicator, with and without compound 7, were measured at 500 nm (peak absorbance of the Fe-phenanthroline complex) and 650 nm (background). The background was subtracted, and all samples were normalized to the standard curve generated from the control with no solvent ($FeCl_3$ and phenanthroline, only).

Quorum Sensing Reporter Assay. Quorum sensing reporter assays were carried out as previously described using *P. aeruginosa* MW1 harboring the pUM15 reporter plasmid.[26,46] The pUM15 plasmid has a *yfp* gene downstream of the *rsal* promoter. The reporter was grown in LB with carbenicillin overnight (~18 h), pelleted, washed three times with phosphate buffered saline, and diluted 100× into fresh TSB. The final concentration of 3OC_{12}−HSL (Cayman Chemicals, Ann Arbor, MI) was brought to 10 μM. The concentration of compound 7 used was 1 mM. The concentration of anthranilate (Sigma-Aldrich) or kynurenine (Sigma) used ranged from 1 mM to 5 mM as indicated. Fluorescence (480 nm excitation/520 nm emission) and cell growth (absorbance at 600 nm) were monitored over time at 37 °C without shaking in a Tecan Infinite M200 microplate reader. Fluorescence intensity values were divided by the A_{600} value at the corresponding time point to obtain a normalized fluorescence value.

Recombinant Kynureninase Cloning, Expression, and Purification. An N-terminal His_6-tagged KynU expression vector was constructed by one-step TOPO cloning using the pEXP5-NT/TOPO vector (Invitrogen) according to the manufacturers protocol. Briefly, the *kynU* coding sequence from PAO1 genomic DNA was *Taq* polymerase-based PCR amplified using the primers 5′-ATGACC-ACTCGTGACGACTG-3′ and 5′-TCAGGTCACCTTGTGCCG-3′ to yield a PCR product of the *kynU* gene with single overhanging deoxyadenosines at the 3′ ends. This was mixed with the pEXP5-NT/TOPO vector to yield pEXP5-NT/*kynU*, which was subsequently transformed into *E. coli* OneShot Top10 and plated on LB-amp agar. Plasmid DNA from viable colonies was isolated using a QIAprep Spin Miniprep kit (Qiagen), and confirmation of full-length *kynU* insertion

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was performed by PCR amplification with the above primers and agarose gel purification. Plasmids with correct length insertions were then sent for sequencing (GENEWIZ, Inc.) to confirm orientation of the *kynU* insertion. Orientation-confirmed pEXP5-NT/*kynU* was then transformed into *E. coli* BL21 (DE3; New England Biolabs) and propagated on LB-amp agar.

A single colony of *E. coli* BL21 (DE3) harboring pEXP5-NT/*kynU* was used to inoculate 5 mL of LB-amp and grown overnight (~18 h). This starter culture was then used to inoculate 500 mL LB, which was shaken at 200 rpm at 37 °C for 3 h. The culture was induced with 200 μM IPTG and allowed an additional 4 h of shaking and incubation (200 rpm, 37 °C). Cultures were then centrifuged for 10 min at 10 000*g* at 4 °C, and pellets were stored overnight at −80 °C.

Pellets were resuspended in 20 mM Tris-HCl at a pH of 8 containing 10 mg mL^{-1} lysozyme, and sonicated on ice with a Branson Sonifier Model 450 at 30% power, in pulse mode (10 s on, 10 s off) for a total sonication time of 5 min. DNase I (New England Biolabs) was added and samples were centrifuged for 10 min at 10 000*g* at 4 °C. Because incomplete lysis was observed, the supernatant was discarded, pellets were resuspended in 10 mL of a solution comprised of 20 mM Tris-HCl at a pH of 8, 0.5 mM NaCl, and 2% Triton-X (w/v) and resonicated under the conditions mentioned above. An equal volume of 1 M NaCl with 40 mM imidazole was added; the solutions were passed through a 0.45 μm filter and then loaded onto a 5 mL HisTrap HP column (GE Healthcare). The column was placed on an ÄKTApurifier liquid chromatography system (GE Healthcare) and washed with a solution comprised of 20 mM $NaPO_4$ at a pH of 7.4, 0.5 M NaCl, and 20 mM imidazole. The protein was eluted by linear ramping using a solution comprised of 20 mM $NaPO_4$ at a pH of 7.4, 0.5 M NaCl, and 500 mM imidazole. Eluents were collected in 1 mL fractions, and the four samples with the highest A_{280} were run on a 12% NuPAGE SDS-PAGE gel (LifeTechnologies) and Coomassie stained to visualize the anticipated protein band (~49 kDa). These samples were pooled and dialyzed against 2 L of H_2O at 4 °C, using SnakeSkin 10 000 molecular weight cutoff dialysis tubing (ThermoScientific), with the water being changed three times over a 48 h period. The final molar concentration of purified rKynU was estimated using the A_{280} and the predicted molar extinction coefficient that was calculated using the ExPASy ProtParam tool.

Kynureninase Assay. Determination of kynureninase activity was performed in a manner similar to that previously described.[23] Solutions of kynurenine with and without compound 7 were exposed to 8.65 μM rKynU, in 40 mM KPO_4, at a pH of 7.8 with 40 μM pyridoxal 5′-phosphate (Alpha Aesar), and incubated at RT. Kynureninase activity was monitored at 350 nm by UV−vis. Enzyme kinetics and inhibition modeling were performed using GraphPad Prism 6 software. Initial velocity was determined from the linear initial change (over at least the first 60 s and up to 180 s for samples that continued the linear trend) at 350 nm absorbance upon introduction of rKynU and converted to molarity values according to the standard curve of known concentrations. To determine if compound 7 inactivates rKynU, 8.65 μM rKynU was pre-exposed to 500 μM compound 7 for several lengths of time (up to 90 min) before introducing 500 μM kynurenine. Upon the addition of kynurenine, the reaction progress was immediately initiated by monitoring at 350 nm for up to 6 h.

Determination of PQS and HHQ Production by High Resolution Direct Analysis in Real Time Mass Spectrometry (DART-HRMS). Extraction of alkyl quinolone (AHQ) signaling molecules from cultures of untreated PAO1, compound-7-treated PAO1, and PW2798 was performed as previously described.[49] Briefly, 10 mL of culture was grown in the presence of compound 7 or with an equal volume of DMSO (vehicle control). Strain PW2798, which has a transposon inserted in the *pqsA* coding region and is therefore deficient in AHQ production, was used as a negative control.[48] After 24 h, cultures were pelleted by centrifugation at 10 000*g* for 10 min. The resulting supernatant was extracted with 10 mL of acidified ethyl acetate (0.01% vol/vol glacial acetic acid in ethyl acetate). The organic phase was collected, and this step was repeated two additional times. The extracts were pooled, and the resulting solution was subjected to rotary evaporation at 37 °C until dryness. Residues were resuspended in 1.5 mL of methanol and stored at −20 °C until analysis. For detection and determination of the relative amounts of AHQ molecules, C-18 SPE-it solid phase microextraction fibers (Ionsense) were suspended in 400 uL of the methanol solutions for 30 min with shaking. The fibers were then removed from the solution and immediately analyzed by MS using an AccuTOF-DART (JEOL USA Inc.) high-resolution time-of-flight mass spectrometer in positive ion mode. The DART ion source grid voltage was 250 V and the mass spectrometer settings were as follows: ring lens voltage, 5 V; orifice 1 voltage, 20 V; orifice 2 voltage, 5 V; and peak voltage, 600 V. Mass spectra were acquired over the m/z range 60−800 at a spectral acquisition rate of 1 spectrum per second. The helium flow rate for the DART ion source was 2.0 L s^{-1}. The resolving power of the mass spectrometer was 6000 fwhm. Analyses were conducted at 250 °C by suspending the fiber between the ion source and the mass spectrometer inlet until analyte peaks were no longer detected. Calibration, spectral averaging, background subtraction, and peak centroiding of the mass spectra were performed using TSSPro3 (Shrader Software Solutions) data processing software. Mass Mountaineer software (www.mass-spec-software.com) was used for mass spectrum analysis, spectral elemental composition, and isotope analysis. Calibration was performed using a polyethylene glycol mixture (PEG 200, 400, 600, and 1000). Measurements were conducted in triplicate. Mass to charge ratio (m/z) values for HHQ and PQS were selected in TSSPro and subjected to peak area integration for each SPME fiber analysis. Total peak area counts for the individual m/z for HHQ and PQS (nominal m/z 244 and 260 respectively for M + H$^+$) were calculated for each sample and then summed to get the overall peak area counts. The three replicate individual peak area counts were averaged, and the average overall peak area count was calculated.

***S. epidermidis* Lysis Assay.** *S. epidermidis* lysis assays were performed as previously described with some modification.[68,69] PAO1 (both untreated and compound-7-treated) was grown in TSB at 37 °C with shaking at 200 rpm. After 24 h, cells were pelleted, and the supernatant was collected, 0.2 μm filtered, and stored at −20 °C until further use. Overnight cultures of *S. epidermidis* 14990 were diluted to OD_{600} of ~0.1 and spread on a TSA plate. After drying, 6-mm-punched disks from sterile filter membrane paper (Whatman, GE Healthcare) were placed on the agar. Supernatants from the *P. aeruginosa* cultures (10 μL) were then deposited onto the filter paper. Plates were incubated at 37 °C for 24 h and then imaged using an Alpha-Innotech AlphaImager.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b01082.

Genes, descriptions, fold changes, p values, iron starvation, protoanemonin-induced, Growth Stage Repressed (C4-/C12-HSL), and MexT transcriptional activator site for PA#'s (XLSX)

Supporting Table 1 and Figures 1−5 (PDF)

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Notes

The authors declare no competing financial interest.

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