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Synthesis and Biological Evaluation of Cajaninstilbene Acid and Amorfrutins A and B as Inhibitors of the *Pseudomonas aeruginosa* Quorum Sensing System

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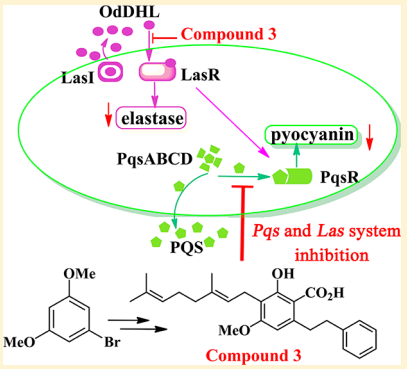
Supporting Information

ABSTRACT: The quorum sensing (QS) system inhibitors of *Pseudomonas aeruginosa* are thought to attenuate bacterial pathogenicity and drug resistance by inhibiting biofilm formation and the production of virulence factors. In this study, a synthetic approach to the natural products cajaninstilbene acid (**1**) and amorfrutins A (**2**) and B (**3**) has been developed and was characterized by the Heck reaction, which was used to obtain the stilbene core and a Pinick oxidation to give the *O*-hydroxybenzoic acid. The biological activities of these compounds against the *P. aeruginosa* quorum sensing systems were evaluated. Amorfrutin B (**3**) showed promising antibiofilm activity against *P. aeruginosa* PAO1 with a biofilm inhibition ratio of 50.3 ± 2.7. Three *lacZ* reporter strains were constructed to identify the effects of compound **3** on different QS systems. Suppression efficacy of compound **3** on the expression of *lasB-lacZ* and *pqsA-lacZ* as well as on the production of their corresponding virulence factors elastase and pyocyanin was observed.



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Multiple drug resistant (MDR) bacterial strains directly cause the death of more than 16 million people annually.[1] At least 65% of these cases are linked to bacterial communities that proliferate by forming biofilms.[2] Bacterial community activities, such as the production of numerous extracellular proteases, biofilm maturation factors, and toxins, are all associated with intercellular communication or "quorum sensing" (QS).[3,4] As an example, one of the most common of the Gram-negative bacteria found in hospital-acquired infections, *Pseudomonas aeruginosa*, has become increasingly resistant to most current antibiotic therapies as a result of its development of QS systems and its acquisition of antibiotic-resistant genes by horizontal gene transfer. *P. aeruginosa* has a relatively complex QS network that includes, at a minimum, *las*, *rhl*, and *pqs* pathways, which can cause pathogenicity. It is considered important to develop approaches to treat *P. aeruginosa* infections by regulating these QS systems.[5]

Chemical strategies to inhibit QS in *P. aeruginosa* have received significant recent attention.[6] Interestingly, a long lipophilic chain, the tail, accompanied by a polar protic moiety, the head, appear to be common structural motifs of QS inhibitors shared across multiple species.[5] The natural products cajaninstilbene acid (**1**) and its analogues amorfrutins A (**2**) and B (**3**) (Figure 1), which have been identified with many biological activities, including antibacterial,[7] cytotoxic,[8] PPARγ agonist,[9] and anti-inflammatory effects,[10] have a long lipophilic isopentenyl or geranyl (tail) and *O*-hydroxybenzoic acid core (head) and thus share the structural characteristics of QS signaling molecules. Consequently, active natural products of this sort are likely to be potential QS inhibitors.

In view of the biological potential of cajaninstilbene acid (**1**) and amorfrutins A (**2**) and B (**3**), several synthetic routes for these compounds have been developed. These are focused on manipulation of the double bond in the stilbene unit parent, and various protocols have been developed, including use of the (1) Wittig reaction,[11] (2) benzylic metalation followed by alkylation,[12] and (3) Sonogashira coupling to link an aryltriflate with a phenylacetylene.[13] In a recent report of the synthesis of cajaninstilbene acid (**1**) and amorfrutin A (**2**) and their analogues, Julia olefination and monobenzylation followed by desulfonylation with SmI_2 were used as key reactions.[14] These methods all must use methyl 2-acetoxy-6-(bromomethyl)-4-methoxybenzoate as the key intermediate, prepared in a four- or five-step synthesis that can be quite expensive. Thus, a concise efficient strategy to synthesize these compounds in fewer steps and under mild conditions may promote the development of such natural products.

In this study, a synthetic approach to the synthesis of cajaninstilbene acid (**1**) and amorfrutins A (**2**) and B (**3**) was developed. The activities of the final compounds and key intermediates against the biofilm formation and quorum sensing systems of *P. aeruginosa* were evaluated. Of these, amorfrutin B (**3**) exhibited good antibiofilm activities. The regulated effects of amorfrutin B on QS systems were investigated using a *lacZ*-based bioreporter strain by monitoring the gene expression of the *las*, *rhl*, and *pqs* QS systems, and these effects were quantified by measurement of the production of related virulence factors.

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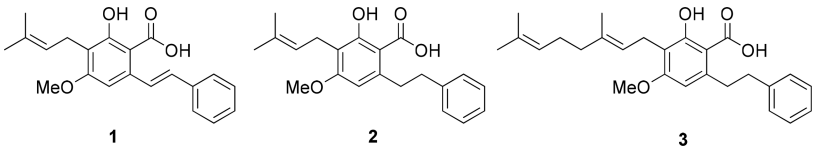
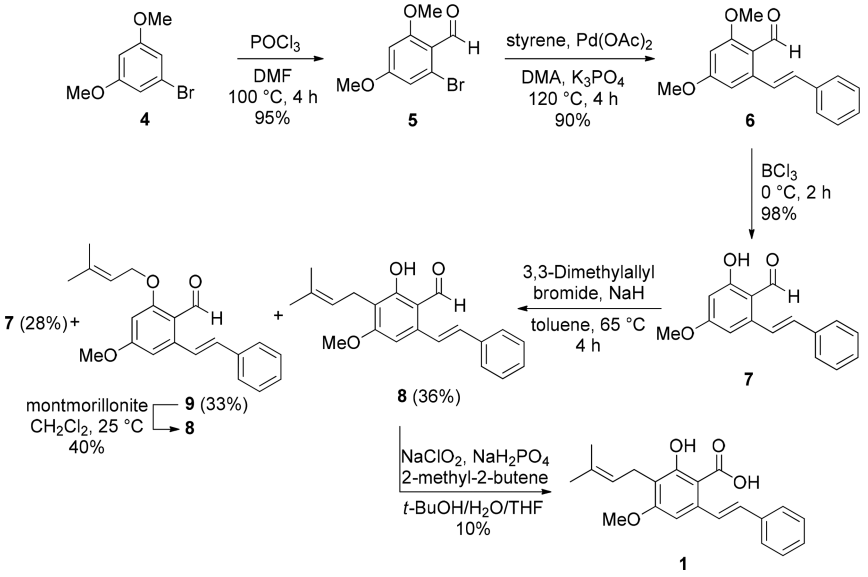


Figure 1. Structures of cajaninstilbene acid (**1**), amorfrutin A (**2**), and amorfrutin B (**3**).

Scheme 1. Synthesis Procedure of Cajaninstilbene Acid (1) with Low Yield

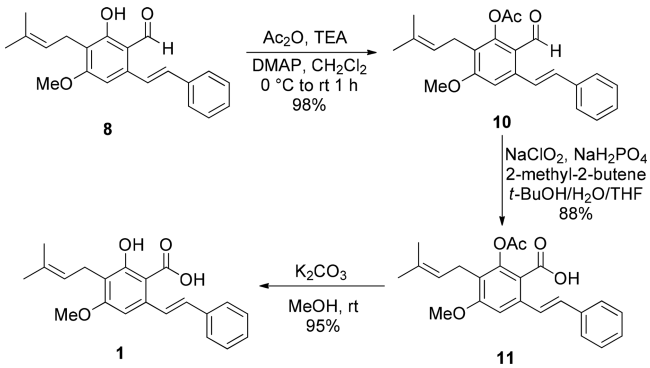


RESULTS AND DISCUSSION

Total Synthesis of Cajaninstilbene Acid (1). An initial approach to the synthesis of cajaninstilbene acid (**1**) is shown in Scheme 1. The benzaldehyde (**5**) was prepared starting from 1-bromo-3,5-dimethoxybenzene (**4**) by a Vilsmeyer formylation[15] that proceeded with high yield (95%). A ligand-free Heck reaction[16] was reported by Yao's group in 2003. Using the same protocol, product **6** was obtained in a 90% yield. Then, ortho-demethylation, giving **7** was achieved with the Lewis acid boron trichloride. With intermediate **7** in hand, a *C*-prenylation reaction was accomplished as the next step. Compound **7** was treated with sodium hydride as a base and prenyl bromide as an electrophile in toluene at 65 °C. This generated the desired *C*-prenylated compound (**8**) with a yield of 36%, the *O*-prenylated compound (**9**) in a 33% yield, and recovered starting material **7** (28%). Although the yield of *C*-prenylated compound (**8**) was acceptable, it was found that the *O*-prenylated compound (**9**) can be converted to the *C*-prenylated compound (**8**) in a 40% yield by a rearrangement induced by Montmorillonite K10.[14] Thus, the conversion of **7** to the *C*-prenylated compound (**8**) can be as high as 49% with these two transformations. The next step was the oxidation of the α-hydroxy benzylaldehyde to α-hydroxybenzoic acid. Pinnick oxidation conditions gave only 10% yield of an impure product, and other oxidation systems, such as $AgNO_3/H_2O_2$ and Ag_2O/NaOH, all failed.[17,18]

The ^{1}H NMR spectrum of compound **8** was measured, and it was found that there was an intramolecular hydrogen bond occurring between the hydroxy group and the aldehyde, which caused the signals from these groups to move downfield by about 2 ppm. In addition, the phenolic hydroxy group could be oxidized to the quinone, and, to avoid this, the *O*-hydroxy group was protected before oxidation. The intermediate **8** was converted to the corresponding acetate **10** in 98% yield under standard conditions. Pinnick oxidation of **10** to the corresponding acid and hydrolysis of the acetate gave the natural product **1**, as shown in Scheme 2, for which the structure was confirmed spectroscopically.

Scheme 2. Optimized Total Synthesis Procedure of Cajaninstilbene Acid (1)



Total Synthesis of Amorfrutins A (2) and B (3). The synthesis protocol of amorfrutins A and B is shown in Scheme 3. Following the same multiple step strategy used in the synthesis of cajaninstilbene acid (**1**), started from the intermediate (**6**), for which the double bond was reduced with hydrogen in the presence of 10% palladium on carbon to give **12** (95%). The dimethyl product (**13**) was produced quantitatively by following the procedure by which **7** was produced from **6**. The subsequent *C*-alkylation reaction with 3,3-dimethylallyl bromide and geranyl bromide as *C*-alkyation reagents, but under the same conditions used for the

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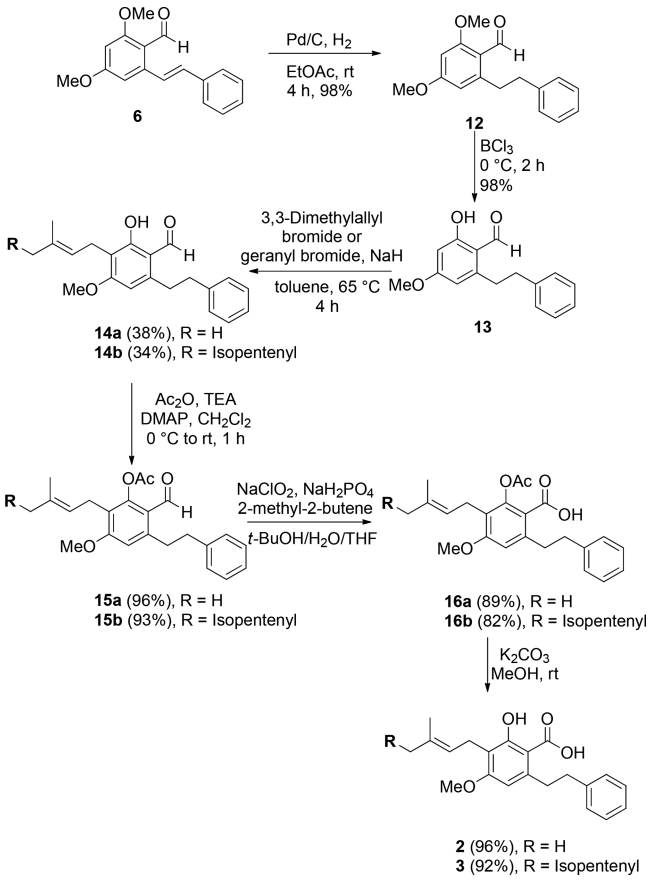
Scheme 3. Total Synthesis of Amorfrutins A (2) and B (3)



conversion of **7** to **8**, gave **14a** (38%) and **14b** (34%), respectively. Compounds **14a** and **14b** were protected separately by acetylation and Pinnick oxidation of the aldehyde group to the acid, and deprotection with base gave amorfrutin A (**2**) and amorfrutin B (**3**). The structures of the amorfrutins were established by NMR and mass spectrometry.

Evaluation of the Antibiofilm Activity of Compounds 1−3 and Some Key Intermediates. A large number of publications have shown that pathogenic bacteria such as *P. aeruginosa* employed quorum sensing to regulate biofilm development. It is becoming increasingly clear that quorum-sensing regulation can control bacterial biofilm formation. Consequently, the antibiofilm activities of all synthesized compounds, including the final compounds **1−3** and some key intermediates, which have many of the structural characteristics of QS regulators, were evaluated initially in vitro with a crystal violet staining assay using a PAO1 strain of *P. aeruginosa*. The results of screening in Table 1 and Figure 2, showed that compounds **3**, **8**, **14a**, **14b**, **15b**, and **16a** actively inhibited the formation of biofilm. Compounds **3** and **14b** showed inhibition of more than 50%. It was found that the *ortho*-hydroxybenzoic acid or the *ortho*-hydroxybenzaldehyde in the structure of cajaninstilbene acid (**1**) and its analogues was key to biofilm inhibition. If the carbon−carbon double bond between the two benzene rings was reduced, the antibiofilm activity was enhanced. Generally, similar compounds with a geranyl substituent showed better antibiofilm activity than the compounds with an isopentenyl group. Amorfrutin B (**3**) is a natural product that exhibits good activity, and it was selected for the further study of its antibiofilm activity. The effect of

Table 1. Biofilm Inhibition Rates of Cajaninstilbene Acid (1), Amorfrutins A (2) and B (3), and Key Intermediates

compound[a]	biofilm inhibition ratio (%)
1	−41.0 ± 5.9
2	−31.3 ± 1.1
3	50.3 ± 2.7***[b]
8	42.4 ± 0.4***
9	−14.3 ± 1.6
10	−12.5 ± 0.7
11	−24.4 ± 1.2
14a	26.9 ± 2.6***
14b	53.1 ± 1.9***
15a	−32.1 ± 0.1
15b	24.7 ± 2.5***
16a	36.9 ± 0.5***
16b	−32.9 ± 2.1

[a]Tested compounds were used at 50 μM. [b]***$p < 0.005$ indicates statistically significant differences from the untreated control group.

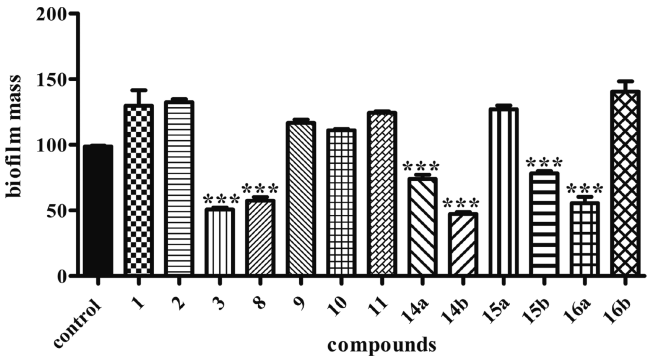


Figure 2. Histogram of biofilm formation rates following treatment with the target compounds cajaninstilbene acid (**1**), amorfrutins A (**2**) and B (**3**), and key intermediates (**8**−**11**, **14a**, **14b**, **15a**, **15b**, **16a**, and **16b**). ***$p < 0.005$ indicates statistically significant differences from the untreated control group.

compound **3** on the *P. aeruginosa* PAO1 biofilm formation was further confirmed by concentration gradient experiments. As shown in Figure 3A, biofilm formation was reduced 20−50% by treatment with compound **3** in a concentration-dependent manner. The toxicity of compound **3** on the growth of PAO1 at the same concentrations was also evaluated. As shown in Figure 3B, the curve representing treatment with compound **3** corresponded well with the control curve. This suggested that compound **3** at a range of concentrations between 1 and 50 μM had no influence on the growth of bacteria. These results demonstrate that compound **3** can serve as a potential specific biofilm formation inhibitor.

Involvement of Compound 3 in Quorum Sensing Inhibition. Quorum sensing circuits, including the *las*, *rhl*, and *pqs* pathways, are the core systems responsible for the regulation of biofilm formation and the secretion of virulence factors in *P. aeruginosa*. To explore the mechanism of inhibition by compound **3**, three reporter strains were used, PAO1-*mini-ctx-lasB-lacZ*, PAO1-*mini-ctx-rhlA-lacZ*, and PAO1-*mini-ctx-pqsA-lacZ*, in which the *lacZ* gene was integrated into the PAO1 genome. Production of β-galactosidase in these strains is known to reflect the activity of the promoters of the *lasB*, *rhlA*, and *pqsA* genes[19,20] and were constructed as illustrated in Figure 4. In this assay, PAO1-*mini-ctx-lacZ* was used as a control in the investigation of the influence of

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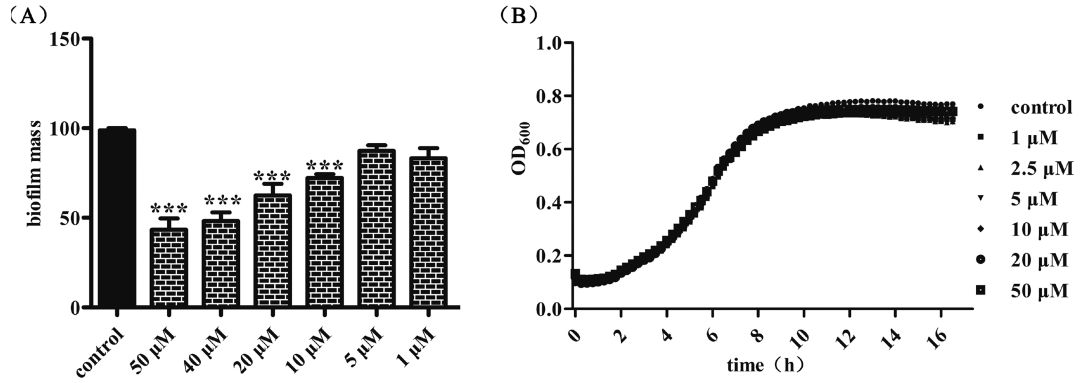

Figure 3. (A) Effects of different concentrations of compound **3** on biofilm formation. (B) Growth curve of *P. aeruginosa* PAO1 after treatment with different concentrations of compound **3**. ***$p < 0.005$ indicates statistically significant differences from the untreated control group.

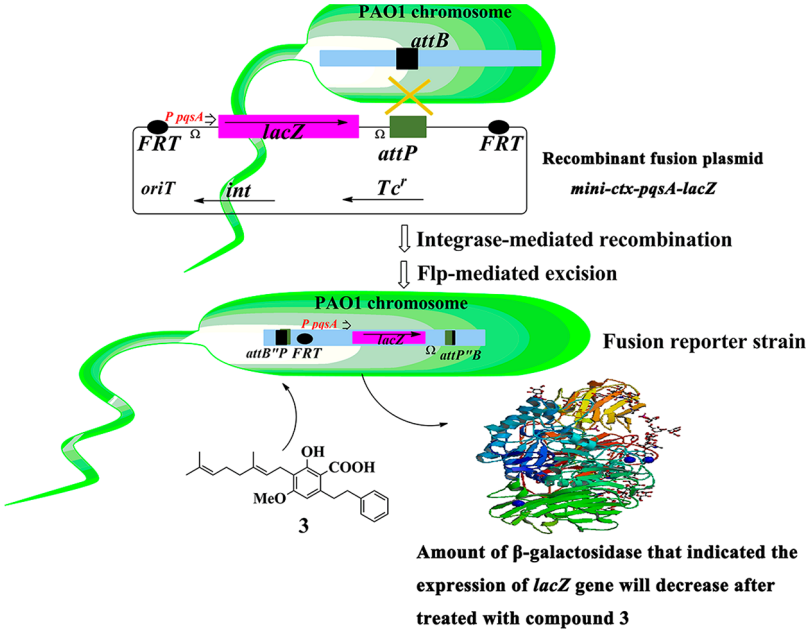

Figure 4. Schematic diagram of reporter strain PAO1-*mini-ctx-pqsA-lacZ* and the mechanism of the effect of compound **3** on the *pqs* system.

compound **3** on the expression of the *lacZ* gene. As shown in Figure 5A, no obvious impact of compound **3** on *β*-galactosidase activity was observed, and consequently the constructed *lacZ* reporter strain could be used to explore the interaction of **3** with this gene. As expected, although compound **3** had little effect on the *rhl* system since it did not depress the expression of *β*-galactosidase except at 50 *μ*M (Figure 5C), the expression of both the *las* and *pqs* systems was inhibited by compound **3** in a concentration-dependent manner (Figure 5B and D). The amount of *β*-galactosidase decreased after the addition of a 5 *μ*M concentration of compound **3**. These findings confirm that compound **3** may play a role in biofilm inhibition by interrupting the *las* and *pqs* systems.

Effect of Compound 3 on Virulence Production. As indicated by the *lacZ* reporter strain, it can be concluded that compound **3** had a significant effect on the *las* and *pqs* systems. To further verify the inhibitory effect of compound **3** on these two systems, the production of the three virulence factors elastase, rhamnolipid, and pyocyanin, which are directly controlled by the *las, rhl, and pqs* system, respectively, was measured. Consistent with the reporter strain assay results, compound **3** at concentrations below 50 *μ*M had no discernible effects on the production of rhamnolipid that is under the control of the *rhl* system. On the other hand, compound **3** reduced the production of elastase and pyocyanin, which are under the control of the *las* and *pqs* systems, respectively. As shown in Figure 6A and C, compound **3** suppressed about 50% of the pyocyanin and elastase expression at 10 *μ*M. These results further demonstrate that compound **3** serves as an inhibitor of the *las* and *pqs* pathways.

Elastase can degrade the elastin, collagen, and other matrix proteins, and consequently, it can delay the healing of wounds,[21,22] while pyocyanin can easily penetrate biological membranes and has crucial roles in *P. aeruginosa* infection. A considerable amount of research has indicated that pyocyanin can interfere with multiple cellular functions in vitro. This can lead to a wide spectrum of cellular damage and can induce neutrophil apoptosis and impair neutrophil-mediated host defenses in vivo.[23,24] More significantly, chronic exposure to pyocyanin can induce destruction of the alveolar airspace in mice, and pyocyanin is closely related to the aggravation of cystic fibrosis.[25] Thus, reduction of the virulence factor itself

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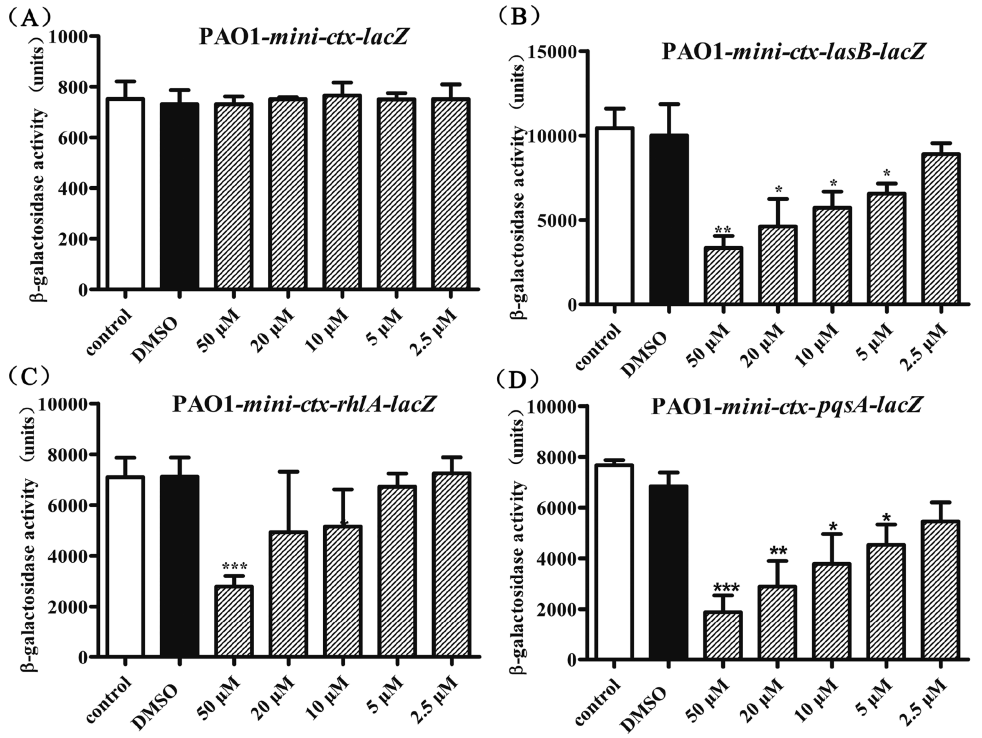

Figure 5. Dose-dependent inhibition curves of compound **3** incubated with the QS monitors PAO1-*mini-ctx-lasB-lacZ*, *rhlA-lacZ*, and *pqsA-lacZ*. PAO1-*mini-ctx-lacZ* was selected as a control to explore the effect on the expression of the *lacZ* gene after treatment with compound **3**. ***p < 0.005, **p < 0.01, *p < 0.05 indicate statistically significant differences from the untreated control group. The experiments were done in triplicate.

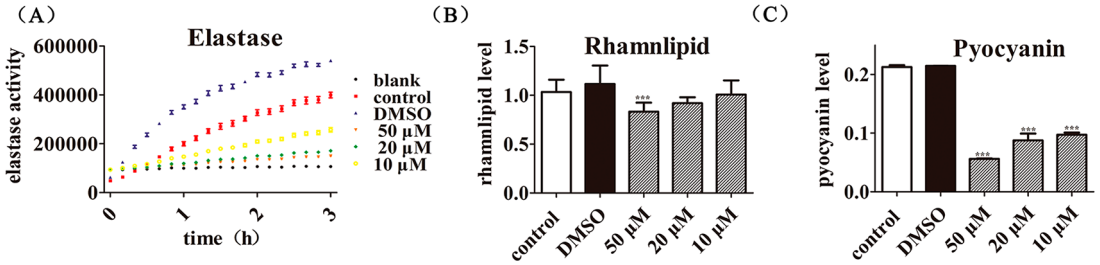

Figure 6. Effects of compound **3** on virulence production. (A) Elastase activity. (B) Rhamnolipid production. (C) Pyocyanin production. PAO1Δ*lasI*Δ*rhlI* was used as a blank, and the control used DMSO only, rather than a test compound under the same conditions. Error bars are means ± SDs. ***p < 0.005 indicate statistically significant differences from the untreated control group.

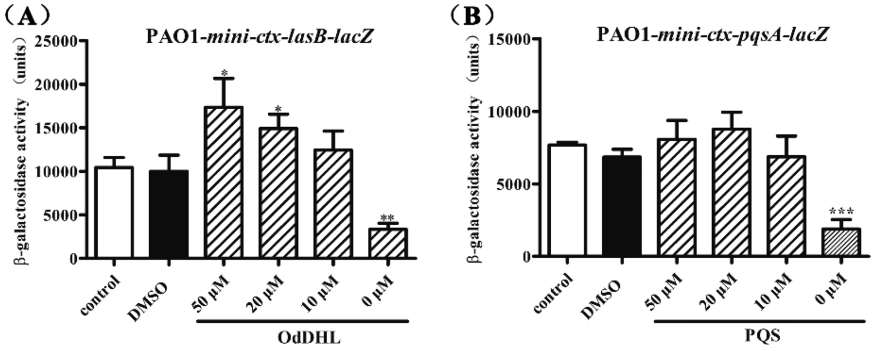

Figure 7. Dose-dependent inhibition histogram of compound **3** incubated with different concentrations of ODdHL and PQS, respectively. ***p < 0.005, **p < 0.01, *p < 0.05 indicate statistically significant differences from the DMSO control group. The experiments were performed in triplicate.

and attenuating bacterial toxicity and infectivity has great clinical significance.

To further confirm that compound **3** can competitively bind the receptors of native AHLs from *P. aeruginosa* such as ODdHL and PQS, competitive assays were conducted. The reporter strains PAO1-*mini-ctx-lasB-lacZ* and PAO1-*mini-ctx-pqsA-lacZ* were treated with compound **3** (50 μM) together with different concentrations of autoinducers. As shown in

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Figure 7, compound **3** did not inhibit *Las* and *Pqs* pathways with increasing concentrations of ODdHL and PQS. These results again support the conclusion that compound **3** inhibits the QS system, which involves competition with the receptors of native AHLs from *P. aeruginosa*.

EXPERIMENTAL SECTION

General Experimental Procedures. All reagents and solvents were purchased from commercial sources and used without further purification. CH_2Cl_2, dimethylacetamide (DMA) and dimethylformamide (DMF) were distilled from CaH_2; toluene was distilled from Na. Thin-layer chromatography (TLC) analysis was conducted on Qingdao Haiyang GF_{254} silica gel plates. Flash chromatography was performed using Qingdao Haiyang 200−300 mesh silica gel. Melting points were measured with an Optimelt automated melting point system and are uncorrected. Unless otherwise specified, ^{1}H NMR and ^{13}C NMR spectra were recorded at room temperature in base-filtered $CDCl_3$ on a Varian spectrometer operating at 300 MHz for protons and 75 MHz for carbon nuclei. The signal due to residual $CHCl_3$ appearing at δ_H 7.26 and the central resonance of the $CDCl_3$ triplet appearing at δ_C 77.16 were used to reference ^{1}H and ^{13}C NMR spectra, respectively. ^{1}H NMR data were recorded as follows: chemical shift (δ) [multiplicity, coupling constant(s) J (Hz), relative integral], where multiplicity is defined as s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet or combinations of the above. Low-resolution ESIMS were recorded on a single quadrupole liquid chromatograph−mass spectrometer, while high-resolution measurements were conducted on a time-of-flight instrument.

Demethylation by BCl_3, General Procedure A. A 1.0 M solution of BCl_3 in CH_2Cl_2 (1.5 equiv) was added dropwise at 0 °C to a solution of substrate (1.0 equiv) in CH_2Cl_2 (0.20 M). After being stirred at the same temperature for 5 min, the mixture was warmed to rt and stirred for 1 h. The reaction mixture was then poured into cold water and extracted with CH_2Cl_2. The combined organic layer was washed successively with saturated $NaHCO_3$ and brine and then dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum, and the crude product was purified by column chromatography on silica gel and eluted with a mixture of hexane and EtOAc.

***C*-Alkylation, General Procedure B.** A solution of the substrate (1.0 equiv) in anhydrous toluene (0.20 M) was added dropwise at 0 °C to a stirred solution of NaH (1.2 equiv) in anhydrous toluene (0.20 M). The combined mixture was stirred for 0.5 h, the *C*-alkylation reagent (1.3 equiv) was added by syringe, and then the solution was heated to 65 °C for 3 h. After cooling to rt, the solution was quenched with saturated NH_4Cl solution and extracted with EtOAc (3 × 50 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated to dryness under vacuum. The crude product was purified by flash chromatography on silica (30:1 hexane−EtOAc).

Protection of Hydroxy Groups by Acetylation, General Procedure C. Triethylamine (1.2 equiv) and Ac_2O (1.1 equiv) were added to a stirred solution of substrate (1.0 equiv) in CH_2Cl_2 (0.20 M) at 0 °C; then dimethylaminopyridine (DMAP) (0.05 equiv) was added. The mixture was warmed to rt and stirred for 1 h. It was quenched with a saturated NH_4Cl solution and extracted with EtOAc (3 × 50 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated to dryness under vacuum. The crude product was purified by flash chromatography on silica (10:1 hexane−EtOAc).

Pinnick Oxidation, General Procedure D. Tetrahydrofuran (1.00 mL/mmol) and H_2O (1.00 mL/mmol) at rt, $NaH_2PO_4{\cdot}2H_2O$ (2.0 equiv), and 2-methyl-2-butene (6.0 equiv) were added to a stirred solution of substrate (1.0 equiv) in *t*-BuOH (5.00 mL/mmol). The mixture was stirred for 5 min, and then $NaClO_2$ (2.0 equiv) was added. The reaction mixture was stirred at rt for 4 h. Then the solvent was removed under vacuum, water was added, and the aqueous phase was extracted with EtOAc (3 × 15 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated to dryness under vacuum. The crude product was purified by flash chromatography on silica (2:1 hexane−EtOAc).

Deprotection of the Hydroxy Group, General Procedure E. K_2CO_3 (2.0 equiv) was added to a stirred solution of substrate (1.0 equiv) in MeOH (0.20 M) at rt. The mixture was stirred at rt for 8 h. The solvent was removed under vacuum, and 2.0 M HCl was added until the pH was 6. The aqueous phase was extracted with CH_2Cl_2 (3 × 15 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated to dryness under vacuum. The crude product was purified by flash chromatography on silica (3:1 hexane−EtOAc).

*2-Bromo-4,6-dimethoxybenzaldehyde (**5**).* $POCl_3$ (6.4 mL, 69.0 mmol) was added dropwise to a stirred solution of 3,5-dimethoxybromobenzene (5.00 g, 23.0 mmol) in DMF (10.8 mL, 138.0 mmol) at 0 °C. The resulting mixture was stirred at rt for 0.5 h, then heated to 100 °C, and stirring was continued for 4 h. The reaction mixture was poured onto crushed ice (100 mL), warmed to rt, left at rt for 16 h, and then filtered. The filter cake was dissolved in ether, and insoluble materials were filtered off. The filtrate was concentrated, and the resulting crude product was purified by flash chromatography on silica (5:1 hexane−acetone) to give pure product **5** as a white solid: mp 94.8−95.7 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.29 (1H, s), 6.77 (1H, d, J = 2.3 Hz), 6.42 (1H, d, J = 2.3 Hz), 3.88 (3H, s), 3.85 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 189.3, 164.6, 163.7, 127.5, 117.0, 111.7, 98.3, 56.2, 56.0; ESIMS m/z 245.0 $[M + H]^+$; HRMS m/z 244.9811 $[M + H]^+$, calcd for $C_9H_9{}^{79}BrO_3$, 244.9808. The spectroscopic data were in agreement with those reported in the literature.[15]

*(E)-2,4-Dimethoxy-6-styrylbenzaldehyde (**6**).* An oven-dried 100 mL Schlenk flask was charged under Ar with K_3PO_4 (5.77 g, 27.2 mmol) and compound **5** (5.20 g, 19.4 mmol) in dimethylacetamide (DMA) (25 mL). Styrene (2.60 mL, 23.3 mmol) was added by syringe. $Pd(OAc)_2$ (0.061 g, 0.27 mmol) was then added, and the Schlenk tube was sealed under Ar and placed in an oil bath preheated to 120 °C; the reaction mixture was stirred for 5 h at 120 °C. After being cooled to rt, the reaction mixture was poured into water (150 mL) and extracted with EtOAc (3 × 100 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, and concentrated to dryness under vacuum. The crude product was purified by flash chromatography on silica (5:1 hexane−EtOAc) to give pure *trans*-stilbene (**6**, 4.68 g, 90%) as white solid: mp 95.1−96.5 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.53 (1H, s), 8.17 (1H, d, J = 16.2 Hz), 7.61−7.52 (2H, m), 7.42−7.32 (2H, m), 7.33−7.22 (1H, m), 7.00 (1H, d, J = 16.2 Hz), 6.75 (1H, d, J = 2.2 Hz), 6.40 (1H, d, J = 2.2 Hz), 3.91 (3H, s), 3.88 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.6, 165.1, 164.7, 142.9, 137.3, 132.5, 128.7, 128.1, 127.9, 127.1, 116.3, 103.8, 97.3, 56.0, 55.7; ESIMS m/z 269.1 $[M + H]^+$; HRMS m/z 269.1184 $[M + H]^+$, calcd for $C_{17}H_{16}O_3$, 269.1172.

*(E)-2-Hydroxy-4-methoxy-6-styrylbenzaldehyde (**7**).* Following procedure A, the substrate, compound **6** (1.50 g, 5.60 mmol), gave compound **7** (1.39 g, 98%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.49 (1H, s), 10.20 (1H, s), 7.56−7.45 (3H, m), 7.45−7.28 (3H, m), 6.98 (1H, d, J = 15.9 Hz), 6.61 (1H, d, J = 2.3 Hz), 6.37 (1H, d, J = 2.3 Hz), 3.87 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.0, 166.7, 166.3, 144.4, 136.4, 135.7, 129.0, 128.8, 127.0, 122.9, 112.5, 107.1, 100.0, 55.8; ESIMS m/z 255.1 $[M + H]^+$.; HRMS m/z 255.1019 $[M + H]^+$, calcd for $C_{16}H_{14}O_3$, 255.1016.

*(E)-2-Hydroxy-4-methoxy-3-(3-methylbut-2-en-1-yl)-6-styrylbenzaldehyde (**8**) and (E)-4-Methoxy-2-((3-methylbut-2-en-1-yl)oxy)-6-styrylbenzaldehyde (**9**).* Following procedure B, the substrate, compound **7** (1.39 g, 5.50 mmol), and 3,3-dimethylallyl bromide as a *C*-alkylation reagent gave the product **8** (0.63 g, 36%) as yellow solid: mp 139.2−141.7 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.41 (1H, s), 10.23 (1H, s), 7.60−7.48 (3H, m), 7.45−7.29 (3H, m), 6.95 (1H, d, J = 15.9 Hz), 6.61 (1H, s), 5.21 (1H, tdt, J = 5.7, 2.8, 1.4 Hz), 3.96 (3H, s), 3.36 (2H, d, J = 7.2 Hz), 1.80 (3H, d, J = 1.4 Hz), 1.69 (3H, d, J = 1.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 193.3, 163.8, 162.4, 142.5, 136.5, 135.2, 132.3, 129.0, 128.7, 126.9, 123.6, 121.8, 116.9, 112.9, 102.0, 56.0, 26.0, 21.6, 17.9; ESIMS m/z 323.2 $[M + H]^+$; HRMS m/z 323.1650 $[M + H]^+$, calcd for $C_{21}H_{22}O_3$, 323.1642.

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The *O*-alkylation compound **9** (0.58 g, 33%) was prepared as yellow solid: mp 75.9−77.3 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.56 (1H, s), 8.19 (1H, d, J = 16.2 Hz), 7.64−7.51 (2H, m), 7.41−7.32 (2H, m), 7.27 (1H, tt, J = 6.4, 1.4 Hz), 7.00 (1H, d, J = 16.2 Hz), 6.76 (1H, d, J = 2.2 Hz), 6.42 (1H, d, J = 2.3 Hz), 5.49 (1H, tdd, J = 6.4, 2.9, 1.4 Hz), 4.60 (2H, d, J = 6.7 Hz), 3.91 (3H, d, J = 0.8 Hz), 1.81 (3H, d, J = 1.4 Hz), 1.76 (3H, d, J = 1.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.0, 164.6, 142.8, 138.9, 137.4, 132.4, 128.7, 128.1, 127.2, 119.0, 116.7, 103.8, 98.5, 65.9, 55.7, 25.9, 18.4; ESIMS m/z 323.2 $[M + H]^+$; HRMS m/z 323.1642 $[M + H]^+$, calcd for $C_{21}H_{22}O_3$, 323.1642.

*(E)-2-Hydroxy-4-methoxy-3-(3-methylbut-2-en-1-yl)-6-styrylbenzaldehyde (**8**).* Montmorillonite K10 (0.59 g) was added to a solution of compound **9** (0.58 g, 1.80 mmol) in anhydrous CH_2Cl_2 (10 mL), and the reaction mixture stirred at rt for 8 h. The progress of the reaction was monitored by TLC. After complete consumption of starting material, the reaction mixture was filtered through a Celite bed and the filtrate obtained was concentrated under vacuum. The crude compound was purified by silica gel column chromatography to obtain the title compound **8** (0.23 g, 40%) as a yellow solid together with the deprenylated compound **7** (0.29 g, 50%) as the major side product.

*(E)-2-Formyl-5-methoxy-6-(3-methylbut-2-en-1-yl)-3-styrylphenyl Acetate (**10**).* Following procedure C, compound **8** (0.50 g, 1.55 mmol) as the substrate gave product **10** (0.55 g, 98%) as a white solid: mp 97.2−99.6 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.23 (1H, s), 7.83 (1H, d, J = 16.1 Hz), 7.59−7.50 (2H, m), 7.43−7.34 (2H, m), 7.34−7.27 (1H, m), 6.93 (1H, s), 6.95 (2H, d, J = 16.1), 5.10 (1H, tt, J = 7.1, 1.4 Hz), 3.97 (3H, s), 3.28 (2H, d, J = 7.0 Hz), 2.40 (3H, s), 1.77 (3H, d, J = 1.4 Hz), 1.69 (3H, d, J = 1.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 188.6, 169.5, 162.2, 151.2, 142.0, 136.7, 134.6, 132.5, 128.9, 128.5, 127.1, 125.5, 123.4, 121.0, 119.2, 107.0, 56.1, 25.8, 23.0, 20.9, 17.9; ESIMS m/z 365.2 $[M + H]^+$; HRMS m/z 365.1759 $[M + H]^+$, calcd for $C_{23}H_{24}O_4$, 365.1747.

*(E)-2-Acetoxy-4-methoxy-3-(3-methylbut-2-en-1-yl)-6-styrylbenzoic Acid (**11**).* Following procedure D, compound **10** (0.55 g, 1.51 mmol) as the substrate gave oxidation product **10** (0.50 g, 88%) as white solid: mp 135.3−138.1 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.76 (1H, s), 7.62 (1H, d, J = 16.1 Hz), 7.53 (2H, d, J = 7.5 Hz), 7.36 (2H, t, J = 7.4 Hz), 7.29 (1H, d, J = 7.2 Hz), 7.07 (1H, s), 7.01 (1H, d, J = 16.1 Hz), 5.13 (1H, t, J = 7.0 Hz), 3.97 (3H, s), 3.28 (2H, d, J = 7.0 Hz), 2.31 (3H, s), 1.77 (3H, s), 1.70 (3H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 169.5, 160.4, 149.1, 138.6, 137.0, 132.3, 131.8, 128.8, 128.2, 127.0, 126.9, 123.5, 121.2, 116.1, 106.2, 56.0, 25.8, 23.5, 20.9, 17.9; ESIMS m/z 379.1 $[M - H]^-$; HRMS m/z 379.1549 $[M - H]^-$, calcd for $C_{23}H_{24}O_5$, 379.1551.

*(E)-2-Hydroxy-4-methoxy-3-(3-methylbut-2-en-1-yl)-6-styrylbenzoic Acid (**1**).* Following procedure E, compound **11** (0.50 g, 1.32 mmol) as the substrate gave product **1** (0.42 g, 95%) as a white solid: mp 170.7−173.8 °C; ^{1}H NMR (300 MHz, acetone-d_6) δ 12.27 (1H, s), 8.08−7.91 (1H, m), 7.69−7.51 (2H, m), 7.38 (2H, ddd, J = 7.9, 6.5, 1.6 Hz), 7.28 (1H, td, J = 7.1, 1.5 Hz), 7.01 (1H, dd, J = 16.1, 1.6 Hz), 6.87 (1H, d, J = 1.5 Hz), 5.22 (1H, tt, J = 5.8, 2.9 Hz), 3.98 (3H, d, J = 1.6 Hz), 3.36 (2H, d, J = 7.3 Hz), 1.77 (3H, s), 1.64 (3H, s); ^{13}C NMR (75 MHz, acetone-d_6) δ 174.2, 163.0, 162.4, 141.8, 138.7, 131.5, 131.2, 131.2, 129.5, 128.5, 127.5, 123.2, 116.9, 104.8, 103.4, 56.2, 25.9, 22.7, 17.9; ESIMS m/z 337.0 $[M - H]^-$; HRMS m/z 337.1441 $[M - H]^-$, calcd for $C_{21}H_{22}O_4$, 337.1445.

*2,4-Dimethoxy-6-phenethylbenzaldehyde (**12**).* Pd/C (0.3 g, 10% by wt) was added to a solution of **6** (3.0 g, 11.18 mmol) in EtOAc (60 mL). The suspension was sealed with a septum under an atmosphere of H_2 supplied via a balloon. The reaction was stirred vigorously for 4 h. Following complete hydrogenation, the suspension was filtered through Celite and the filtrate was concentrated in vacuo. The crude product was purified by flash chromatography on silica (15:1 hexane−EtOAc) to give **12** (2.96 g, 98%) as white solid: mp 81.3−83.3 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.53 (1H, d, J = 1.0 Hz), 7.40−7.23 (4H, m), 7.24−7.15 (1H, m), 6.35 (1H, d, J = 2.3 Hz), 6.23 (1H, d, J = 2.3 Hz), 3.89 (3H, s), 3.80 (3H, s), 3.36- 3.19 (2H, t, J = 7.5 Hz), 2.92- 2.81 (2H, t, J = 7.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 190.3, 165.6, 164.6, 148.3, 142.2, 128.8, 128.3, 125.9, 116.9, 108.4, 96.3, 55.9, 55.5, 37.6, 37.3; ESIMS m/z 271.0 $[M + H]^+$; HRMS m/z 271.1335 $[M + H]^+$, calcd for $C_{17}H_{18}O_3$, 271.1329.

*2-Hydroxy-4-methoxy-6-phenethylbenzaldehyde (**13**).* Following procedure A, compound **12** (2.70 g, 10.00 mmol) as the substrate gave 2.51 g (98%) of compound **13** as a white solid: mp 131.1−134.0 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.53 (1H, s), 10.01 (1H, s), 7.41−7.23 (5H, m), 7.22−7.09 (3H, m), 6.43−6.05 (3H, m), 3.84 (4H, s), 3.43- 3.06 (2H, m), 3.05−2.89 (2H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.5, 166.8, 166.7, 147.8, 140.5, 128.7, 128.5, 126.6, 112.6, 109.9, 99.0, 55.7, 38.7, 33.9; ESIMS m/z 257.1 $[M + H]^+$; HRMS m/z 257.1183 $[M + H]^+$, calcd for $C_{16}H_{16}O_3$, 257.1172.

*2-Hydroxy-4-methoxy-3-(3-methylbut-2-en-1-yl)-6-phenethylbenzaldehyde (**14a**).* Following procedure B, compound **13** (1.00 g, 3.90 mmol) as the substrate and 3,3-dimethylallyl bromide as a *C*-alkylation reagent gave product **14a** (0.48 g, 38%) as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.43 (1H, s), 10.06 (1H, s), 7.37−7.23 (3H, m), 7.19−7.09 (2H, m), 6.22 (1H, s), 5.20 (1H, dddd, J = 8.5, 5.7, 2.8, 1.4 Hz), 3.83 (3H, s), 3.40−3.27 (2H, m), 3.16 (2H, dd, J = 9.2, 6.5 Hz), 2.94 (2H, dd, J = 9.2, 6.5 Hz), 1.79 (3H, d, J = 1.3 Hz), 1.69 (3H, d, J = 1.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.8, 163.9, 162.8, 145.9, 140.6, 132.0, 128.7, 128.6, 126.5, 122.0, 115.5, 112.9, 104.9, 55.8, 39.2, 34.2, 25.9, 21.4, 17.9; ESIMS m/z 325.2 $[M + H]^+$; HRMS m/z 325.1808 $[M + H]^+$, calcd for $C_{21}H_{24}O_3$ 325.1798.

*(E)-3-(3,7-Dimethylocta-2,6-dien-1-yl)-2-hydroxy-4-methoxy-6-phenethylbenzaldehyde (**14b**).* Following procedure B, compound **13** (1.00 g, 3.90 mmol) as the substrate and geranyl bromide as a *C*-alkylation reagent gave product **14b** as a yellow oil (0.52 g, 34%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.47 (1H, s), 10.09 (1H, s), 7.37−7.25 (4H, m), 7.20−7.14 (2H, m), 6.25 (1H, s), 5.23 (1H, q, J = 1.3 Hz), 5.11 (1H, tt, J = 5.5, 3.1 Hz), 3.86 (3H, s), 3.36 (2H, d, J = 7.1 Hz), 3.24−3.15 (2H, m), 2.98 (2H, d, J = 8.6 Hz), 2.14−2.05 (3H, m), 2.05−1.96 (3H, m), 1.82 (3H, d, J = 1.3 Hz), 1.69 (3H, d, J = 1.3 Hz), 1.62 (3H, d, J = 1.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.7, 163.9, 162.8, 145.8, 140.6, 135.4, 131.2, 128.6, 128.5, 126.5, 124.5, 121.8, 115.5, 112.8, 104.8, 55.8, 39.9, 39.2, 34.2, 26.8, 25.8, 21.3, 17.8, 16.2; ESIMS m/z 393.2 $[M + H]^+$; HRMS m/z 393.233 $[M + H]^+$, calcd for $C_{26}H_{32}O_3$ 393.2424.

*2-Formyl-5-methoxy-6-(3-methylbut-2-en-1-yl)-3-phenethylphenyl Acetate (**15a**).* Following procedure C, compound **14a** (0.48 g, 1.48 mmol) as the substrate gave the product **15a** (0.52 g, 96%) as a colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.18 (1H, s), 7.35−7.26 (2H, m), 7.25−7.17 (3H, m), 6.50 (1H, s), 5.07 (1H, tt, J = 7.0, 1.5 Hz), 3.82 (3H, s), 3.33−3.18 (4H, m), 2.91 (2H, dd, J = 9.3, 6.6 Hz), 2.39 (3H, s), 1.75 (3H, d, J = 1.3 Hz), 1.68 (3H, d, J = 1.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 188.3, 169.5, 162.1, 152.0, 146.0, 141.2, 132.3, 128.7, 128.5, 126.2, 122.0, 121.2, 119.3, 110.8, 56.0, 38.3, 35.9, 25.8, 22.9, 20.9, 17.9; ESIMS m/z 367.2 $[M + H]^+$; HRMS m/z 367.1914 $[M + H]^+$, calcd for $C_{23}H_{26}O_4$, 367.1904.

*(E)-2-(3,7-Dimethylocta-2,6-dien-1-yl)-6-formyl-3-methoxy-5-phenethylphenyl Acetate (**15b**).* Following procedure C, compound **14b** (0.52 g, 1.32 mmol) as the substrate gave product **15b** (0.54 g, 93%) as a colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.19 (1H, s), 7.35−7.25 (2H, m), 7.22 (3H, d, J = 7.0 Hz), 6.50 (1H, s), 5.08 (2H, ddd, J = 6.9, 5.4, 2.9 Hz), 3.81 (3H, s), 3.33−3.19 (4H, m), 2.92 (2H, dd, J = 9.3, 6.6 Hz), 2.38 (3H, s), 2.07 (2H, q, J = 7.6, 6.4 Hz), 2.01−1.92 (2H, m), 1.75 (3H, d, J = 1.4 Hz), 1.66 (3H, d, J = 1.4 Hz), 1.59 (3H, d, J = 1.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 188.3, 169.5, 162.1, 152.1, 145.9, 141.2, 135.9, 131.4, 128.7, 128.5, 126.2, 124.3, 122.1, 121.2, 119.3, 110.8, 55.9, 39.7, 38.2, 35.8, 26.7, 25.8, 22.8, 20.8, 17.8, 16.2; ESIMS m/z 435.3 $[M + H]^+$; HRMS m/z 435.2536 $[M + H]^+$, calcd for $C_{28}H_{34}O_4$ 435.2535.

*2-Acetoxy-4-methoxy-3-(3-methylbut-2-en-1-yl)-6-phenethylbenzoic Acid (**16a**).* Following procedure D, compound **15a** (0.52 g, 1.42 mmol) as the substrate gave the oxidation product **16a** (0.48 g, 89%) as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30−7.22 (2H, m), 7.19 (3H, dt, J = 8.0, 2.0 Hz), 6.52 (1H, s), 5.09 (1H, tt, J = 5.6, 1.8 Hz), 3.78 (3H, s), 3.22 (2H, d, J = 7.1 Hz), 3.17−3.07 (2H, m), 2.95 (2H, dd, J = 9.9, 6.0 Hz), 2.28 (3H, s), 1.73 (3H, d, J = 1.3 Hz), 1.67 (3H, d, J = 1.4 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 169.5, 160.1, 149.0, 142.6, 141.7, 132.1, 128.7, 128.5, 126.1, 121.9, 121.5,

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116.7, 110.5, 55.9, 38.1, 37.3, 25.8, 23.4, 21.0, 17.9; ESIMS m/z 381.0 $[M - H]^-$; HRMS m/z 381.1697 $[M - H]^-$, calcd for $C_{23}H_{26}O_5$, 381.1707.

*(E)-2-Acetoxy-3-(3,7-dimethylocta-2,6-dien-1-yl)-4-methoxy-6-phenethylbenzoic Acid (**16b**).* Following procedure D, compound **15b** (0.54 g, 1.24 mmol) as the substrate gave the oxidation product **16b** (0.46 g, 82%) as yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31−7.24 (2H, m), 7.23−7.15 (3H, m), 6.54 (1H, s), 5.17−5.02 (2H, m), 3.79 (3H, s), 3.25 (2H, d, J = 6.9 Hz), 3.14 (2H, dd, J = 9.5, 5.7 Hz), 2.97 (2H, dd, J = 9.9, 6.1 Hz), 2.29 (3H, s), 2.06 (2H, s), 1.99 (2H, d, J = 7.2 Hz), 1.74 (3H, d, J = 1.3 Hz), 1.69−1.63 (3H, m), 1.60 (3H, d, J = 1.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.1, 169.5, 160.1, 149.1, 142.5, 141.7, 135.6, 131.4, 128.7, 128.5, 126.1, 124.4, 122.0, 121.4, 116.7, 110.4, 55.8, 39.8, 38.1, 37.3, 26.8, 25.8, 23.2, 20.9, 17.8, 16.2; ESIMS m/z 449.1 $[M - H]^-$; HRMS m/z 449.2324 $[M + H]^-$, calcd for $C_{28}H_{34}O_5$, 449.2333.

*2-Hydroxy-4-methoxy-3-(3-methylbut-2-en-1-yl)-6-phenethylbenzoic Acid (**2**).* Following procedure E, compound **16a** (0.48 g, 1.26 mmol) as the substrate gave a deprotected product (**2**, 0.41 g, 96%) as a white solid: mp 139.2−141.2 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.60 (1H, s), 7.38−7.28 (2H, m), 7.26−7.19 (3H, m), 6.24 (1H, s), 5.30−5.16 (1H, m), 3.81 (3H, s), 3.37 (2H, d, J = 7.1 Hz), 3.34−3.23 (2H, m), 2.95 (2H, dd, J = 9.6, 6.3 Hz), 1.81 (3H, d, J = 1.3 Hz), 1.71 (3H, d, J = 1.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.4, 163.0, 162.3, 146.0, 142.0, 131.9, 128.7, 128.5, 126.1, 122.3, 115.5, 106.7, 103.8, 55.7, 39.4, 38.3, 26.0, 22.1, 17.9; ESIMS m/z 339.0 $[M - H]^-$; HRMS m/z 339.1605 $[M - H]^-$, calcd for $C_{21}H_{24}O_4$ 339.1602.

*(E)-3-(3,7-Dimethylocta-2,6-dien-1-yl)-2-hydroxy-4-methoxy-6-phenethylbenzoic Acid (**3**).* Following procedure E, compound **16b** (0.46 g, 1.02 mmol) as the substrate gave deprotection product **3** (0.38 g, 92%) as a white solid: mp 80.2−83.1 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.60 (1H, s), 7.31 (2H, dd, J = 7.9, 6.8 Hz), 7.23 (3H, d, J = 7.0 Hz), 6.24 (1H, s), 5.22 (1H, tt, J = 5.7, 3.2 Hz), 5.09 (1H, tt, J = 5.5, 2.6 Hz), 3.80 (3H, s), 3.38 (2H, d, J = 7.1 Hz), 3.33−3.22 (2H, m), 2.95 (2H, dd, J = 9.6, 6.3 Hz), 2.07 (2H, t, J = 7.4 Hz), 1.99 (3H, dd, J = 9.6, 5.0 Hz), 1.81 (3H, d, J = 1.3 Hz), 1.66 (3H, d, J = 1.3 Hz), 1.59 (3H, d, J = 1.3 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 176.4, 163.0, 162.3, 146.0, 142.0, 135.3, 131.3, 128.7, 128.5, 126.1, 124.6, 122.1, 115.6, 106.7, 103.8, 55.7, 40.0, 39.4, 38.3, 26.9, 25.8, 22.0, 17.8, 16.3; ESIMS m/z 407.1 $[M - H]^-$; HRMS m/z 407.2216 $[M - H]^-$, calcd for $C_{26}H_{32}O_4$, 407.2228.

Biofilm Formation Inhibition Assay by Crystal Violet Staining. The steps to measure biofilm inhibition used the method developed earlier[26] with some modifications. Thus, *P. aeruginosa* PAO1 cultured overnight in LB broth was diluted in ABTGC medium[27] (B-medium (0.1% $MgCl_2$, 0.1% $CaCl_2$, 0.1% $FeCl_3$) supplemented with 10% A10, 0.2% glucose, and 0.2% casamino acids). Test compounds were mixed with ABTGC medium, and 100 μL was added to each well. An equal amount of the bacterial suspension was added to the wells to give a final concentration of 50 μM. DMSO and medium served as a negative control and a blank, respectively. The plates were sealed with preservative film and then incubated at 37 °C for 24 h without agitation. After incubation, the supernatant was removed, and each well was washed gently with phosphate-buffered saline (PBS) three times before adding MeOH (200 μL) for 20 min. The MeOH was discarded, and 200 μL of 0.1% w/v crystal violet solution, previously filtered, was loaded into each well for staining lasting 20 min. The staining solution was removed after 20 min, and the plate was washed gently with PBS three times. Finally, 33% glacial AcOH solution was used to dissolve the crystal violet stain absorbed into the biofilm matrix. The OD_{570} was determined using a microplate reader, and the biofilm inhibitory effect was calculated and compared with a negative control. The experiment was repeated at least three times.

Construction of *lacZ* Reporter Strains and β-Galactosidase Measurement. *Mini-ctx-lacZ* plasmid was a kind gift from Singapore Centre for Environmental Life Sciences Engineering of Nanyang Technology University. PAO1-*mini-ctx-lasB-lacZ*, PAO1-*mini-ctx-rhlA-lacZ*, and PAO1-*mini-ctx-pqsA-lacZ* reporter strains were constructed according to previous documents.[28,29] First, the promoters of *lasB*, *rhlA*, and *pqsA* were amplified by following specific primers. *lasB*-F: 5′-GGGGTACCGCGGCCAGGAAAGCGTGCAA-3′, *lasB*-R: 5′-CGGGATCCTTGTTCAGTTCTCCTGGT-3′. *rhlA-F*: 5′-GGGGTACCTCCAATACCACCAACCTGCC-3′, *rhlA-R*: 5′-CGGGATCCTTCACACCTCCCAAAAATTTTCG-3′. *pqsA-F*: 5′-GGGGTACCATGCCGTCGCCCCCTTGGA-3′, *pqsA-R*: 5′-AACTGCAGCATGACAGAACGTTCCCTCTT-3′. T_4 ligase was used to link the plasmid *mini-ctx-lacZ* and promoter sequences, which were cut by restriction enzymes (*Kpn*I and *Pst*I for *PqsA*, *Kpn*I and *BamH1* for *lasB* and *rhlA*). The recombinant plasmids were transferred to DH5α by heat shock and then conjugated to the PAO1 with the helper plasmid RK600 (a kind gift from Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University). A control strain, PAO1-*mini-ctx-lacZ*, was constructed using the same technique. These recombined strains were used to evaluate the effect of synthetic compounds on different QS systems by measuring the activity of β-galactosidase. Briefly, a 2 mL bacterial culture in ABTGC medium (OD_{600} = 0.1) was shaken for 6 h at 37 °C with different concentrations of compound **3**. Then, the amount of β-galactosidase was measured using a published method.[30]

Elastase Assay. Overnight cultures of *P. aeruginosa* wild-type PAO1 were diluted in ABTGC medium to a final optical density at 600 nm of 0.01. Cultures were grown for 24 h at 37 °C with shaking (200 rpm). The cultures were centrifuged, and the amount of elastase in the supernatants was measured by using the EnzChek elastase assay kit (Invitrogen).

Pyocyanin Quantification Assay. Pyocyanin activity was estimated by measuring OD at 695 nm according to the protocol of O'Loughlin with some modifications.[31] The overnight-cultured *P. aeruginosa* PAO1 was diluted to OD_{600} = 0.01 with LB medium, and then 20 mL was inoculated into the 50 mL conical flask with shaking at 37 °C. After 20 h of incubation, the culture was centrifuged at 1500*g* for 10 min, and the absorbance at 695 nm of the supernatant was read before obtaining the final cell density by reading the OD_{600}. Data were normalized by dividing this absorbance value by the final OD_{600}.

Rhamnolipid Quantification Assay. Rhamnolipid quantification was carried out using orcinol according to an original method established by Koch and associates.[32] A fresh minimal medium (49.3 mM Na_2HPO_4, 5 mM KH_2PO_4, 4.8 mM $MgSO_4$, 7.6 mM $(NH_4)_2SO_4$, 0.6 mM $CaCl_2$, 25 μM $FeSO_4$, 0.162 μM $(NH_4)_6Mo_7O_{24}$, 38 μM $ZnSO_4$, 14 μM $MnCl_2$, 1.6 μM $CuSO_4$, 0.86 μM $CoCl_2$, 1.9 μM boric acid, 5.5 μM $NiCl_2$, 6.72 μM EDTA, 0.6% glycerol in 18 MΩ deionized water) was prepared as described previously.[33] The overnight cultured *P. aeruginosa* PAO1 was diluted with minimal medium, and then 5 mL was inoculated into the 15 mL glass tube with different concentrations of compound under shaking at 37 °C for 20 h. DMSO was selected as a control to measure whether the solvent affects the production of rhamnolipid. The culture was then centrifuged at 12000*g* at 4 °C for 5 min. Then supernatant (2 mL) was extracted with Et_2O (6 mL) with complete mixing. The organic extract (1 mL) was transferred to a 1.5 mL tube and placed in the fume cupboard while evaporating the solvent. Crude rhamnolipid was redissolved by deionized water (40 μL) and orcinal solution (360 μL, 0.19% in 53% H_2SO_4) to measure the production. The tube was mixed thoroughly, kept in an 80 °C heating block for 30 min, and then cooled to room temperature. Data were normalized by dividing the absorbance value at 421 nm by the final OD_{600}.

Competition Assays. Overnight cultures of reporter strains PAO1-*mini-ctx-lasB-lacZ* and PAO1-*mini-ctx-pqsA-lacZ* were diluted with ABTGC medium to a final OD_{600} of 0.01. Then, compound **3** (50 μM) was added to the dilute culture together with ODdHL or PQS, respectively, to give final concentrations of 50, 20, 10, and 0 μM. The amount of β-galactosidase was measured when the OD_{600} reached 1.0.

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■ ASSOCIATED CONTENT

Supporting Information

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NMR and mass spectra of compounds **1**−**3**, **5**−**13**, **14a**−**16a**, and **14b**−**16b** (PDF)

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Notes

The authors declare no competing financial interest.

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