Flavonoids Suppress Pseudomonas aeruginosa Virulence through Allosteric Inhibition of Quorum-sensing Receptors*

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Quorum sensing is a process of cell-cell communication that bacteria use to regulate collective behaviors. Quorum sensing depends on the production, detection, and group-wide response to extracellular signal molecules called autoinducers. In many bacterial species, quorum sensing controls virulence factor production. Thus, disrupting quorum sensing is considered a promising strategy to combat bacterial pathogenicity. Several members of a family of naturally produced plant metabolites called flavonoids inhibit Pseudomonas aeruginosa biofilm formation by an unknown mechanism. Here, we explore this family of molecules further, and we demonstrate that flavonoids specifically inhibit quorum sensing via antagonism of the autoinducer-binding receptors, LasR and RhlR. Structure-activity relationship analyses demonstrate that the presence of two hydroxyl moieties in the flavone A-ring backbone are essential for potent inhibition of LasR/RhlR. Biochemical analyses reveal that the flavonoids function non-competitively to prevent LasR/RhlR DNA binding. Administration of the flavonoids to P. aeruginosa alters transcription of quorum sensing-controlled target promoters and suppresses virulence factor production, confirming their potential as anti-infectives that do not function by traditional bacteriocidal or bacteriostatic mechanisms.

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Quorum sensing (QS)<sup>1</sup> is a bacterial cell–cell communication process that controls collective behaviors (1). QS relies on the production, accumulation, detection, and population-wide response to extracellular signaling molecules called autoinducers (AIs) (2). The Pseudomonas aeruginosa QS circuit consists of two primary AI synthase/receptor pairs, LasI/R and RhlI/R, which produce and detect 3OC<sub>12</sub>-homoserine lactone (3OC<sub>12</sub>HSL) and C<sub>4</sub>-homoserine lactone (C<sub>4</sub>HSL), respectively (3–6). At high cell density, LasR and RhlR, which are members of the large family of LuxR-type proteins, bind their cognate AIs, dimerize, bind DNA, and activate expression of genes encoding functions required for virulence and biofilm formation as well as other processes not involved in pathogenicity (7).

<i>P. aeruginosa</i> is a pathogen of clinical relevance that affects cystic fibrosis sufferers, burn victims, immunocompromised individuals, and patients with implanted medical devices, such as intubation tubes (8, 9). <i>P. aeruginosa</i> frequently forms biofilms on medical surfaces, leading to nosocomial infections. <i>P. aeruginosa</i> has acquired resistance to commonly used antibiotics and is now a priority pathogen on the Centers for Disease Control and Prevention ESKAPE pathogen list (10, 11). New anti-infective approaches are urgently needed for <i>P. aeruginosa</i>, and targeting bacterial behaviors, such as QS, rather than targeting bacterial growth, represents an attractive alternative for exploration in anti-microbial research (1, 12). Such therapies could minimize selection for drug resistance, potentially endowing these medicines with extended functional lifetimes.

Previous efforts to develop <i>P. aeruginosa</i> QS inhibitors include screening of natural products, screening of small molecule libraries, <em>in silico</em> screening, and synthesis of focused libraries based on the native AI structures (13–16). These efforts resulted in the discovery of several competitive LasR inhibitors that function <em>in vitro</em> but not <em>in vivo</em> in an animal infection model (17). However, one <i>P. aeruginosa</i> QS inhibitor, <em>meta</em>-bromothiolactone (mBTL), discovered through synthesis of focused libraries, inhibits QS both <em>in vitro</em> and <em>in vivo</em> in a <i>Caenorhabditis elegans</i> model of infection; mBTL inhibits LasR and RhlR via competition with the natural AIs for occupancy of the ligand binding sites (18).

Flavonoids are a group of natural products that exhibit broad pharmacological activities ranging from anti-microbial to anti-inflammatory (19). Recently, multiple flavonoids were reported to inhibit <i>P. aeruginosa</i> biofilm formation, raising the possibility that they function by affecting QS signaling (20–22). How-
ever, their mechanisms of action were not investigated. Here, we show that novel flavonoids possessing dihydroxyl moieties in the flavone A-ring backbone, as well as the previously identified flavonoids baicalein and quercetin, bind to the QS receptors, LasR and RhlR, and significantly reduce their ability to bind to DNA encoding QS-regulated promoters. Structure-activity relationship (SAR) analyses indicate that the presence of two hydroxyl groups in the flavone A-ring is necessary for inhibition of LasR and RhlR. Using LasR as the representative receptor, we show that the flavonoids act by an allosteric mechanism. The flavonoids inhibit virulence factor production and swarming in a LasR/RhlR-dependent manner. These compounds are the first noncompetitive QS inhibitors identified that target LasR/RhlR and prevent DNA binding. Halogenated furanones have been discovered that function non-competitively by destabilizing LasR, promoting its degradation (23–25). Many flavonoids are GRAS (generally recognized as safe) compounds and thus could immediately be explored for uses in industry, agriculture, and animal husbandry. Our results support the general notion that targeting QS represents a viable route for controlling *P. aeruginosa* pathogenicity. Presumably, strategies analogous to those presented here could be used to control other pathogens that use QS to regulate virulence, biofilm formation, or other traits for which inhibition on demand would be useful (26).

**Results**

**Discovery of Flavonoid LasR and RhlR Inhibitors**—To screen for and characterize *P. aeruginosa* QS inhibitors, we constructed an *Escherichia coli* strain harboring arabinose-inducible *lasR* and a plasmid containing *plasB-luxCDABE* (black, called the LasR reporter strain) or arabinose-inducible *rhlR* and *phlA-luxCDABE* (gray, called the RhlR reporter strain). 0.1% (v/v) arabinose, 100 μM 3OC12HSL (LasR experiments), and 100 μM C4HSL (RhlR experiments) were provided as designated. RLU are defined as light production [absorbance units (lumi)] divided by A600. A, response of the LasR reporter strain to different concentrations of 3OC12HSL in the presence of 0.1% arabinose. The EC75 for 3OC12HSL is 2.5 nm, which is the concentration used for screening. C, flavonoid nomenclature and the A-, B-, and C-rings are shown for the backbone molecule as well as the three flavonoid compounds identified in the high throughput screen. D, response of the LasR reporter strain to phloretin (1) (blue), chrysin (2) (brown), and narigenin (3) (red) in the presence of 2.5 nm 3OC12HSL and 0.1% arabinose. % Control Activity, data normalized to data obtained in the absence of inhibitor compound (n = 3). The dotted line shows 100%. E, as in D using the RhlR reporter strain and 10 μM C4HSL. Error bars, S.E.
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We obtained 32 hit molecules for follow-up analysis. The set of LasR hit compounds contained three flavonoids, phloretin (1), chrysin (2), and naringenin (3) (Fig. 1, C and D). All three compounds also reduced light production in the E. coli reporter strain containing RhlR and rhlA-luxCDABE (Fig. 1E) but displayed no activity in the constitutive light-producing reporter strain (supplemental Fig. 1). Together, these results suggest that these flavonoids are dual LasR/RhlR inhibitors.

SAR of Flavonoid Inhibition of LasR and RhlR—All three flavonoid compounds identified above contain hydroxyl groups in the 5- and 7-positions of the A-ring (Fig. 1C). Additionally, phloretin (1) does not contain the conformational constraint of the chromanone/chromene C-ring present in chrysin (2) and naringenin (3). To ascertain the importance of the hydroxyl moieties in the A- and B-ring and the contribution of the C-ring to inhibition of LasR and RhlR, we conducted a focused structure-activity study. We tested 20 structural analogs at high concentration (100 μM) against each receptor (Fig. 2A). Six flavonoid compounds (Fig. 2B), in addition to the three discovered in the screen, exhibited inhibition and were subsequently examined in 10-point antagonist dose-response assays against each receptor to define potency and efficacy (Fig. 2C and supplemental Table 1). All nine compounds inhibited both receptors in dose-dependent manners. We do note that at low con-

![Diagram](image_url)
centrations, and only in the presence of AlS, the flavonoids modestly activate the reporter strains (Figs. 1D and 2C). This feature will need to be considered if these compounds are further explored for applications.

The A-ring SAR—We first performed a systematic survey of the A-ring hydroxyl groups to determine their importance in LasR/RhlR inhibition. The three monohydroxy flavones, 5-hydroxyflavone (7), 6-hydroxyflavone (13), 7-hydroxyflavone (8), and the flavone lacking any A-ring hydroxyl group, flavone (4), were all inactive (Fig. 2 (A and B) and supplemental Table 1). Of the dihydroxy analogs tested, chrysin (2) and 7,8-dihydroxyflavone (17) are potent LasR/RhlR inhibitors, whereas 5,6-dihydroxyflavone (16) displayed no activity. The corresponding 6,8-dihydroxyflavone was not available for testing. These data suggest that 1) two hydroxyl groups are necessary for potent inhibition and 2) one of them must be at position 7 on the A-ring. The presence of three hydroxy groups on the A-ring (i.e. baicalein (9)) is tolerated but does not increase inhibitory potency. A free hydroxy group is essential for activity, because the methyl ether analogs 5,7-dimethoxyflavone (12), 5,7,4′-trimethoxyflavone (14), and 5,7-dimethoxy-4′-hydroxyflavone (15) were all inactive. Thus, either the binding pocket that the A-ring fits into is small or the presence of hydrogen bond donors on the A-ring is important for activity.

The C-ring SAR—None of the modifications that we examined in the C-ring appeared to significantly influence LasR/RhlR inhibition. Specifically, the double bond between positions 2 and 3 is not a strict requirement for activity because naringenin (3) and pinocembrin (19) lack the C-ring double bond and possess inhibitory capability (Fig. 2 (A and B) and supplemental Table 1). The presence of a C-3 hydroxyl moiety is tolerated, because quercetin (11) and 3,5,7-trihydroxyflavone (18) are LasR/RhlR inhibitors (Fig. 2 (A and B) and supplemental Table 1). Finally, as noted above, phloretin (1) does not contain the C-ring chromanone structure yet retains activity. This result suggests that it is able to adopt a conformation similar to that of the chromanone/chromenone-based flavonoids when binding to LasR/RhlR.

The B-ring SAR—Hydroxyl groups on the B-ring are not absolutely required for LasR/RhlR inhibitory activity because a number of analogs with a simple phenyl B-ring are active (e.g. quercetin (11)). The presence of 3′- and 4′-hydroxyl groups in the B-ring is tolerated, but these groups do not enhance LasR/RhlR inhibition. The presence of methyl ether groups, by contrast (acacetin (5) and diosmetin (10)), eliminated inhibitory activity, which suggests that there is a specific steric constraint within the protein binding pocket that the B-ring occupies (Fig. 2 (A and B) and supplemental Table 1).

Taken together, our data reveal that, with respect to LasR/RhlR inhibition by the representative set of flavonoids tested here, there is a requirement for a hydroxyl group at position 7 of the A-ring combined with at least one other hydroxyl group elsewhere on the A-ring. Additional hydroxyl groups in the A-ring are tolerated, whereas larger methyl ethers are not. The C- and B-rings can accommodate many substitutions, with the exception that methyl groups on the B-ring are not tolerated. Some of the flavonoids that inhibit LasR are also capable of inhibiting RhlR (e.g. 7,8-dihydroxyflavone (17)), whereas some are not (e.g. pinocembrin (19); Fig. 2 (A and B) and supplemental Table 1).

Diaparoxyl Flavonoids Show Cross-species Receptor-Inhibitory Activity—We wondered whether flavonoid inhibition was restricted to the P. aeruginosa LasR and RhlR receptors or whether flavonoids generally inhibit AI binding QS receptors. To explore specificity, we examined several flavonoids for inhibition of another LuxR type protein, CviR, which, when bound to its cognate AI, C4HSL, activates expression of the vioA promoter. We measured a vioA-gfp transcriptional fusion as the readout (Fig. 3A). As a control, we used C10HSL, which is a competitive inhibitor of C4HSL (27). None of the flavonoids significantly inhibited CviR function (Fig. 3A). Conversely, C4HSL could also not activate LasR in our reporter assay (Fig. 3B). Thus, there appears to be no cross-activation and no cross-inhibition between these receptors at least with respect to the molecules under study here. We also examined the flavonoids for inhibition of LuxN, the Vibrio harveyi AI receptor that detects the AI 3OHC4HSL (28). Unlike LasR, RhlR, CviR, and other LuxR-type receptors, LuxN is a transmembrane protein that transduces the AI binding event into the cell via a phosphorylation-dephosphorylation cascade. In V. harveyi, bioluminescence is the endogenous output of QS signal transduction. Specifically, the AI, 3OHC4HSL, stimulates light production, and this activity depends on LuxN. Phloretin (1) and quercetin (11) inhibited LuxN-dependent light production, although less potently than does the previously characterized inhibitor, chlorolactone (CL) (Fig. 3C) (28, 29). Thus, both types of antagonists that we have discovered, structural analogs of HSL AIs and the structurally distinct flavonoid analogs, inhibit LuxN. By contrast, CviR and LasR/RhlR show specificity with respect to antagonism, in agreement with previous findings that CL specifically inhibited CviR but not RhlR and LasR (18). These results indicate that flavonoids could conceivably be used to target QS networks of multiple bacterial species. However, their ability to do so must be evaluated on a case-by-case basis because their SARs may differ depending on receptor type.

Flavonoids Inhibit LasR and RhlR through a Non-competitive Mechanism—The discovery of flavonoids as a new class of LasR/RhlR inhibitors is intriguing because they are not structurally similar to the native AIs or to previously reported competitive LasR/RhlR inhibitors, which are all structural analogs of the AIs (13, 14, 30). It is possible that the flavonoids function competitively by occupying the ligand-binding site. We call this mechanism 1. Other potential mechanisms of inhibition are equally plausible and include inhibition of receptor stability/solubility (mechanism 2), disruption of receptor dimerization (mechanism 3); impairment of DNA binding (mechanism 4); and interference with RNA polymerase engagement (mechanism 5). Here, using LasR as the representative receptor, we examine possible mechanisms to characterize how the flavonoid inhibitors function.

We first tested whether the flavonoids are competitive inhibitors of LasR (mechanism 1). We assayed the E. coli bioluminescent reporter strain for inhibition of light production by 100 μM flavonoid in the presence of different concentrations of the native AI, 3OC12HSL. If the inhibition mechanism is competi-
tive, the expectation is that for each 10-fold increase in AI concentration assayed, a corresponding 10-fold decrease in inhibitor potency should occur. However, Fig. 4A shows that phloretin (1), chrysin (2), baicalein (9), quercetin (11), and 7,8-dihydroxyflavone (17) inhibited light production in a manner that was independent of AI concentration. These results suggest that the flavonoid inhibitors do not act via competition with the AI for the ligand-binding pocket.

LasR and related proteins, including RhlR, do not fold and are thus insoluble in the absence of their cognate AI ligands (31–35). Exogenously supplied AIs, synthetic agonists, or antagonists that bind in the ligand-binding pockets typically allow LasR-type proteins to fold and therefore become soluble. We reasoned that if the addition of flavonoids stabilized LasR in a soluble form, it would indicate that flavonoids functioned by a competitive inhibition mechanism. If not, it would further confirm the results in Fig. 4A indicating that the flavonoids do not act via competition with the AI for the ligand-binding pocket.

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**Flavonoids Prevent LasR from Binding DNA**—We next explored the possibility that the flavonoids function by mechanism 3, disruption of LasR dimerization (36). To do this, we expressed and purified full-length LasR bound to 3OC12HSL in the presence and absence of flavonoids. As expected, administration of exogenous 3OC12HSL at 100 μM caused a significant fraction of the LasR protein to become soluble. Similarly, mBTL, which binds in the ligand binding site, also solubilizes LasR (Fig. 4B). By contrast, the addition of chrysin (2) or 7,8-dihydroxyflavone (17) did not cause LasR to be stabilized (Fig. 4B). These results indicate that flavonoids do not bind in the ligand binding pocket in LasR, validating the data in Fig. 4A. We conclude that flavonoids do not function competitively (i.e., they do not function by mechanism 1 above). Of note, the flavonoids also do not decrease LasR solubility in the presence of 3OC12HSL (Fig. 4B), showing that they also do not function by mechanism 2 above. Together, the dose-response and solubility analyses eliminate mechanism 1 (competitive inhibition) and mechanism 2 (inhibition of receptor stability/solubility).

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that we demonstrated in Fig. 1A is sufficient for binding by LasR and activation of transcription. LasR-3OC12HSL bound to the lasB promoter with high affinity, causing the DNA probe to shift (supplemental Fig. 2) (35). Incubation of LasR-3OC12HSL with a 100 μM concentration of the test flavonoids phloretin (1), chrysin (2), baicalein (9), quercetin (11), and 7,8-dihydroxyflavone (17) prevented LasR-3OC12HSL DNA binding by ~50% or more (Fig. 5B and supplemental Fig. 2). Inhibitor
activity in the lasB-luxCDABE reporter assay correlated well with the ability of each compound to prevent LasR-3OC_{12}HSL from binding to DNA. Consistent with these data, flavonoids that did not inhibit LasR-dependent transcription in the luciferase assay were likely to not affect LasR-3OC_{12}HSL DNA binding (Fig. 5B and supplemental Fig. 2; see 5,7-dimethoxyflavone (12) and baicalin (20)).

To ensure that the flavonoids were not binding to the DNA substrate itself and in so doing preventing LasR-3OC_{12}HSL from binding, we used thermal shift assays to assess flavonoid binding to LasR-3OC_{12}HSL. We incubated 5 μM LasR-3OC_{12}HSL with 1% DMSO, 100 μM phloretin (1), quercetin (11), 7,8-dihydroxyflavone (17), baicalin (20), C_{4}HSL, or 4,5-dihydroxy-2,3-pentanedione (DPD; an AI made by a variety of bacteria that is commonly called AI-2). The final three molecules, baicalin (20), C_{4}HSL, and DPD, represent non-binding control molecules. We melted the protein with a temperature gradient of 0.05 °C/s from 25 to 99 °C. No shift in ΔT occurred upon the addition of the DMSO control solvent. A large shift in ΔT (5.434 °C) (Fig. 5C and Table 1) occurred when 3OC_{12}HSL was added to LasR-3OC_{12}HSL, indicating that as LasR unfolds and releases prebound 3OC_{12}HSL, additional AI can bind to and restabilize LasR. The addition of C_{4}HSL, DPD, or baicalin (20) did not result in a shift in ΔT (Fig. 5C and Table 1). By contrast, phloretin (1), quercetin (11), and 7,8-dihydroxyflavone (17) caused intermediate thermal shifts with ΔT values of 1.055, 1.480, and 1.318 °C, respectively (Fig. 5C shows quercetin (11), and Table 1 provides the data for all test compounds). These results show that the active flavonoids bind directly to the LasR protein. We conclude that flavonoids act as inhibitors via mechanism 4, by preventing LasR from binding to promoter DNA.

We did not test mechanism 5, impairment of the ability of LasR to interact with RNA polymerase, because our findings show that DNA binding, the step in the LasR-transcriptional activation process preceding LasR-RNA polymerase engagement, is disrupted by the flavonoids.

**TABLE 1**

| Protein and molecule | Melting temperature* °C | ΔT °C |
|----------------------|-------------------------|-------|
| LasR                 |                         |       |
| DMSO                 | 53.959                  | 0     |
| 3OC_{12}HSL          | 59.393                  | 5.343 |
| C_{4}HSL             | 53.858                  | −0.101|
| DPD                  | 54.122                  | 0.163 |
| I                    | 55.014                  | 1.055 |
| II                   | 55.439                  | 1.48  |
| III                  | 55.277                  | 1.318 |
| 20                   | 54.122                  | 0.163 |
| mBTL                 | 60.711                  | 6.752 |
| 3OC_{12}HSL + I      | 60.211                  | 6.252 |
| 3OC_{12}HSL + II     | 60.447                  | 6.488 |
| 3OC_{12}HSL + 17     | 60.711                  | 6.752 |
| 3OC_{12}HSL + mBTL   | 59.129                  | 5.17  |
| LasR LBD             |                         |       |
| DMSO                 | 51.486                  | 0     |
| 3OC_{12}HSL          | 55.967                  | 4.481 |
| C_{4}HSL             | 51.757                  | 0.264 |
| 11                   | 54.649                  | 3.163 |
| 3OC_{12}HSL + 11     | 58.075                  | 6.589 |

* The melting temperature (°C) was averaged from six replicates, and the ΔT (°C) was calculated using the melting temperature of the DMSO control for both full-length LasR and the LasR LBD.
AI and mBTL together, $\Delta T = 5.434$ for AI alone) (Table 1). The differing $\Delta T$ values for the competitive compound mBTL and the flavonoids reinforce our conclusion that flavonoids do not use the AI binding site.

**Flavonoids Inhibit QS-dependent Transcription and QS-directed Behaviors in Vivo**—The above results show that particular flavonoids inhibit LasR and RhlR in recombinant *E. coli*, and they inhibit purified LasR protein in vitro. To examine the in vivo consequences of flavonoid inhibition of QS receptors, we first assayed regulation of the QS-controlled promoter rhlA fused to mNeonGreen (mNG) in *P. aeruginosa* PA14. We confirmed that our chromosomal reporter, PrhlA-mNG, was QS-regulated by assaying its expression in different QS mutant backgrounds. As expected, compared with wild-type *P. aeruginosa* PA14, the $\Delta$lasR and $\Delta$rhlR mutants exhibited reduced PrhlA-mNG expression (Fig. 6A). The addition of 100 $\mu$M phloretin (1) or 7,8-dihydroxyflavone (17) to wild-type *P. aeruginosa* caused 15 and 24% decreases, respectively, of PrhlA expression. These results indicate that both molecules function in vivo to inhibit QS-controlled transcription of a target gene. Only modest inhibition is expected in this assay because complete loss of rhlA expression does not occur in QS mutants (Fig. 6A); nor does complete inhibition of rhlA expression occur in the *E. coli* reporter assay when phloretin (1) or 7,8-dihydroxyflavone (17) is provided (Fig. 2A).

We also measured production of pyocyanin, a QS-controlled virulence factor. All of the flavonoids that we characterized as LasR/RhlR inhibitors significantly decreased pyocyanin pro-
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duction in *P. aeruginosa* PA14 (Fig. 6B). Flavonoids that were characterized as inactive in our above tests did not reduce pyocyanin production. As a reference, we have been unable to show this directly. We have shown that the flavonoids inhibit the LasR LBD and prevent the protein from binding to DNA. RhlR has, for 2 decades, proven intractable to purification and biochemical analysis. We expect our LasR mechanistic interpretations to extend to RhlR, given the similarity between LasR and RhlR; however, we are unable to show this directly. We have shown that the flavonoids inhibit both LasR and RhlR in our recombinant *E. coli* reporter assays. *In vivo*, the flavonoids inhibit transcription of rhlA, reduce pyocyanin production, and prevent swarming. A previous report noted that dihydroxyflavonoids repress certain QS behaviors, such as pyocyanin production, elastase production, and biofilm formation in *P. aeruginosa* and other bacteria (20–22). However, the flavonoid mechanism of action was not studied before the present work. Our findings underpin those earlier results with mechanistic insight.

Our findings are also consistent with previous results showing that flavonoids can bind to particular transcription factors that contain variable LBDs and canonical helix-turn-helix DNA-binding domains. One example is the TetR-type transcription factor, TtgR. The crystal structure of TtgR bound to quercetin (11) and narigenin (3) revealed that binding relied on six amino acids forming hydrogen bonds with the hydroxyl groups on the quercetin (11) and narigenin (3) rings. The structure of TtgR bound to phloretin (1) showed that phloretin (1) uses a binding pocket similar to that used by narigenin (3) and quercetin (11) as well as a second, adjacent binding pocket (37). However, phloretin (1) could not occupy both binding sites in the same monomer. The binding of these flavonoids to TtgR disrupted the ability of TtgR to bind to DNA, similar to our findings with LasR (37). Simulations of flavonoids binding to other transcription factors indicate that π–π stacking interactions could stabilize flavonoid–protein interactions (38, 39). For example, the intermolecular forces stabilizing the interaction between nsP3 (non-structural protein 3) from the Chikungunya virus and baicalein (9) were calculated, and extensive hydrogen bonding with the hydroxyl groups in the A-ring of baicalein (9) was predicted. An important π–π interaction was also predicted to occur between a tryptophan residue on nsP3 and the B-ring of baicalein (9) (39). Consistent with these data suggesting that flavonoid ligands employ multiple amino acids for binding, we have been unable to identify any single missense mutation in LasR that confers resistance to the flavonoids.

All LasR and RhlR inhibitors reported previously function by binding in the ligand binding site of the receptor LBD (18, 40). Our understanding of the mechanistic consequences of such competitive inhibitory mechanisms in this protein family was accelerated by the solution of the structure of CviR from *Chromobacterium violaceum* bound to the competitive inhibitor CL (27). CL, when in the AI binding site, induces a closed domain, closed conformation that locks the CviR DNA binding helices into a configuration that is incompatible with DNA binding (27). Although not competitive, the flavonoids also prevent DNA binding. It is possible that by binding in the LBD, the flavonoids cause long range conformational changes that also lock the DNA-binding domain into an unfavorable configuration.

Flavonoids may present an exciting avenue for future pharmacological development, given that the work described here provides a new mechanism of action for them. For example, approaches similar to those employed here could be used to explore flavonoid inhibition of QS systems in other pathogenic bacteria (21). With respect to *P. aeruginosa*, potential applications of the present findings are enhanced by a recent report showing that QS activates CRISPR-Cas (clustered regularly interspaced short palindromic repeats) adaptive immunity in *P. aeruginosa* PA14 (41). *P. aeruginosa* PA14 uses the CRISPR-Cas system to eliminate invading phages. QS activates CRISPR-Cas expression, increases activity, and enhances adaptation, presumably optimizing the timing and level of deployment of this defensive mechanism. Inhibition of QS through small molecules, such as flavonoids, could effectively repress virulence factor production while simultaneously rendering *P. aeruginosa* more susceptible to phage infection through suppression of QS-directed activation of CRISPR-Cas immunity (41). Phage therapy is not widely used in the United States; however, it is being revisited in light of current developments in antibiotic resistance, and it is an accepted antimicrobial therapy in other nations. We suggest that phage therapy coupled to QS inhibition in *P. aeruginosa* could be explored as a combination therapy with far reaching implications, perhaps beyond medicine, for animal husbandry, agriculture, and engineering.

Flavonoids are produced by plants as secondary metabolites, and they have a range of pharmacological effects (19, 42). For instance, chrysin (2), described here as an inhibitor of LasR and RhlR, is an inhibitor of glycogen phosphorylase and is proposed to have the potential to control hyperglycemia in type 2 diabetes patients (43). Other flavonoids possess traditional antibiotic activity (44, 45). Flavonoids combined with conventional antibiotics can enhance the efficacy of the antibiotic, as is the case with 6,7-dihydroxyflavone and β-lactam antibiotics in methicillin-resistant *Staphylococcus aureus* (46). Thus, exploration of flavonoids, either alone or in combination with existing therapies, for new uses, such as we have shown here for QS, seems a promising route.

In their native roles as plant metabolites, flavonoids are crucial for root nodule development (47). Specifically, some actinorhizal plants require nitrogen-fixing rhizobial symbionts. In this relationship, the rhizobia convert atmospheric nitrogen
Flavonoids activate *nod* gene expression in rhizobia, and the bacterial *nod* components are required for nodule development in the plant, establishing the location for nitrogen fixation (48, 49). Flavonoids can also repress bacterial *nod* gene expression, allowing the plant and bacteria to fine tune their interactions such that overexpression of *nod* factors does not occur, which avoids initiation of the host plant’s defense response (45).

One flavonoid, nariginenin (3), induces *nod* gene expression in rhizobia, and here we show that it is also a potent LasR and RhlR inhibitor (50). Given that we and others have shown that flavonoids function as transcription factor modulators, we speculate that flavonoids could have evolved as a consequence of the plants’ need to influence transcriptional regulation in bacterial symbionts and, perhaps, in pathogens as well. Indeed, *P. aeruginosa* is a ubiquitous bacterium, and it is found in the rhizosphere, where it acts as a pathogen that relies on QS for virulence (51, 52).

Flavonoid production in plants and AI signaling in bacteria have other known links because certain legumes produce flavonoids in response to the presence of bacterial AIs (53). Additionally, in a few instances, plants produce QS mimics in response to bacterial cues. For instance, *p*-coumaric acid is exuded by legume roots, and this molecule can alter QS signaling in bacteria that use *p*-coumaroyl HSL as an AI (54, 55). It is particularly fascinating to envision instances of co-evolution in which plants produce flavonoids that function to maintain symbiotic bacteria via enhancement of QS while simultaneously suppressing potential pathogens through inhibition of QS (56). Our findings indicate that such natural products have promise as plausible alternatives/supplements to traditional antibiotics and as possible new stand-alone medicines (24).

**Experimental Procedures**

*P. aeruginosa* Strain Construction—To construct the PrhlA-mNeonGreen transcriptional reporter, 500 bp of DNA upstream of the *rhlA* gene and the mNeonGreen open reading frame were amplified using *P. aeruginosa* PA14 genomic DNA and the plasmid pmNeonGreen-N1 (licensed from Allele Biotech) (57) as templates, respectively. Next, two DNA fragments of ~730 bp, one corresponding to the intergenic region ~700 bp downstream of the PA14_20500 gene and the other corresponding to ~1000 bp upstream of PA14_20510, were amplified using PA14 genomic DNA as templates. The four DNA fragments were stitched together by Gibson assembly and the plasmid pmNeonGreen-N1 was transformed into TOP10 *E. coli* cells using 1 mM IPTG at 30 °C. Stationary phase cultures were back-diluted 1:1000 in 250 ml of fresh LB medium containing antibiotics and grown at 37 °C until *A*$_{600}$ = 0.5 or for ~4 h. For inhibition assays, arabinose was added to a final concentration of 0.1%. AIs were used at 2.5 nM and 10 μM for LasR and RhlR, respectively. For dose-response assays, 100× stocks were generated in DMSO. These stocks were assayed at various dilutions in the reporter assays, starting at 100 μM. Assays were carried out in 96-well plates (Corning). Plates were incubated at 30 °C for 4 h, and bioluminescence was measured on an Envision 2103 Multilabel Reader (PerkinElmer Life Sciences) with a measurement time of 0.1 s. *A*$_{600}$ was measured using a photometric 600-nm filter at 100% light emission. Relative light units (RLU) were calculated by dividing the bioluminescence measurement by the *A*$_{600}$ measurement. To construct the constitutively bioluminescent reporter strain, the DNA encoding the *tac* promoter was amplified and cloned upstream of the *luxCDABE* operon from *V. harveyi* to make plasmid pCS26. pCS26 was transformed into TOP10 *E. coli*, which does not possess the *lacI*Q genes, resulting in constitutive expression of *luxCDABE*. For consistency with our primary screen, the *lasR*-containing plasmid pJP100 was transformed into this strain. Dose-response assays were performed on hit compounds as described above. Compounds that reduced light production in this strain were eliminated from further analyses. The CviR and LuxN reporter assays were performed as described previously (28).

*High Throughput Small Molecule Screen*—The plasmids carrying *lasR* and *lasB-luxCDABE* were co-transformed into TOP10 *E. coli* cells (Invitrogen) and grown as described above. Stationary phase cultures were back-diluted 1:1000 in 250 ml of fresh LB medium containing antibiotics and grown at 37 °C until *A*$_{600}$ = 0.5 or for ~4 h with shaking. Arabinose was added to a final concentration of 0.1% (v/v), and AI was added to 2.5 nM. 384-well plates (Corning) were supplied with 200 nl of 10 mM stock compounds dissolved in DMSO from the Princeton University Small Molecule Screening Center. 20 μl of the reporter strain culture was next added by an automatic plate filler (Thermo Multidrop Combi). The first two and final two columns of each plate were left blank as controls. Positive control wells received arabinose, AI, and DMSO but no small molecule. Negative control wells received no AI. Plates were incubated at 30 °C for 4 h, bioluminescence and *A*$_{600}$ were measured, and RLU were calculated as described above. The average and S.D. were calculated for the positive control group. For each plate, the *Z*’ was calculated, and hits were only considered for wells with *Z*’ > 0.7. Compounds that caused bioluminescence reductions at least three S.D. values from the mean were considered hits and were retested from frozen stocks. Once confirmed, candidate hit compounds were obtained as powders (Sigma and Cayman Chemical) and retested.

*LasR Protein Purification*—Full-length LasR (cloned into pET23b) was produced in BL21 *E. coli* cells using 1 mM IPTG at
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18 °C overnight in the presence of 100 μM 3OC₁₂HSL. Cells were pelleted at 3000 rpm and resuspended in lysis buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8, 20 mM imidazole, 1 mM EDTA, 1 mM DTT, 5% glycerol). The cell resuspension was lysed using sonication (1-s pulses for 15 s with a 50% duty cycle). The soluble fraction was isolated using centrifugation at 32,000 × g. To prepare the protein for heparin column binding, the soluble fraction was diluted 5-fold in buffer A (20 mM Tris-HCl, pH 8, 1 mM DTT). The protein was loaded on to a heparin column (GE Healthcare) and eluted using a linear gradient from buffer A to buffer B (1 mM NaCl, 20 mM Tris-HCl, pH 8). Peak fractions were collected and assessed by SDS-PAGE analysis. Fractions were pooled and again diluted 5-fold in buffer A and then loaded onto a MonoQ column (GE Healthcare) and eluted using a linear gradient from buffer A to buffer B. Peak fractions were collected for SDS-PAGE analysis, pooled, and concentrated for size exclusion chromatography on a GE Healthcare S200 column in 20% buffer B. Peak fractions were pooled, concentrated to 2 mg/ml, flash-frozen, and stored at −80 °C. His₆-tagged LasR LBD was produced as described for the full-length protein. The soluble fraction was applied to a nickel-nitrotriacetic acid column and eluted using a linear gradient of buffer C (200 mM NaCl, 20 mM Tris-HCl, pH 8, 20 mM imidazole, 1 mM DTT) to buffer D (200 mM NaCl, 20 mM Tris-HCl, pH 8, 1 mM imidazole, 1 mM DTT). Peak fractions were pooled, and protein homogeneity was verified on a S200 size exclusion gel filtration column as described above.

Electrophoretic Mobility Shift Assay—The lasB promoter sequence (−250 to −20) was amplified using PCR and end-labeled with ³²P using PNK enzyme (Fermentas). The labeled probe was incubated with 0, 25, and 50 ng of purified LasR-3OC₁₂HSL in binding buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 50 mM KCl, 1.5 mg/ml poly(I-C), 50 μg/ml BSA, and 10% glycerol) containing DMSO or 100 μM flavonoids, back-diluted 1:50 into fresh medium containing appropriate test compounds. The cultures were grown for 5 h and back-diluted 1:50 into fresh medium containing test compounds. The cultures were grown for 18 h. The cells were pelleted by centrifugation, and the cell-free culture fluids were passed through 0.22-μm filters into clear plastic cuvettes. The A₅₉₀ of the filtered culture fluids was measured on a spectrophotometer (Beckman Coulter DV 730).

Swarming Assay—Cultures of *P. aeruginosa* PA14 and the ΔlasRΔrhlR mutant were grown overnight in LB with DMSO, 100 μM phloretin (1), or 100 μM 7,8-dihydroxyflavone (17). 2 μl of the stationary phase cultures were spotted onto swarming agar medium (Luria-Bertani broth (Thermo) with 0.5% (w/v) casamino acids, 0.5 (w/v) glucose, and 0.5% Bacto agar) that had been supplemented with 1% DMSO or with 10 μM or 100 μM phloretin (1) or with 10 μM or 100 μM 7,8-dihydroxyflavone (17). The plates were incubated overnight at 37 °C and imaged after 24 h using an Image Quant LAS4000 gel doc system using the trans-illumination setting (GE Healthcare).

Growth Curve—Cultures of *P. aeruginosa* PA14 were grown overnight in LB medium with 1% DMSO or 100 μM test flavonoids, back-diluted 1:1000 into LB containing DMSO or a 100 μM concentration of the same flavonoid, and transferred to a 96-well plate. The plate was incubated in a BioTek Eon plate reader overnight with shaking at 37 °C. A₆₅₀ was measured every 15 min.

Author Contributions—J. E. P. conceived and coordinated the study; wrote the paper; and designed, performed, and analyzed the experiments shown in Figs. 1–6. S. M. provided technical assistance, constructed mutants, and performed experiments shown in Fig. 6. A. R. M. provided technical assistance and contributed to the preparation of the figures. J.-P. C. provided technical assistance and performed the experiments in Figs. 1 and 2. B. R. H., C. J. A., H. K., and C. D. S. assisted in the conception and coordination of the study and provided technical assistance. H. K., B. R. H., and C. D. S. provided important intellectual input. B. L. B. conceived and coordinated the study, analyzed data, and wrote the paper.

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