Synthesis and Biological Evaluation of Novel L-Homoserine Lactone Analogs as Quorum Sensing Inhibitors of Pseudomonas aeruginosa

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In this study, we synthesized four series of novel L-homoserine lactone analogs and evaluated their in vitro quorum sensing (QS) inhibitory activity against two biofilm strains, Chromobacterium violaceum CV026 and Pseudomonas aeruginosa PA01. Studies of the structure–activity relationships of the set of L-homoserine lactone analogs indicated that phenylurea-containing N-dithiocarbamated homoserine lactones are more potent than (Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone (C30), a positive control for biofilm formation. In particular, compared with C30, QS inhibitor 11f significantly reduced the production of virulence factors (pyocyanin, elastase and rhamnolipid), swarming motility, the formation of biofilm and the mRNA level of QS-related genes regulated by the QS system of PA01. These results reveal 11f as a potential lead compound for developing novel antibacterial quorum sensing inhibitors.

Key words quorum sensing; L-homoserine lactone; Pseudomonas aeruginosa; dithiocarbamate; phenylurea; benzothiazolyl

Introduction

N-Acyl homoserine lactones (AHLs) are well-known quorum sensing molecules of Gram-negative bacteria.1,2 For example, Pseudomonas aeruginosa regulates its physiological activity by releasing N-(3-oxododecanoyl)-1-homoserine lactone3,4 (OdDHL, Fig. 1). Previous studies have shown that AHLs regulate the production of biofilm and virulence factors that include elastase and pyocyanin.5–7 Thus, many bacteria tend to express virulence factors when the concentration of AHLs they have generated is high enough to overcome inherent host defenses.8,9

Expression of virulence genes is also connected with las, rhl and Pseudomonas quinolone signal (PQS) systems which are important quorum sensing (QS) systems.10–13 LasI, for example, directs the synthesis of OdDHL in the las QS system. Once OdDHL reaches a critical threshold concentration, it binds to transcriptional regulatory protein lasR.14 The QS network of P. aeruginosa is very complicated, with each signaling mechanism related to and affecting another. The las system is thought to be at the top of the regulatory network, which can positively regulate expression of the rhl/R gene, the pqs1ABCDE gene cluster and the pqsR gene.3 Bacteria gain resistance to antibiotics due to the ease with which their biofilm and virulence factors induce mutations that allow them to evade the toxic effects of antibiotics.15,16 Quorum sensing inhibitors (QSIs) are thus considered a new direction for antibacterial drug discovery because they only inhibit the virulence factors of bacteria and do not interfere with the normal physiological activities of pathogens, so a resistance response is not triggered.17–19

Aromatic thioethers are a class of important sulfur-containing derivatives with biological activities, and have attracted the interest of scientific researchers. For example, cefazolin exhibits a potent inhibitory effect against Pneumococcus and Haemophilus influenza.20 Moreover, the aromatic thioether group has long been used as a linker to connect different biologically active moieties in the design of novel compounds.21–23 The phenylurea moiety has biological activities, including antibacterial, anticancer and insecticidal24 activities, and phenylurea derivatives have been found to be fungicidal against Rhizoctonia solani and Pellicularia spp.25

Brominated furanones (BFs) are a leading class of anti-virulence compounds and have been proven to prevent the normal operation of the QS system, which competes with AHLs for a receptor protein.26 We chose the frequently used (Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone (C30, Fig. 1) as a positive control for inhibition of the las system for our evaluation of the anti-biofilm formation activity of our analogs.27

Inspired by previous research about the antibacterial evaluation of AHL analogs containing thioester linkages,28 we herein report the design and synthesis of novel L-homoserine lactone analogs by introducing a thioether group (Fig. 1 7a–d, 8a–c) or a phenylurea group (Fig. 1 11a–i, 12a–e) at the side chain terminus, and the antibacterial activities of these analogs against the P. aeruginosa QS system. Structure–activity relationships (SARs) and molecular docking studies were conducted to rationalize the mechanism of the action of these QSIs.

Results and Discussion

Chemistry The L-homoserine lactone hydrochloride 4 was efficiently synthesized from methionine (1) following previously reported methods29 (Chart 1). The reported optical rotation of compound 4 ([α]20 = −27.5° (c 0.2, water)) was used
to confirm its stereochemistry.\textsuperscript{30} With crucial intermediate 4 in hand, four series of novel \textit{i}-homoserine lactone (HL) analogs were synthesized by introducing different moieties onto the amine.

Acyl compounds 5 and 6 were formed by reacting 4 with chloroacetyl chloride and 3-chloropropionyl chloride, respectively, under Schotten–Baumann conditions at room temperature.\textsuperscript{31,32} Compound series I and II were prepared by reacting different mercapto compounds with 5 and 6, respectively, in the presence of \( \text{K}_2\text{CO}_3 \). Compound series III were synthesized by one-pot reaction with compounds 10a–i\textsuperscript{34} (Chart 2). Compound series IV were synthesized by coupling compounds 10a–c with compound 4\textsuperscript{35} (Chart 3). The structures and yields of these four synthetic series are shown in Table 1 and Table 2.

**Biological Evaluation** A QSI screening model (\textit{las} system model: QSIS-\textit{las}\textsuperscript{I\textdegree}) was used to evaluate the inhibitory activity of newly synthesized compounds against \textit{P. aeruginosa}. This screening model is based on the sucrose lethal gene \textit{ sacB} being activated after self-expressed or externally added signal molecules bind to the \textit{ LasR} protein expressed by the \textit{P. aeruginosa las} system. The growth of bacteria can be inhibited in LB medium containing sucrose. Thus, if QSIs are added into LB medium, the expression of the \textit{ sacB} gene will be inhibited, meaning the bacteria can grow normally. This screening principle is consistent with the series of high throughput QSI screening models constructed by Rasmussen’s laboratory.\textsuperscript{36} The inhibitory effects of all compounds of the four series against the \textit{P. aeruginosa} PAO1 is shown in Fig. 2A.

It can be seen from Fig. 2 that analogs 7b, 8b, 7c and 8c containing acyl homoserine lactones with benzothiazolyl or 4-chlorophenyl substituents showed significant inhibitory activity of the \textit{las} QS system, and were more potent than the positive control (C30). However, analogs 7a, 7d and 8a had no effect on QS. 7b, 8b and 8c showed excellent inhibitory activity against CV026 in Figure S1. According to the biological data for series I and II against CV026, it can be seen that the extra carbon atom of 8c increased its inhibitory activity compared with that of its analog 7c.

Surprisingly, almost all compounds of series III, containing both phenylurea groups and dithiocarbamate groups, exhibited remarkable QS inhibitory (QSI) activity against CV026 (Fig. S1) and PAO1 (Fig. 2). However, only the analogs with the electron-donating groups on the terminal phenyl group (11b–f, 11h and 11i) showed powerful inhibitory activity against the \textit{las} system. These results indicated that the position and electronic character of the substituents on the terminal phenyl homoserine lactone analogs series III are critical for this series’ QSI activity. Specifically, 2-methoxy (11b) and 2-methyl (11b) analogs were more potent than 4-methoxy (11d) and 3-methyl (11i) analogs. Biological evaluation of compounds of series IV revealed that they had weak QSI activity. This clearly shows that the dithiocarbamate group of series III is critical to its QSI activity.

To explore the mechanism of action of active compounds, we tested the effects of analogs 7b, 8b, 8c, 11b, 11f and 11i on the production of virulence factors such as the inhibition of pyocyanin production, reduction of elastase activity and reduction of rhamnolipid production. Meanwhile, we examined the effects of these active compounds on the formation of biofilm and swarming motility. As shown, analogs 7b, 8b, 8c, 11b, 11f, 11i and the positive control C30 had no significant effects on the growth of PAO1 at 15 \( \mu \text{M} \) (Fig. 3A). Thus, we decided to examine their impact on virulence factors at this concentration and found that analogs 7b, 8b, 8c, 11b, 11f and 11i could inhibit the production of pyocyanin. Specifically, these analogs decreased the production of pyocyanin in PAO1 by 14.2, 15.1, 27.5, 33.0, 34.5 and 30.3%, respectively, at sub-minimum inhibitory concentrations (sub-MIC), with the inhibitory activity of analogs 8c, 11b, 11f and 11i being greater than that of C30 (31.6%) (Fig. 3B). 11b, 11f and 11i decreased the production of elastase by 14.3, 13.7 and 18.4%, respectively, almost as much as the reduction by C30 (16.5%) (Fig. 3C).

11b, 8b, 8c, 11f, 11i and C30 decreased the production of rhamnolipid in PAO1 by 20.5, 22.2, 17.6, 20.8, 28.1, 16.7 and 25.8%, respectively. Overall, 11f was the most potent against virulence factor production compared with C30 (Fig. 3D).

Interestingly, all active compounds also inhibited biofilm formation (Fig. 3E), with this decreased by 28.6, 38.0, 34.1, 35.3, 36.2, 40.3 and 28.6% by 7b, 8b, 8c, 11b, 11f, 11i and C30, respectively. Moreover, analogs 7b, 11f and 11i showed stronger inhibitory effects on the swarming motility of \textit{Pseudomonas} than C30 (Fig. 3F), with 11f being the most active. These results verified that analogs 7b, 8b, 8c, 11b, 11f and 11i...
inhibited the bacterial QS system by selectively attenuating its production of virulence factors, especially those that promoted PAO1 biofilm formation.

To further verify the mechanism of action of analogs 7b, 8b, 8c, 11b, 11f and 11i, the mRNA expression of QS-related genes rhlI, lasI, pqsA, rhlR, lasR and pqsR was analyzed by real-time fluorescence quantitative PCR. These genes are related to the synthesis of QS signal molecules (rhlI, lasI, pqsA) and the regulation of QS proteins (rhlR, lasR, pqsR). The results showed that the analogs inhibited the expression of lasI, lasR, pqsA, pqsR, rhlR and rhlR. For example, the expression of lasI was downregulated by 50.5, 32.9, 47.9, 38.9, 55.1, 59.2 and 26.9% by analogs 7b, 8b, 8c, 11b, 11f and 11i, respectively (Fig. 4A). 8b was almost equipotent with C30 for downregulation of the expressions of lasA, and 11f (71.0%) and 11i (64.1%) were superior to C30 (58.5%) (Fig. 4B). 7b, 8c and 11f were most effective at inhibiting expression of pqsA, with 11f downregulating pqsA expression by 67.7% (Fig. 4C).

Table 1. The Structures and Yields of Compounds of Series I and II

| Compd. | R   | Yield | Compd. | R   | Yield |
|--------|-----|-------|--------|-----|-------|
| 7a     |     | 58%   | 8a     |     | 55%   |
| 7b     |     | 44%   | 8b     |     | 50%   |
| 7c     |     | 53%   | 8c     |     | 58%   |
| 7d     |     | 52%   |        |     |       |

Table 2. The Structures and Yields of Compounds of Series III and IV

| Compd. | R   | Yield | Compd. | R   | Yield |
|--------|-----|-------|--------|-----|-------|
| 11a    | 3-Cl | 44%   | 11g    | 4-i-Pr | 49% |
| 11b    | 2-OCH3 | 51%  | 11h    | 2-CH3 | 42%  |
| 11c    | 4-CH3 | 61%   | 11i    | 3-CH3 | 55%  |
| 11d    | 4-OCH3 | 40%  | 12a    | 3-Cl  | 45%  |
| 11e    | 2-OC3H7 | 45%  | 12b    | 2-OCH3 | 37% |
| 11f    | 2,3-Ar | 47%  | 12c    | 4-CH3 | 56%  |
(Fig. 4D), but surprisingly only 11f achieved equipotency with C30 for inhibition of rhlI expression (Fig. 4E). Furthermore, 11f and 11i were only slightly more inhibitory of rhlR expression (58.8 and 55.5%, respectively) than C30 (48.4%) (Fig. 4F).

In summary, analog 11f was the most potent inhibitor of the expression of most QS-regulated genes in P. aeruginosa PAOI. However, 11f showed more powerful inhibitory activity of lasR, pqsR and rhlR expression than it did of lasI, pqsA and rhlI expression. These results confirmed that active compounds inhibited virulence expression by controlling the expression of related genes (Fig. 4).

Analysis of Molecular Docking Results

To verify the results of this bioassay evaluation, we used AutoDock molecular simulation software (Molecular modeling was performed using the autodock 4 software from the Scripps Research Institute) to simulate the binding of the active compounds to the receptor protein LasR.30) OdDHL and analogs 7b and 8b interacted with Thr-75, Tyr-93 and Ser-129 groups which are located inside the receptor pocket of LasR, via three hydrogen bonds (i.e., 7b and 8b each formed one hydrogen bond with each of the three residues) (Figs. 5A–C). 8c interacted with the amino acid residues Tyr-93, Thr-75, Asp-73 and Ser-129 to form four hydrogen bonds (Fig. 5D); 11b formed two hydrogen bonds by interacting with Asp-73 and Ser-129 (Fig. 5E); 11f linked with Ser-129 and Arg-61 to form three hydrogen bonds (Fig. 5F); 11i interacted with Tyr-56, Tyr-47 and Ser-129 via three hydrogen bonds (Fig. 5G).

The fact that 11f was the most potent compound and also the only analog that interacted with Arg-61 suggested that Arg-61 may be more important for QS than other amino acid residues in the active site of LasR. Thus, the molecular docking results correlated well with the biological evaluation results.

Conclusion

In conclusion, we have designed and synthesized four series of novel L-homoserine lactone analogs and evaluated their inhibitory activity against QS in P. aeruginosa. The results indicated that 7b and 7c of series I and 8b and 8c of series II, representing L-homoserine lactone analogs with benzothiazolyl and 4-chlorophenyl substituents, respectively, showed greater inhibitory activity against CV026 and PAO1 than 7a, 7d and 8a, with 4,5-dihydro-thiazol or 1,3,4-thiadiazole substituents. In addition, most compounds of series III with a sidechain comprising a phenylurea and dithiocarbamate group exhibited remarkable inhibition of QS in CV026 and PAO1. Compounds of series IV, with a sidechain comprising only a phenylurea group, had weak QSI activity. These results indicated that the dithiocarbamate group was critical to the QSI activity of series III.

The results of mechanism of action studies verified that analogs 7b, 8b, 8c, 11b, 11f and 11i inhibited the P. aeruginosa QS system by selectively attenuating the expression of virulence factors, especially biofilm formation by PAO1. The activity of compound 11f was superior to that of all other analogs, and the results of molecular docking indicated that 11f competed with OdDHL to bind with LasR. This 11f–LasR interaction inhibited the expression of the las system genes, PQS system genes and rhl system-related genes, thereby inhibiting the production of virulence factors and the formation of PAO1 biofilm.

Our research has enriched the chemical space around L-homoserine lactones, and afforded a new lead compound (11f)
for the development of QS inhibitors as antibacterial agents with less likelihood of triggering the development of resistance. We expect that further investigation and expansion of the SAR of 11f will afford more efficient and highly selective QSI for antibacterial drug development.

Experimental

General Remarks

All chemical reagents and solvents were purchased from commercial sources and used without further purification. $^1$H-NMR (400 MHz) and $^1$C-NMR spectra (100 MHz) were recorded on a Bruker (DPX-400) spectrometer. High resolution (HR)-MS were recorded on a Waters Micromass Q-Tof Micromass spectrometer by electrospray ionization (ESI). Melting points were determined on X-5 Microdigital melting point (mp) apparatus and are reported uncorrected.

Synthesis of L-Homoserine Lactone Hydrochloride (4)

L-Homoserine lactone hydrochloride (4) was efficiently synthesized from methionine following our previously reported methods.28) L-Homoserine lactone hydrochloride 4, white solid, (3 steps, yield 59%), mp 218–220°C, $[\alpha]_{D}^{20} = -27.5^\circ$, (c 0.2, water) $^3$O.

$^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta$: 9.04 (s, 3H, NH$_2$·HCl), 4.45 (t, J = 8.8 Hz, 1H, -O-CH$_2$), 4.37–4.21 (m, 2H, -O-CH$_2$-CO), 2.62–2.51 (m, 1H, -O-CH$_2$-CH$_2$-CH), 2.42–2.27 (m, 1H, -O-CH$_2$-CH$_2$-CH). 

General Procedure for the Synthesis of Compounds 5 and 6

Compounds 5 and 6 were efficiently synthesized from L-homoserine lactone hydrochloride 4 following our previously reported methods.39) The acyl group of 5 and 6 was introduced by condensation with chloroacetyl chloride and 3-chloropropionyl chloride, respectively.

(S)-2-Chloro-N-(2-carbonyl Tetrahydrofuran-3-yl)acetamide 5

White solid: (TLC: acetone : petroleum ether 2 : 1), (yield 68.2%), mp 121–122°C; $^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta$: 8.76 (d, J = 8.0 Hz, 1H, -CO- NH), 4.63 (dd, J = 20.0 Hz, J = 8.0 Hz, 1H, -O-CH$_2$), 4.35 (t, $J = 8.0$ Hz, 1H, -CH-NH), 4.21 (dd, $J = 20.0$ Hz, $J = 8.0$ Hz, 1H, -O-CH$_2$), 4.14 (s, 2H, -CH$_2$-Cl), 2.44–2.36 (m, 1H, -O-CH$_2$-CH$_2$-CH), 2.24–2.15 (m, 1H, -O-CH$_2$-CH$_2$-CH). 

(S)-2-Chloro-N-(2-carbonyl Tetrahydrofuran-3-yl)propylamide 6

White solid (TLC: acetone : petroleum ether 2 : 1), (yield 64.5%), mp 127–129°C; $^1$H-NMR (400 MHz, DMSO-$d_6$) $\delta$: 8.50 (d, J = 7.8 Hz, 1H, -CO-NH), 4.52 (dt, $J = 10.8$ Hz, 8.9 Hz, 1H, -O-CH$_2$), 4.28 (td, $J = 8.8$, 1.8 Hz, 1H, -CH-NH), 4.15 (ddd, $J = 10.4$, 8.8, 6.5 Hz, 1H, -O-CH$_2$), 3.73–3.69 (m, 2H, -CH$_2$-Cl), 2.56 (td, $J = 6.2$, 1.7 Hz, 2H, -CH$_2$-CH$_2$-Cl), 2.39–2.31 (m, 1H, -O-CH$_2$-CH$_2$-CH), 2.12–2.01 (m, 1H, -O-CH$_2$-CH$_2$-CH).
**General Procedure for the Synthesis of Compounds 7a–d**

A solution of HS-R₂ (0.92 mmol) and K₂CO₃ (120 mg) in acetone was added to compound 5 (0.84 mmol). After 5 h stirring at 50°C, the solvent was removed under vacuum, and the resulting residue was extracted with ethyl acetate and water. The organic layer was dried over MgSO₄ and evaporated under vacuum, and the resulting residue was purified by column chromatography, affording white solid 7a–d.

**(S)-2-((4,5-Dihydrothiazol-2-yl)thio)-N-(2-oxotetrahydrofuran-3-yl) Cetamide 7a**

White solid (TLC: petroleum ether : EtOAc 1 : 6), (yield 58%), mp 114–116°C; ¹H-NMR (400 MHz, DMSO-d₆) δ: 8.65 (d, J = 7.8 Hz, 1H, -CO- NH-), 4.60–4.53 (m, 1H, -O- CH₂-), 4.34 (td, J = 8.8, 1.7 Hz, 1H, - CH-NH-), 4.20 (ddd, J = 10.4, 8.8, 6.5 Hz, 1H, -O-CH₂-), 4.12 (t, J = 8.0 Hz, 2H, -CO- CH₂-S-), 3.94–3.86 (m, 2H, - CH₂-S-), 3.46 (t, J = 8.0 Hz, 2H, - CH₂-N), 2.44–2.36 (m, 2H, -CH₂-CH₂-CH₂-CH₂-, 2.18–2.08 (m, 1H, -O-CH₂-CH₂-CH₂-CH₂-). ¹³C-NMR (100 MHz, DMSO-d₆) δ: 174.9, 166.8, 162.6, 65.3, 63.8, 48.29, 35.55, 35.42, 28.11. HRESI-MS m/z calcld for [M+H]+ C₉H₁₂N₂O₃S₂: 261.0362. Found: 261.0368.

**(S)-2-(Benzo[d]thiazol-2-ylthio)-N-(2-oxotetrahydrofuran-3-yl) Cetamide 7b**

White solid (TLC: petroleum ether:EtOAc 1:3), (yield 44%), mp 133–135°C; ¹H-NMR (400 MHz, DMSO-d₆) δ: 8.88 (d, J = 7.8 Hz, 1H, -CO-NH-), 8.02 (d, J = 8.0 Hz, 1H, Ar-H-), 7.84 (d, J = 8.1 Hz, 1H, Ar-H-), 7.49–7.45 (m, 1H, Ar-H-), 7.37 (m, 1H, Ar-H-), 4.63 (dt, J = 10.7, 8.8 Hz, 1H, -O-CH₂-), 4.35 (td, J = 8.8, 1.5 Hz, 1H, -CH-NH-), 4.25–4.17 (m, 3H, -O-CH₂-, -CH₂-S-), 2.46–2.39 (m, 2H, -O-CH₂-CH₂-CH₂-, 2.17 (m, 1H, -O-CH₂-CH₂-CH₂-CH₂-). ¹³C-NMR (100 MHz, DMSO-d₆) δ: 174.9, 166.6, 165.9, 152.5, 134.8, 126.4, 124.5, 121.8, 121.1, 65.3, 48.4, 36.2, 28.1. HRESI-MS m/z calcld for [M+Na]+ C₁₃H₁₂N₂O₃S₂: 331.0182. Found: 331.0198.

**(S)-2-((4-Chlorophenyl)thio)-N-(2-oxotetrahydrofuran-3-yl) Cetamide 7c**

White solid (TLC: petroleum ether:EtOAc 1:6), (yield 53%), mp 139–141°C; ¹H-NMR (400 MHz, DMSO-d₆) δ: 8.69 (d, J = 7.8 Hz, 1H, -CO-NH-), 7.40–7.36 (m, 4H, Ar-H-), 4.57 (dt, J = 10.9, 8.9 Hz, 1H, -O-CH₂-), 4.34 (td, J = 8.8, 1.5 Hz, 1H, -CH-NH-), 4.20 (ddd, J = 10.4, 8.8, 6.5 Hz, 1H, -O-CH₂-), 2.40–2.33 (m, 1H, -O-CH₂-CH₂-CH₂-).

**Fig. 4. Inhibition of QS-regulated Gene ((A)–(F)) Expression in P. aeruginosa PAO1 by Active Compounds**

(A) lasI. (B) lasR. (C) pqsA. (D) pqsR. (E) rhlI. (F) rhlR.
2.11 (m, 1H, -O-CH<sub>2</sub>-CH<sub>2</sub>-CH=). 13C-NMR (100 MHz, DMSO-d<sub>6</sub>) δ: 174.9, 167.9, 134.9, 130.7, 129.9, 128.8, 65.3, 48.2, 36.2, 28.0. HRESI-MS m/z calcd for [M + Na]<sup>+</sup> C<sub>12</sub>H<sub>12</sub>ClNO<sub>3</sub>S: 308.0124. Found: 308.0117. 

(S)-2-((1,3,4-Thiadiazol-2-yl)thio)-N-(2-oxotetrahydrofuran-3-yl)acetamide 7d

White solid (TLC: acetone : EtOAc 1 : 6), (yield 52%), mp 96–97°C; 1H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ: 9.52 (s, 1H, -CH-N-), 8.84 (d, J = 7.8 Hz, 1H, -CO-NH-), 4.64–4.57 (m, 1H, -O-CH<sub>2</sub>-), 4.35 (td, J = 8.8, 1.7 Hz, 1H, -CH-NH-), 4.24–4.18 (m, 1H, -O-CH<sub>2</sub>-), 4.15 (d, J = 2.5 Hz, 2H, -CH<sub>2</sub>-S-), 2.45–2.38 (m, 1H, -O-CH<sub>2</sub>-CH<sub>2</sub>-CH-), 2.20–2.09 (m, 1H, -O-CH<sub>2</sub>-CH<sub>2</sub>-). 13C-NMR (100 MHz, DMSO-d<sub>6</sub>) δ: 174.8, 166.5, 164.9, 154.2, 65.3, 48.4, 37.0, 28.1. HRESI-MS m/z calcd for [M + Na]<sup>+</sup> C<sub>8</sub>H<sub>9</sub>N<sub>3</sub>O<sub>3</sub>S<sub>2</sub>: 281.9978. Found: 281.9977. 

General Procedure for the Synthesis of Compounds 8a–c

A solution of the mercapto compound (0.92 mmol) and K<sub>2</sub>CO<sub>3</sub> (120 mg) in acetone was added to compound 6 (0.78 mmol). After stirring at 50°C for 5 h, the solvent was removed under vacuum and the resulting was extracted with ethyl acetate and water. The organic layer was dried over MgSO<sub>4</sub> and evaporated under vacuum to give a residue, which was then purified by column chromatography, affording a white solid (8a–c).

(S)-3-((4,5-Dihydrothiazol-2-yl)thio)-N-(2-oxotetrahydrofuran-3-yl)propanamide 8a

White solid (TLC: petroleum ether : EtOAc 1 : 10), (yield 55%), mp 128–130°C; 1H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ: 8.59 (d, J = 7.9 Hz, 1H, -CO-NH-), 4.55 (dt, J = 10.8, 8.8 Hz, 1H, -O-CH<sub>2</sub>-), 4.35 (td, J = 8.7, 1.5 Hz, 1H, -CH-NH-), 4.24–4.19 (m, 1H, -O-CH<sub>2</sub>-), 4.14 (t, J = 7.9 Hz, 2H, -CH=N-), 3.88 (t, J = 6.9 Hz, 2H, -CH<sub>2</sub>-CH<sub>2</sub>-N), 3.33–3.29 (m, 2H, -CH<sub>2</sub>-S-), 2.53–2.50 (m, 2H, -CH<sub>2</sub>-CH<sub>2</sub>-S-), 2.43–2.36 (m, 1H, -O-CH<sub>2</sub>-CH<sub>2</sub>-CH=). 13C-NMR (100 MHz, DMSO-d<sub>6</sub>) δ: 195.0, 175.2, 170.0, 65.3, 57.3, 48.0, 45.1, 32.2, 28.0, 27.1. HRESI-MS m/z calcd for [M + Na]<sup>+</sup> C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub>S<sub>2</sub>: 297.0338. Found: 297.0331. 

White solid (TLC: petroleum ether : EtOAc 1 : 4), (yield 50%), mp 137–138°C; 1H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ: 8.53 (d, J = 7.9 Hz, 1H, -CO-NH-), 8.01 (d, J = 8.0 Hz, 1H, Ar-H), 7.88 (d, J = 8.0 Hz, 1H, Ar-H), 7.49–7.45 (m, 1H, Ar-H), 7.39–7.35 (m, 1H, Ar-H), 4.59 (dt, J = 10.7, 8.8 Hz, 1H, -O-CH<sub>2</sub>-), 4.35 (td, J = 8.8, 1.6 Hz, 1H, -CH-NH-), 4.22 (ddd, J = 10.3, 8.8, 6.5 Hz, 1H, -O-CH<sub>2</sub>-), 3.54 (t, J = 6.9 Hz, 2H, -CH<sub>2</sub>-S-), 2.78–2.69 (m, 2H, -CH<sub>2</sub>-CH<sub>2</sub>-S), 2.46–2.38 (m, 1H, -O-CH<sub>2</sub>-CH<sub>2</sub>-CH=), 2.14 (m, 1H, -O-CH<sub>2</sub>-CH<sub>2</sub>-). 13C-NMR (100 MHz, DMSO-d<sub>6</sub>) δ: 175.7, 170.5, 167.0, 153.2, 135.0, 126.8, 124.9, 122.3, 121.6, 65.8, 48.5, 35.0, 28.9, 28.8. HRESI-MS m/z calcd for [M + Na]<sup>+</sup> C<sub>14</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub>S<sub>2</sub>: 345.0338. Found: 345.0346. 

White solid (TLC: petroleum ether : EtOAc 1 : 3), (yield 58%), mp 88–89°C; 1H-NMR (400 MHz, DMSO-d<sub>6</sub>) δ: 8.47 (d, J = 7.9 Hz, 1H, -CO-NH-), 7.41–7.35 (m, 4H, Ar-H), 4.57 (dt, J = 10.8, 8.8 Hz, 1H, -O-CH<sub>2</sub>-), 4.35 (td, J = 10.8, 1.7 Hz, 1H, -CH-NH-), 4.21 (ddd, J = 10.4, 8.7, 6.5 Hz, 1H, -O-CH<sub>2</sub>-), 3.16 (t, J = 7.2 Hz, 2H, -CH<sub>2</sub>-S-), 2.46 (t, J = 7.3 Hz, 2H, -CH<sub>2</sub>-CH<sub>2</sub>-S-), 2.42–2.36 (m, 1H, -O-CH<sub>2</sub>-CH<sub>2</sub>-CH=), 2.17–2.06 (m, 1H, -O-CH<sub>2</sub>-CH<sub>2</sub>-CH=). 13C-NMR (100 MHz, DMSO-d<sub>6</sub>) δ: 175.2, 170.1, 155.0, 130.4, 129.9, 129.0, 65.3, 47.9, 34.5, 28.3, 28.0. 

Fig. 5. The Results of the Docking Pose of OdDHL (A) and Compounds 7b (B), 8b (C), 8c (D), 11b (E), 11f (F) and 11i (G) with the Receptor Protein LasR. 

Hydrogen bonds are shown as green balls.
HRESI-MS m/z calcd for [M + Na]+ C_{15}H_{17}N_{3}O_{5}S_{2}: 437.1312. Found: 437.1876.

11c -tolyl)ureido)ethyl(2-oxotetrahydrofuran-3-yl)carbamodithioate

White solid (TLC: petroleum ether:EtOAc 1:1), (yield 4%), mp 151–153°C; 1H-NMR (400 MHz, DMSO-d$_6$-d$_6$) $\delta$: 8.9, 2H, -O-CH$_2$-CH$_3$), 2.58 (m, 3H, -O-CH$_2$-CH$_2$-CH$_3$-), 1.37 (t, $J$ = 7.0 Hz, 3H, -O-CH$_2$-CH$_3$). 13C-NMR (100 MHz, DMSO-d$_6$) $\delta$: 203.7, 173.7, 173.5, 170.9, 156.9, 151.4, 146.2, 137.4, 126.5, 121.6, 119.6, 116.9, 55.4, 54.7, 50.2, 39.7, 37.9. HRESI-MS m/z calcd for [M + Na]+ C$_{17}$H$_{21}$N$_3$O$_4$S$_2$: 426.0585. Found: 426.0462.

12-(3-(2-Isopropylphenyl)ureido)-2-oxoethyl(2-oxotetrahydrofuran-3-yl)carbamodithioate

White solid (TLC: petroleum ether:EtOAc 1:1), (yield 4%), mp 142–144°C; 1H-NMR (400 MHz, DMSO-d$_6$-d$_6$) $\delta$: 7.7, 2H, -O-CH$_2$-CH$_2$-CH$_3$), 2.58 (m, 3H, -O-CH$_2$-CH$_2$-CH$_3$-), 1.37 (t, $J$ = 7.0 Hz, 3H, -O-CH$_2$-CH$_3$). 13C-NMR (100 MHz, DMSO-d$_6$) $\delta$: 197.3, 173.3, 170.9, 156.9, 154.4, 137.4, 126.5, 121.6, 119.6, 55.4, 54.7, 50.2, 39.7, 37.9. HRESI-MS m/z calcd for [M + Na]+ C$_{17}$H$_{21}$N$_3$O$_4$S$_2$: 426.0585. Found: 426.0462.

2-Oxo-2-(3-(p-toly)lureido)ethyl(2-oxotetrahydrofuran-3-yl)carbamodithioate
White solid ( TLC: petroleum ether: EtOAc 1:1), (yield 55%), mp 130–131°C; \(^1\)H-NMR (400 MHz, DMSO-\(_d6\)) \(\delta\): 10.93 (s, 1H, \(-CO-NH-Co-\)), 10.51 (d, \(J = 7.9\) Hz, 1H, \(-CS-NH-)\), 10.27 (s, 1H, \(-Ar-NH-Co-\)), 7.36 (s, 3H, \(-O-CH_3\)), 2.59 (m, 1H, \(-O-CH_2-CH2-CH-\)), 2.00 (m, 1H, \(-O-CH_2-CH2-CH-\)). \(^13\)C-NMR (100 MHz, DMSO-\(_d6\)) \(\delta\): 150.5, 135.9, 130.3, 127.4, 126.4, 123.9, 120.7, 65.5, 54.7, 39.0, 29.5. HRESI-MS calcd for [M+Na]+ \(C_{13}H_{24}N_3O_3\): 390.0558. Found: 390.0560.

2-Oxo-2-((3-(m-toly)-ureido)ethyl)-(2-oxotetrahydrofuran-3-yl)carbamoylthioate 11i

White solid ( TLC: petroleum ether:EtOAc 1:1), (yield 55%), mp 130–131°C; \(^1\)H-NMR (400 MHz, DMSO-\(_d6\)) \(\delta\): 10.93 (s, 1H, \(-CO-NH-Co-\)), 10.51 (d, \(J = 7.9\) Hz, 1H, \(-CS-NH-)\), 10.27 (s, 1H, \(-Ar-NH-Co-\)), 7.36 (s, 3H, \(-O-CH_3\)), 2.59 (m, 1H, \(-O-CH_2-CH2-CH-\)), 2.00 (m, 1H, \(-O-CH_2-CH2-CH-\)). \(^13\)C-NMR (100 MHz, DMSO-\(_d6\)) \(\delta\): 150.5, 135.9, 130.3, 127.4, 126.4, 123.9, 120.7, 65.5, 54.7, 39.0, 29.5. HRESI-MS calcd for [M+Na]+ \(C_{13}H_{24}N_3O_3\): 390.0558. Found: 390.0489.

General Procedure for the Synthesis of Compounds 12a–c

In a 25mL round bottom flask, the solution of compound \(\delta\)(150 mg, 1.09mmol) in acetonitrile was added to corresponding compounds 13a–c (2.0mmol) and NaHCO\(_3\) (230mg, 2.74mmol). After stirring for 8h at 75°C, the solvent was removed under vacuum, extracted with ethyl acetate and water. The organic layer was dried over MgSO\(_4\) and evaporated under vacuum, extracted with ethyl acetate and evaporated under vacuum to give a residue, which was then subject to column chromatography, affording a white solid (12a–c).

(S)-\(N-(3-Chlorophenyl)carbamoyl)-2-(2-oxotetrahydrofuran-3-yl)amino)acetamide 12a

White solid ( TLC: petroleum ether:EtOAc 1:2), (yield 45%), mp 133–134°C; \(^1\)H-NMR (400 MHz, DMSO-\(_d6\)) \(\delta\): 10.48 (s, 2H, \(-CO-NH-Co-\)), \(-Ar-NH-Co-)\), 7.78 (d, \(J = 1.9\) Hz, 1H, \(-Ar-H\)), 7.41–7.34 (m, 2H, \(-Ar-H\)), 7.16 (d, \(J = 7.5\) Hz, 1H, \(-Ar-H\)), 4.33 (td, \(J = 8.6, 2.2\) Hz, 1H, \(-O-CH_2-CH2-CH\)), 4.20–4.13 (m, 1H, \(-O-CH_2-\)), 3.68 (t, \(J = 9.2\) Hz, 1H, \(-\text{CH-NH}\)), 3.55 (s, 2H, \(-\text{CH}_2-\)), 2.94 (s, 1H, \(-\text{CH-NH}\)), 2.45 (m, 1H, \(-O-\text{CH}_2-\)), 2.03–1.98 (m, 1H, \(-O-\text{CH}_2-\)). \(^13\)C-NMR (100 MHz, DMSO-\(_d6\)) \(\delta\): 177.2, 174.0, 150.4, 147.2, 136.8, 134.7, 129.2, 128.8, 124.4, 120.1, 116.7, 65.5, 54.6, 39.0, 27.9, 21.0. HRESI-MS m/z calcd for [M+Na]+ \(C_{12}H_{22}N_3O_3\): 390.0588. Found: 390.0489.

Growth Curve Analysis

P. aeruginosa PAO1 was cultured overnight, diluted to OD600 = 0.05, and then transferred into 96-well plates with active compounds (to give a final concentration of 15\(\mu\)M). Bacterial cultures were incubated for 24h at 37°C, and optical density was measured at 600nm every 2h.

Detection of Pyocyanin Production

P. aeruginosa PAO1 was cultured overnight, diluted with LB medium, and then transferred into 96-well plates with active compounds (to give a final concentration of 15\(\mu\)M). Bacterial cultures were incubated at 37°C, and optical density was measured at 600nm every 2h.
Bacterial cultures were centrifuged at 4°C for 10 min, and then 500 µL of Congo red buffer was added into 500 µL of the liquid supernatant. After reacting for 8 h at 37°C, the mixture was centrifuged at 4°C for 10 min, and sample of the supernatant analyzed by OD490 to reveal the elastase activity.

Determination of Rhamnolipid Production

PAO1 bacterial cultures (OD ≈ 1.0) was diluted, and transferred to LB medium. When OD600 ≈ 0.2, the active compounds were added to PTBS medium containing PAO1, then cultured in the shock conditions. The sample was then centrifuged at 4°C for 10 min, and a 1 mL of the liquid supernatant was added to 1 mL of ether, and shaken for 1 min. A sample of the resulting ether phase (800 µL) was transferred to a new tube, then the remaining aqueous solution was extracted once with ether (1 mL). The ether phases were combined and taken to dryness, and the resulting residue was dissolved in sterile water (100 mL). The OD420 was measured after the sterile water solution reacted with the orcin sulfuric acid reagent.

Swarming Movement Detection

P. aeruginosa PAO1 was cultured overnight, diluted with LB medium, and then centrifuged at 4000rpm for 4 min. Active compounds were added into the melted swarming medium to give a final concentration of 15 µM, and the resulting mixture was dried for 3 min, and then incubated with 1 µL of concentrated bacterial cultures at 37°C for 24 h.

Biofilm Formation Assay

P. aeruginosa PAO1 was cultured overnight, diluted with LB medium, and then transferred into 96-well plates with active compounds (15 µM; final concentration). The bacterial cultures were incubated at 37°C for 12 h. The cultures were dyed with crystal violet (0.1%) for 20 min, and were then washed with normal saline. After drying, acetic acid glacial (150 µL, 33%) was added, so that OD600 could be measured.

The mRNA Expression of QS-Related Genes Assay

(1) Extraction of total bacterial RNA: P. aeruginosa PAO1 was cultured overnight, then transferred to LB medium and cultured in the shock conditions. When OD600 = 0.2, the test group and control group were respectively added active compounds and blank solvent, then incubated for 6 h. According to the kit instructions, the RNA mass was measured by agarose gel electrophoresis. (2) Reverse transcription to synthesize cDNA: total RNA was used as template, primer (1 µL, 25 µM) and of diethyl pyrocarbonate (DEPC) water (1 µL) were added, and the mixture was heated at 70°C for 10 min and quenched at 0°C for 3 min. 5X Moloney Mouse Leukemia Virus (MMLV) buffer (2 µL), deoxyribonucleoside triphosphate (DNTP) (10 mM, 0.5 µL), of ribonuclease (RNase) inhibitor RRI (0.25 µL), reverse transcriptase MMLV (0.5 µL), and DEPC water (0.75 µL) were added to the system. The mixture was reacted under the following conditions: 30°C, 10 min; 42°C, 60 min; 70°C, 10 min; after the end of the procedure, the system was placed on ice. (3) Real-time fluorescence quantitative PCR: a cDNA template was diluted 10 times, the reaction system was set according to the kit instructions: the conserved gene 16SrDNA as the internal reference for amplification, 95°C, 10 min; 95°C, 30 s, 60°C, 30 s; 4°C, 10 min, but 95°C, 30 s, 60°C, 30 s; 40 cycles were run. Plot of the dissolution curve: 95°C, 30 s; 60°C, 30 s; 95°C, 30 s. ABI 7500 RT-PCR software was used to analyze the amplification results.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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