Inhibition of Biofilm Formation, Quorum Sensing and Infection in *Pseudomonas aeruginosa* by Natural Products-Inspired Organosulfur Compounds

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Abstract

Using a microplate-based screening assay, the effects on *Pseudomonas aeruginosa* PAO1 biofilm formation of several S-substituted cysteine sulfoxides and their corresponding disulfide derivatives were evaluated. From our library of compounds, S-phenyl-L-cysteine sulfoxide and its breakdown product, diphenyl disulfide, significantly reduced the amount of biofilm formation by *P. aeruginosa* at levels equivalent to the active concentration of 4-nitropyridine-N-oxide (NPO) (1 mM). Unlike NPO, which is an established inhibitor of bacterial biofilms, our active compounds did not reduce planktonic cell growth and only affected biofilm formation. When used in a *Drosophila*-based infection model, both S-phenyl-L-cysteine sulfoxide and diphenyl disulfide significantly reduced the *P. aeruginosa* recovered 18 h post infection (relative to the control), and were non-lethal to the fly hosts. The possibility that the observed biofilm inhibitory effects were related to quorum sensing inhibition (QSI) was investigated using *Escherichia coli*-based reporters expressing *P. aeruginosa* *lasR* or *rhlR* response proteins, as well as an endogenous *P. aeruginosa* reporter from the *lasI/lasR* QS system. Inhibition of quorum sensing by S-phenyl-L-cysteine sulfoxide was observed in all of the reporter systems tested, whereas diphenyl disulfide did not exhibit QSI in either of the *E. coli* reporters, and showed very limited inhibition in the *P. aeruginosa* reporter. Since both compounds inhibit biofilm formation but do not show similar QSI activity, it is concluded that they may be functioning by different pathways. The hypothesis that biofilm inhibition by the two active compounds discovered in this work occurs through QSI is discussed.

Introduction

Biofilm formation by many pathogens is intimately linked to a form of inter-bacterial communication known as quorum sensing (QS), in which small diffusible signaling molecules called autoinducers globally regulate gene expression. Using quorum sensing, bacterial populations can switch from acting as individual cells to operating in a concerted, multi-cellular fashion [1]. In a clinical setting, a major challenge presented by biofilms is that bacteria living within them enjoy increased protection against host immune responses [2–8] and are markedly more tolerant to various anti-microbial treatments. A case in point is the opportunistic human pathogen *Pseudomonas aeruginosa*. *P. aeruginosa* is a Gram negative, biofilm-forming bacterium that has been shown to exhibit quorum sensing behavior using two distinct acyl-homoserine lactone (AHL) based pathways: the *rhl*/*rhlR* pathway, which uses butyryl acyl homoserine lactone (C4-HSL), and the *lasI/lasR* pathway that uses 3-oxo-dodecanoyl homoserine lactone (3-oxo C12-HSL). A third signaling molecule, 2-heptyl-3-hydroxy-4(1H)-quinolone, has been identified [9], and plays a role in *P. aeruginosa* virulence and possibly inter-species communication [10]. Multiple studies have shown that *P. aeruginosa* defective in QS may be compromised in their ability to form biofilms [11–14]. However, media composition and hydrodynamic conditions (independent of QS parameters) may also play a role in biofilm quality and stability [15–17].

One implication of the aforementioned observations is that the use of quorum sensing inhibitors (QSI) may have the potential to circumvent the challenge of the development of multidrug resistance in the bacteria to which they are exposed. It is also conceivable that QSIs could be developed as an adjuvant to the administration of antibiotics, with the former serving to increase the susceptibility of infecting bacteria to cell death by exposure to antibiotics. Preliminary findings by Brackman *et al.* have demonstrated that QIS increase the susceptibility of bacterial biofilms (including *P. aeruginosa* biofilms) to multiple types of antibiotics [18]. It has recently been demonstrated that some biofilm-associated bacteria return to the planktonic state through the secretion of D-amino acids [19] and cis-2-decenioic acid [20] natural products, both of which trigger biofilm disassembly. This
Results and Discussion

Effect of Compounds on Biofilms

Both in vitro and in vivo studies have shown the potential of garlic extracts to attenuate the virulence of *P. aeruginosa*. Harjai et al. [21] have observed that in a mouse model of nosocomial catheter-associated urinary tract infections, oral treatment with crude garlic extracts significantly lowered renal bacterial counts and protected mouse kidney from tissue destruction. The further observation of decreased production of virulence factors and reduced production of quorum-sensing signals by *P. aeruginosa* was interpreted to suggest that garlic exhibits QS activity. Symth et al. observed a trend towards improvement in lung function in cystic fibrosis patients with garlic therapy. However, the sample size was too small to demonstrate statistically significant improvement in clinical outcomes [22]. Garlic derived natural products 1–6 (Figure 1) have been reported to inhibit las/luxR-based QS systems that are found in *Pseudomonas* species [23], as well as the lux/luxR systems found in other bacteria, such as *Vibrio spp.* [24,25]. Both lux and las QS systems utilize acyl homoserine lactone (AHL) compounds as autoinducers, although genetic regulation by these systems varies widely between different species. Bioassay guided fractionation of garlic extracts has revealed that the derivatives responsible for QS activity include ajoene, as well as sulfides and polysulfides 1–4, and vinyl diithiins 5 and 6 (Figure 1) [25–27]. Of these, 1–4 antagonized LuxR but were also toxic to bacteria. However, 5 and 6 possessed QS activity exclusively in a LuxR monitor system [23]. All six of these compounds are derivatives of the cysteine sulfoxide alliin (Figure 1). These compounds are derivatives of a reaction cascade that begins in macerated garlic tissue with alliinase-catalyzed breakdown of the precursor S-alk(en)yl cysteine sulfoxide alliin (Figure 1). Similar alliinase-mediated chemistry occurs in the Amazonian medicinal plant *Petiveria alliacea* L. (Phylolaccaceae) to afford a variety of organosulfur derivatives with similar functionalities but different structures from those observed in garlic [28,29]. A combination of natural and unnatural cysteine sulfoxides with which the *P. alliacea* alliinase reacts (7–11) [30] and several of the organosulfur compounds downstream of the action of its alliinase (12–16) [25,28,31,32] are also shown in Figure 1. The demonstration that garlic-derived natural products inhibit lux and las-based QS systems [23] and the observation of the presence of the chemistry that produces these compounds in an increasing number of plants [28,29,31,33,34], implies that plants may have evolved to produce these secondary metabolites in order to serve as quorum sensing antagonists that prevent the establishment of infections by pathogenic microbes.

To begin our investigations into this possibility, we determined the effect of five cysteine sulfoxides with which the *P. alliacea* alliinase has been shown to react (7–11, Figure 1) [30], as well as several of the sulfide and disulfide derivatives of the alliinase-mediated reactions (12–16, Figure 1), on biofilm formation and QS-based signaling in *P. aeruginosa*. The effects of these compounds on *P. aeruginosa* biofilm formation were initially assessed using a crystal violet-based biomass staining assay, with the results shown in Figure 2A. 4-Nitropyridine-N-oxide (NPO), a known inhibitor of biofilm formation in *P. aeruginosa*, was used as a positive control [32,33]. Of the compounds tested, only 3-phenyl-L-cysteine sulfoxide (7), diphenyl disulfide (12) and NPO demonstrated significant biofilm inhibitory activity (Dunnett’s test, p < 0.01), each at a concentration of 1 mM. These results were confirmed in optical micrographs of the resultant biofilms (Figure 3A), which show a significant difference in biofilm density on the bottom surface of the microplates in the presence of 7, 12 and NPO as compared to the “no-inhibitor” control. Further, laser scanning confocal microscopy of a biofilm grown in the presence of compound 7 showed significantly altered 3D morphology, as compared to a no-inhibitor control (Figure 3B). In particular, the biofilm exposed to compound 7 is sparse and has limited 3D projections off of the glass substrate (Figure 3B, right), while the control biofilm (Figure 3B, left) is densely packed and extends vertically with multiple 3D projections.

Following this initial screening of compound effectiveness, compounds 7, 12 and NPO were tested in a concentration dependent manner. Figure 2B shows the results of these experiments in which the concentrations of compounds 7, 12 and NPO were varied from 0.01–1 mM. All three compounds exhibited significant (p < 0.01) inhibitory activity against biofilm formation at 1 mM, but had diminished activity at lower concentrations. Of the three compounds, 12 retained significant inhibitory activity at 0.1 mM. To distinguish between the effects of 7, 12 and NPO on cell growth vs. cellular biofilm forming ability, planktonic cell density was determined by measuring the OD of each microwell prior to biofilm staining with crystal violet. Our results show that, in addition to inhibiting biofilm formation, NPO significantly inhibits planktonic cell growth (Figure 2, panels A & B). In contrast, planktonic cell density was not significantly affected by compounds 7 and 12. We further confirmed these results by performing growth curve analysis for compounds 7, 12, and NPO (Figure 4). *P. aeruginosa* cells exposed to 1 mM of compounds 7 and 12 showed no lag in growth, as compared to control cultures [containing only media and the solvent (DMSO) used for compound resuspension], and reached an optical density of >0.4 within 10 h. A parallel experiment using concentrations of up to 0.1 M of compounds 7 and 12 showed no difference in growth behavior (data not shown). In contrast, cells exposed to NPO showed no increase in OD, indicating complete inhibition of cell growth. These data suggest that NPO primarily affects biofilm formation by inhibiting bacterial growth, while compounds 7 and 12 affect biofilm formation independent of cell growth/propagation.

To further evaluate the effects of the compounds on *P. aeruginosa* biofilms, the formazan-based 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay was performed on biofilms that were exposed to compounds 7, 12 and NPO. This assay measures enzymatic activity in actively respiring cells and is therefore a measure of cell viability and/or relative numbers of viable cells. The analysis showed that biofilms grown in the presence of compounds 7 and 12 had significantly lower activity (41% and 45%, respectively) as compared to a no inhibitor control (Figure 5). In contrast, exposure to NPO reduced cell activity in the biofilm by >99%. These data further corroborate the results of the crystal violet-based staining assays, which showed a distinct decrease in stained biomass for biofilms grown in the presence of compounds 7, 12 and NPO. The compounds did not however, inhibit planktonic cell growth, as demonstrated in the planktonic cell OD measurements shown in Figure 2, panels A & B, as well as the growth curve analysis shown in Figure 4. This is in stark contrast to the previously reported biofilm inhibitor NPO, which
appears to inhibit both planktonic cell growth and biofilm formation. This distinction is important, since inhibition of planktonic growth and biofilm formation are decoupled for our compounds, and this inhibition does not rely upon strict biocidal activity.

Effect of Compounds on Quorum Sensing

As described earlier, garlic-derived structural relatives of our active compounds demonstrated QSI activity in lux-based QS systems [23]. To determine if our active compounds behaved similarly, we evaluated their effects on QS using previously described quorum sensing reporter plasmids, pFNK202 and pFNK503 in E. coli, and in a P. aeruginosa reporter strain (PAO-MW1 with pUM15) [36]. The results of these experiments are shown in Figures 6 and 7. Compound 7 was shown to significantly affect the quorum sensing response (p<0.01), as measured by GFP expression, for both pFNK202 and pFNK503. This indicates that compound 7 can antagonize both the lasI/lasR and rhlI/rhlR quorum sensing systems in this artificial reporter strain. Compound 7 also significantly (p<0.01) affected quorum sensing in the P. aeruginosa PAO-MW1 reporter, further supporting the conclusion that it is operating through antagonism of the lasI/R quorum sensing system. To further examine the effect of compound 7 on quorum sensing, we used the PAO-MW1 reporter containing pUM15 in our biofilm inhibition assay. The reporter itself is deficient in both rhlI and lasI (rhlI::Tn501 lasI::tetA) which

Figure 1. C-S lyase (i.e. alliinase) mediated cleavage of cysteine sulfoxides. For both the onion and P. alliacea alliinases, reaction with a cysteine sulfoxide derivative yields a fleeting sulfenic acid, two molecules of which can condense to give thiosulfimates. The thiosulfimates react further to yield a variety of organosulfur compounds. Compounds 1–6 from garlic have been found to inhibit lasR- and lasR-based QS systems. The P. alliacea alliinase has been shown to have broad substrate specificity and degrade a variety of cysteine sulfoxide derivatives such as compounds 7–11.

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makes it a QS mutant when exogenous autoinducer is not provided. We exposed this strain to both exogenous autoinducer (3-oxo C12 HSL) alone, and to 3-oxo C12 HSL plus compound 7, and evaluated biofilm formation. This experiment showed that exposure to compound 7 reduced biofilm formation by >50% (data not shown). When no autoinducer was added (completely shutting off quorum sensing), biofilm formation by both the control (no compound added) and compound 7 exposed cells was reduced by 80% (data not shown). Further, there was no significant difference between biofilm formation in these two exposure groups. This follows previous studies in which elimination of quorum sensing significantly affected biofilm formation in P. aeruginosa [11–14]. We conclude that complete inhibition of quorum sensing masks the effect of compound 7 on biofilm formation and cannot distinguish between QSI or another mechanism responsible for biofilm inhibition.

Unlike compound 7, compound 12 did not significantly affect the quorum sensing response for the E. coli reporters pFNK202 or pFNK503, but did show significant antagonism in the P. aeruginosa reporter (12% reduction in fluorescence). None of the other compounds significantly affected the quorum sensing response (data not shown). The control inhibitor, NPO, did not show an effect on quorum sensing in either of the E. coli reporters, but did reduce quorum sensing in the P. aeruginosa reporter by 99%. This is likely due to the difference between the assays used for E. coli vs. P. aeruginosa, since the P. aeruginosa assay was dependent upon cell growth and expression of the fluorescent reporter protein. As shown previously, NPO inhibits growth of P. aeruginosa, which would likewise prevent expression of the reporter (YFP) in this assay. Since both compounds 7 and 12 were shown to inhibit biofilm formation, but do not have similar QSI activity, they may be functioning via different pathways, or may be additionally

Figure 2. Panel A: Inhibition of P. aeruginosa PA01 biofilm formation by small molecule inhibitors. Panel B: Concentration dependence for inhibition of P. aeruginosa PA01 biofilm formation by small molecule inhibitors. For 2A and 2B, average OD_{600nm} measurements of crystal violet stained biofilms (top) and planktonic cells (bottom) are shown with error bars representing one standard deviation (n = 3). ANOVA (p < 0.0001) was performed, followed by Tukey’s test, with asterisks (*) indicating significant (p < 0.01) reduction in planktonic cell density or biofilm, and (+) representing a significant increase.
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Figure 3. Panel A: Optical micrographs of bacterial biofilms. The films were grown in the presence of small molecule inhibitors 7 and 12 at 1 mM final concentration. Scale bar = 100 μm. Panel B: Laser scanning confocal micrographs of biofilms. The films were grown in the presence of 1 mM compound 7 (right) or without inhibitor (left). Top down images are shown in the upper views, with vertical and horizontal cross-sections shown to the right and below, respectively. Three dimensional reconstructions are shown in the bottom views. Scale bars for top down and 3D views are 50 μm, while scale bars for cross sections are 10 μm.

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Effect of Compounds on Infection in D. melanogaster

It has been observed that biofilm formation can have profound effects on infection dynamics and pathogenesis [37]. Recently, the model organism Drosophila melanogaster has been used to study in vivo P. aeruginosa infection dynamics, and in particular, the relationship between biofilm formation and pathogenicity [38,39]. Thus, Estin et al. have shown that P. aeruginosa infection and lethality in Drosophila is QS-dependent and that expression of a human-derived autoinducer degrading enzyme (paraoxonase 1) can act as a protective agent to reduce infection [38]. Fly mortality (reflecting pathogen virulence) is a composite measure of host defense incorporating both resistance and tolerance to the infection. Previous experiments in our labs (using Pseudomonas aeruginosa infections) have shown a strong positive correlation between CFU counts and fly mortality (data not shown). In this study, we used the Drosophila infection model to assess the effect of compounds on patterns of resistance (which is defined as the inverse of bacterial load). We observed a dramatic decrease in the number of bacteria recovered from flies treated with either compound 7 or 12 compared to controls (Figure 8). Both compounds had similar effects on slowing bacterial growth in flies. The solvent used to dissolve the compounds (DMSO) appears to slow bacterial growth during infection, but to a much lesser extent than either compound 7 or 12 (Figure 8). Similar effects were seen for both male and female flies, and therefore no sex bias was observed. For these experiments, bacteria were suspended in a 0.1 M concentration of compound that was then injected into the flies. Bacteria were only exposed to this high concentration for 5 min or less, since injected material is expected to be diluted inside of the flies. Further, 0.1 M concentrations of compounds 7 and 12 were not shown to affect P. aeruginosa cell growth dynamics (as determined by growth curve analysis). Therefore the reduced bacterial load in affected flies is attributed to increased ability of the flies to mitigate infection, and not compound induced reduction in bacterial growth rate.

Our results indicate that the disruption of biofilm formation (or QS) may significantly alter infection dynamics in acute systemic infections in D. melanogaster. We propose that the inability to form a biofilm during acute systemic infections may make the growing bacterial population more susceptible to the innate immune defense of the fly. Our results seem in contrast to those of a recent study that found that strains of P. aeruginosa not capable of producing a biofilm showed increased virulence in the fly [39]. However, in that study, flies were fed bacteria that resulted in chronic infections maintained largely in the crop. Further, strains capable of biofilm formation were better inducers of local antimicrobial peptide production in the crop, indicating that the biofilm is more immunogenic than planktonic cells [39]. In acute systemic infections such as those carried out here, innate immune induction due to both wounding and the recognition of the bacteria are likely to occur, and the inability to form a biofilm may leave the population more prone to attack by these defenses.

The structural relationship between the two active compounds 7 and 12 is noteworthy because of the clues it provides on how the active compounds may be metabolized in P. aeruginosa. When a C-S lyase such as the P. alliacea alliinase encounters 7 or another cysteine sulfoxide of appropriate structure, the C=S(=O) bond that is β- to the amino acid moiety is cleaved to yield a sulfenic acid, two molecules of which rapidly condense to yield a thiosulfinate (Figure 1). The labile thiosulfinates can then undergo further transformations to yield disulfides. In the case of 7, the corresponding disulfide is 12. C-S lyase mediated decompositions of cysteine sulfoxides are marked by the emergence of a strong sulfurous odor as the disulfide is generated. The smell is characteristic and easily detectable since the precursor cysteine sulfonates are non-volatile compounds. Over the course of our experiments, we observed that with time, the P. aeruginosa samples that were incubated with cysteine sulfones 7–11 all began to emit an organosulfur odor reminiscent of fresh P. alliacea macerates. The implication of this observation is that the bacteria possess a C-S lyase enzyme analogous to those observed in garlic and P. alliacea, that can degrade cysteine sulfoxides to ultimately yield sulfides and/or disulfides that might themselves exhibit biofilm inhibitory activity. Although there are no literature reports that show the PAO1 strain of P. aeruginosa to possess cysteine sulfoxide lyases, a cysteine sulfoxide lyase has been isolated from Pseudomonas crucivora [40]. An amino acid BLAST search [41] of the P. aeruginosa PAO1 genome yields a hypothetical protein sequence with 26% sequence similarity (and total alignment score of 31.6) to the Allium cepa (onion) C-S lyase (GenBank: AF126049.1). The sequence similarity is relatively low; however, this putative protein could indeed have C-S lyase enzymatic activity. This possibility would need to be demonstrated or confirmed in a follow-on study. A further implication of our findings is that the cysteine sulfoxides enter into the bacterial cells, since their decomposition requires the action of lyase enzymes that would not likely be present in the extracellular milieu, but rather in the cytosol.

Since it has been demonstrated that structurally similar garlic-derived organosulfur compounds can inhibit lux-based bacterial quorum sensing [23] and that compound 7 identified in this study...

Figure 4. Growth curve analysis (OD500nm) for P. aeruginosa. The bacteria were grown in the presence of 1 mM of compounds 7, 12, NPO and DMSO. Error bars represent the standard deviation (n = 3).

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inhibits las and rhl-based quorum sensing, we hypothesize that its inhibition of biofilm formation in P. aeruginosa may occur through antagonism of quorum sensing pathways. The fact that compound 12 did not show significant quorum sensing inhibition, but did inhibit biofilm formation, suggests that it is functioning via a different mechanism, or its QSI activity is obscured by the particular QSI assays that were used. Nevertheless, our results reveal a new class of bacterial biofilm inhibitors, and further support an approach to biofilm inhibition via antagonism of quorum sensing behavior. Further, we have demonstrated that these compounds can significantly affect the bacterial load in a Drosophila based infection model, which suggests their applicability for mitigating P. aeruginosa infections. Studies are underway to elucidate the specific mechanism of action of these compounds in biofilm inhibition, to identify their mode of action during Drosophila infection, and to further characterize their QSI activity.

**Materials and Methods**

**Instrumentation**

NMR spectra were recorded on a Bruker 400 MHz spectrometer. IR spectra were recorded using a Perkin Elmer Spectrum 100 FT-IR spectrophotometer.

**Compounds**

The S-phenyl- and S-benzyl-L-cysteines (precursors to compounds 7 and 8 respectively), as well as diphenyl disulfide, dibenzyl sulfide and dibenzyl disulfide (compounds 12, 13, and 14 respectively) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were used without further purification. Compound 8 was synthesized as previously described [29]. S-β-Phenylethyl-, S-[(4-methylphenyl)methyl]- and S-[(4-chlorophenyl)methyl]-L-cysteines (precursors to compounds 9, 10, and 11 respectively) were synthesized as described by Kubec and Musah [29]. Briefly, to a stirred solution of L-cysteine (0.1 mol) in 150 ml of 1 M NaOH and 200 ml of ethanol was added the corresponding bromide (1.15 equiv.). After 2 h, the solution was acidified with conc. HCOOH to pH 5–6. The precipitated product was filtered off and washed thoroughly with acetone and diethyl ether. Cysteine sulfoxides 7 and 9–11 were prepared by oxidation of the corresponding S-substituted L-cysteines with H2O2. Bis[(4-methylphenyl)methyl] disulfide and bis[(4-methylphenyl)methyl] disulfide (compounds 15 and 16 respectively) were synthesized by NaIO4 oxidation of the corresponding thiols as described by Montazerozohori et al. [42]. Briefly, 0.5 equivalents of NaIO4 was placed in a mortar and moistened with water. Then 1 equivalent of the thiol was added and the mixture was triturated for 2 min. The solid material was taken up in CH2Cl2 and the resulting solution filtered. The filtrate was dried over MgSO4 and the solvent was evaporated under reduced pressure to yield the disulfide product.

![Graph](image_url)

**Figure 5. Cell viability within microplate-established biofilms as determined by the MTT assay.** Biofilms were grown in the presence of 1 mM of compounds 7, 12 and NPO. ANOVA (p<0.0001) was performed, followed by Tukey’s test, with asterisks (*) indicating significant (p<0.01) reduction in viability as compared to the no inhibitor control (n = 5).

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**S-Phenyl-L-cysteine sulfoxide (7):** white solid; mp 158–160°C (boiling water); IR ν\text{max} \text{ cm}^{-1}: 2476–2316 (m, br), 1533 (s), 1516 (m), 1401 (m), 1392 (s, sh), 746 (m, sh), 688 (s, sh); 13C NMR (400 MHz; D2O, NaOD): 38.8, 54.9, 126.5, 129.1, 129.5, 134.5, 180.5; HR ESI-TOF [MH\text{+}]: 214.0533 (calc. for C9H11NO3S: 214.0532).

**S-[(4-Methylphenyl)methyl]-L-cysteine sulfoxide (10):** white solid; mp 169–171°C; IR ν\text{max} \text{ cm}^{-1}: 2530–3170 (m, br), 1583 (s), 1514 (m, sh), 1420 (m, sh), 1356 (m, sh), 1016 (s, sh), 817 (m, sh); 13C NMR (400 MHz; D2O/NaOD; DSS): δ 20.22, 50.88, 55.59, 125.93, 129.49, 130.34, 138.93, 179.79; HR ESI-TOF [MH\text{+}]: 242.0842 (calc. for C11H15NO3S: 242.0845).

**S-[(4-Chlorophenyl)methyl]-L-cysteine sulfoxide (11):** white solid; mp 169–172°C; IR ν\text{max} \text{ cm}^{-1}: 2521–3163 (m, br), 1577 (s, sh), 1492 (m, sh), 1388 (m, sh), 1019 (s, sh), 824 (m); 13C NMR (400 MHz; D2O, NaOD): δ 31.0, 55.6, 55.7, 127.8, 128.8.

### Table 1: Fluorescence Intensity of pFNK202 and pFNK503 Constructs

|          | pFNK 202 Mean Fluor. (A.U.) | % Reduction pFNK 202 QS | pFNK 503 Mean Fluor. (A.U.) | % Reduction pFNK 503 QS |
|----------|----------------------------|-------------------------|----------------------------|-------------------------|
| 7        | 2,943 +/- 311              | -79.8%                  | 2,237 +/- 784              | -87.2%                  |
| 12       | 13,622 +/-1,643            | -6.6%                   | 12,985 +/-1,097            | -24.0%                  |
| NPO      | 13,760 +/-425              | -5.7%                   | 13,326 +/-923              | -                |
| No Inhibtor | 14,587 +/-623           | -                   | 17,532 +/-3,498            | -                |
| No Autinducer | 1,564 +/-46             | -89.3%                  | 2,564 +/-46               | -85.4%                  |

Figure 6. Inhibition of quorum sensing. In these experiments, *E. coli* pFNK202 (rhlI/rhlR - C4 HSL autoinducer) and pFNK503 (lasI/lasR - 3-oxo C12 autoinducer) reporters were used. Fluorescence intensity (480 nm/520 nm Ex/Em) of the resulting QS induced GFP expression was measured, following 24 h exposure to 1 mM of compounds 7, 12 and NPO. ANOVA (p < 0.0001) was performed, followed by Tukey’s test, with asterisks (*) indicating significant (p < 0.01) reduction in fluorescence as compared to the no inhibitor control (n = 3).

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**Figure 7. Inhibition of quorum sensing for the* P. aeruginosa* PAO-MWI pUM15 (lasI/lasR -3-oxo C12 autoinducer) reporter.** Fluorescence intensity (480 nm/520 nm Ex/Em) of the resulting QS induced YFP expression was measured, following 24 h exposure to 1 mM of compounds 7, 12 and NPO. ANOVA (p<0.0001) was performed, followed by Tukey’s pairwise comparison test, with asterisks (*) indicating significant (p<0.01) reduction in fluorescence as compared to the no-compound control (n = 3).

|                     | 7               | 12              | NPO             | No Inhibitor | No Autoinducer |
|---------------------|-----------------|-----------------|-----------------|-------------|----------------|
| Mean Fluorescence (A.U.) | 6,037 +/- 367   | 19,292 +/- 392  | 132 +/- 5      | 21,974 +/- 247 | 784 +/- 48    |
| % Reduction in QS Response | -72.5%         | -12.2%          | -99.4%         | -           | -96.4%        |

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**Figure 8. Effects of compounds 7 and 12 on* D. melanogaster* infected with* P. aeruginosa* PAO1.** Treatment groups not connected by the same symbol are significantly different from each other, as determined by ANOVA (p<0.01) and Tukey’s test (p<0.05).

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Biofilm Inhibition

P. aeruginosa PAO1 was propagated on tryptase soy agar (TSA) for plate-based assays or in tryptase soy broth (TSB) for liquid culture. M9 growth media supplemented with 0.4% (w/v) glucose and 0.4% (w/v) casamino acids was used for biofilm formation experiments. Culture media (TSB, TSA, M9 salts and casamino acids) were obtained from Difco/Becton Dickinson (Franklin Lakes, NJ, USA) and all other reagents (phosphate buffered saline, glucose, ethanol and crystal violet) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Corning 35–1172 flat-bottomed polystyrene 96-well plates were used for biofilm formation experiments and optical density measurements were performed in a Tecan M-200 (Durham, NC, USA) plate reader. Optical micrographs of biofilms were obtained using a Nikon Eclipse 80i microscope.

A microplate based assay, modified from Junker et al. [32] was used to screen compounds for QSI. Briefly, P. aeruginosa PAO1 was grown in TSB for 18 h at 37°C which was then centrifuged at 14,000 rpm and rinsed with phosphate buffered saline (PBS, pH 7.4) three times, then was resuspended in M9 minimal growth media to approximately 10^8 cfu/ml (determined by OD and plate count assay). Test compounds were dissolved in DMSO and were added to sterile distilled water to achieve concentrations ranging from 0.1–10 mM while keeping DMSO at a maximum of 1% (v/v). P. aeruginosa inocula (360 μl) were then pre-mixed with 40 μl of the test compound solutions to achieve final compound concentrations ranging from 0.01–1 mM. An aliquot (100 μl) of this cell compound mixture was then added to three separate wells in a 96-well microplate for replicate testing. For control wells (no inhibitor), dilute DMSO was added to the inocula instead of test compounds, to a final concentration of 1% (v/v). Optical density (OD600nm) measurements were performed immediately after inoculation and after 24 h incubation at 37°C (without shaking) to monitor planktonic cell growth. To determine the amount of biofilm formation, supernatant from the microplate wells was gently removed and the wells were washed twice with 150 μl of PBS using a multichannel pipette. The remaining biofilm was then stained using 100 μl of a 0.2% (w/v) crystal violet solution for 15 min at room temperature. The crystal violet was then removed from the wells, the wells were rinsed four times with PBS, and then 100 μl of 95% ethanol was added to extract the crystal violet solution from the biofilm. The OD600nm of the extracted crystal violet was then measured, yielding a measure of biofilm formation (relative to the control). For optical imaging, crystal violet stained biofilms were washed with distilled water and no ethanol extraction was performed.

In addition to crystal violet based quantification of biofilm biomass, cell viability within biofilms exposed to inhibitor compounds was determined using the formazan dye-based MTT assay (Cell Proliferation Kit I, Roche Diagnostics, Mannheim, Germany). This assay has previously been described for determination of biofilm cell viability [43–45]. Briefly, biofilms were grown in 96 well microplates for 24 h as described above, in the presence and absence of inhibitor compounds. After this initial inoculation period, planktonic cells were removed and the remaining biofilm was gently rinsed three times with 100 μl of PBS. After rinsing, 100 μl of PBS and 10 μl of the MTT labeling reagent were added and the suspension was incubated for 4 h at 37°C, followed by addition of 100 μl of solubilization solution. Plates were then incubated for 24 h at 37°C and absorbance measurements were taken using a Tecan M-200 plate reader at 560 nm (peak absorbance for the formazan dye breakdown product) and at 700 nm (reference wavelength for the intact dye). This assay (Cell Proliferation Kit I, Roche Diagnostics, Mannheim, Germany) uses pUM15 (:rasL::gfp reporter strain previously described by Muh et al. [33]) as a host for the gfp reporter. Plasmids pFNK503 contains part of the P. aeruginosa lasI/R pathway, including the lasI gene and the green fluorescent protein (gfp) gene under the control of the βLai promoter. Cells hosting this plasmid respond to 3-oxo C12-HSL by producing GFP. Plasmid pFNK202 contains part of the P. aeruginosa rhlI/R pathway, including the rhlI gene and the green fluorescent protein (gfp) gene under the control of the pRhl promoter. Cells hosting this plasmid respond to 3-oxo C12-HSL by producing GFP. Both strains pFNK503 and pFNK202 were maintained on trypticase soy agar (TSA) plates containing 0.5 μg/ml kanamycin (Kan) at 37°C. QSI studies in P. aeruginosa were performed using a quorum sensing reporter strain previously described by Muh et al. [36]. This reporter strain, obtained from Dr. Peter Greenberg (University of Washington, Washington State, USA) uses pUM15 (:rasL::gfp) transcriptional fusion, Bo) in a P. aeruginosa PAO-MW1 (βLai::Tn501 lasE::tetA) background. This reporter expresses YFP when exposed to 3-oxo C12-HSL at concentrations as low as 0.3 μM [36]. Cultures were maintained on Luria-Bertani Broth (LB) plates containing 150 μg/ml carbenicillin at 37°C.

Quorum Sensing Inhibition

Quorum sensing inhibition (QSI) studies were performed in both E. coli and P. aeruginosa biosensor strains which respond to exogenously added autoinducers by expressing fluorescent proteins (GFP or YFP). Experiments using E. coli were performed using quorum sensing reporter plasmids pFNK-503 (abbreviated pFNK503) or pFNK-202-esc119 (abbreviated pFNK202) in E. coli JM2.300, as previously reported by Bremer et al. [46]. These plasmids were kindly provided by Dr. Ron Weiss (Massachusetts Institute of Technology, Cambridge, MA, USA). Plasmid pFNK503 contains part of the P. aeruginosa lasI/R pathway, including the lasI gene and the green fluorescent protein (gfp) gene under the control of the βLai promoter. Cells hosting this plasmid respond to 3-oxo C12-HSL by producing GFP. Plasmid pFNK202 contains part of the P. aeruginosa rhlI/R pathway, including the rhlI gene and the green fluorescent protein (gfp) gene under the control of the pRhl promoter. Cells hosting this plasmid respond to 3-oxo C12-HSL by producing GFP. Both strains pFNK503 and pFNK202 were maintained in trypticase soy agar (TSA) plates containing 0.5 μg/ml kanamycin (Kan) at 37°C. QSI studies in P. aeruginosa were performed using a quorum sensing reporter strain previously described by Muh et al. [36]. This reporter strain, obtained from Dr. Peter Greenberg (University of Washington, Washington State, USA) uses pUM15 (:rasL::gfp) transcriptional fusion, Bo) in a P. aeruginosa PAO-MW1 (βLai::Tn501 lasE::tetA) background. This reporter expresses YFP when exposed to 3-oxo C12-HSL at concentrations as low as 0.3 μM [36]. Cultures were maintained on Luria-Bertani Broth (LB) plates containing 150 μg/ml carbenicillin at 37°C.

Liquid cultures of E. coli reporter strains were grown in TSB +0.5 μg/ml Kan at 37°C with shaking at 225 rpm. For QSI experiments, strains were grown for 12 h to an OD_600 of 0.7. Aliquots of cells (80 μl) were then added to individual wells of a sterile 96 well microplate. Autoinducers (10 μl of 1 mM 3-oxo C12-HSL or C4-HSL, Gayman Chemicals, Ann Arbor, MI) were then added to each well. This addition was followed by addition of test compounds (10 μl of a 10 mM stock solution) to yield a final concentration of 1 mM, prepared as described above. Control wells contained TSB only, cells without autoinducer, cells with autoinducer but no test compound, or cells with autoinducer and 1 mM DMSO (to determine if the DMSO component of the test compound stocks affected quorum sensing response). P. aeruginosa QSI experiments were performed using a similar protocol with the following modifications. The P. aeruginosa reporter was grown in LB with 150 μg/ml carbenicillin, and 10 μl of a mid-log phase culture was added per microwell. Microwells were brought to 100 μl total volume with sterile LB and the autoinducer 3-oxo-
C12-HSL was added to a final concentration of 0.1 mM. For both E. coli and P. aeruginosa reporters, an initial fluorescence reading (400 nm excitation/520 nm emission) was then taken for each well (in a Tecan M200 microplate reader), followed by incubation at 37°C without shaking. Fluorescence readings were then repeated at 24 h to determine the amount of GFP expression for each experimental condition. The percent change in fluorescence intensity was determined for each test condition and a minimum of three replicate samples was used for all experiments.

Effect of Compounds on Infections in Drosophila Melanogaster

In recent years D. melanogaster has emerged as a powerful model system for understanding P. aeruginosa pathogenicity [39,47]. Thus, the effects of compounds on in vitro infections were tested using D. melanogaster infected with P. aeruginosa strain PAO1. For infections, cells were grown to log-phase in LB broth at 37°C in a shaking incubator. Cultures were diluted using sterile LB to a concentration of 5 x 10^6 cfu/ml for infections. There were three treatment groups: (i) cells alone; (ii) cells with DMSO (10% v/v final concentration); and (iii) cells and 0.1 M compounds. Stock solutions of compounds were made in DMSO. Growth curve analysis, as described above, was performed using 0.1 M concentrations of compounds to demonstrate P. aeruginosa viability at elevated compound concentrations. A volume of 54 μl for each treatment culture was injected into the thorax of flies using a Nanoject II nanoliter injector (Drummond Scientific), corre-

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