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## 3-Aminooxazolidinone AHL analogs as hydrolytically-stable quorum sensing agonists in Gram-negative bacteria†

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Synthetic molecules that modulate quorum sensing, QS, in bacteria have great potential for use in synthetic biology applications as well as acting as anti-virulence and anti-biofilm agents. Acylhomoserine lactone (AHL)-based autoinducer analogs have been extensively developed as QS modulators but these suffer from both chemical and enzymatic degradations. Here, we reveal that 3-aminooxazolidinone acylhomoserine lactone analogs are hydrolytically stable and are as potent in activating LuxR-type receptors. Docking analysis revealed that 3-oxo-C12-3-aminooxazolidinone docked in LasR of *P. aeruginosa*, making similar interactions with the protein's active-site residues to the native ligand, 3-oxo-C12 HSL. Experimentally, 3-oxo-C12-3-aminooxazolidinone was equally as potent as the natural ligand in inducing bioluminescence in *E. coli* carrying a bioluminescent gene that was under the control of LasR. In *C. violaceum* CV026, the 3-aminooxazolidinone analogs could also modulate pigment (violacein) formation, albeit this time not as potent as the natural AHL ligands.

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### Introduction

The old view that bacteria live in solitary mode has now been replaced with a community-based bacterial lifestyle, whereby most bacteria live on surfaces as part of polymicrobial biofilms<sup>1</sup> and communicate with neighbors using diffusible molecules. Bacteria also communicate *via* contact, using surface associated receptors<sup>2</sup> or connecting nanotubes.<sup>3</sup> Even in the planktonic state, bacteria can still communicate with neighboring self and non-self cells and react to population density *via* response to small molecule autoinducers secreted by other bacteria.<sup>4</sup> The cell-to-cell communication between bacteria, called quorum sensing (QS), regulates diverse phenotypes, including biofilm formation, competence, bioluminescence, virulence factors production and antibiotic synthesis.<sup>5</sup> Additionally, both plant and animal hosts respond to bacterial signaling molecules and some QS molecules have been shown to promote apoptosis or programmed cell death in diverse eukaryotic cell types.<sup>6–9</sup>

In the past decade, many small molecules that modulate quorum sensing have been developed.<sup>10–13</sup> These QS modulators have been either agonists or antagonists and have the potential for use in diverse applications, ranging from

inhibition of bacterial toxin production and biofilm formation (QS antagonists),<sup>14–18</sup> manipulation of bacterial behavior and synthetic biology applications (both agonists and antagonists)<sup>19–21</sup> to the inhibition of cancer (by 3-oxo-C12 HSL of *Pseudomonas aeruginosa*).<sup>22</sup> Thus far, acylhomoserine lactone (AHL)-based QS modulators have been the most rigorously pursued by many groups. The majority of these compounds have targeted LasR from *P. aeruginosa*.<sup>9–15</sup>

Most of the AHL analogs developed to date have kept the acylhomoserine lactone head group and modified the acyl chain. A few lactone head group modifications have also been reported but often, modification of the head group usually leads to a dramatic reduction of activity.<sup>23</sup> Unfortunately  $\gamma$ -lactones are not chemically stable and can hydrolyze in mild acidic or basic environments.<sup>24</sup> Additionally bacterial, plant or animal lactonases<sup>25–28</sup> and acylases<sup>29–32</sup> have been shown to readily inactivate AHLs so there is clearly a need for an AHL head group that is resistant to hydrolysis and at the same time maintains the high QS modulatory activity seen with homoserine lactones.

We docked several lactone mimics into the active site of *P. aeruginosa* LasR and found that oxazolidinone-based AHL analogs had similar conformation in the binding site of LasR to the native 3-oxo-C12-HSL. The docking results were somehow surprising to us because many reports have documented the importance of the chirality at the C3 position for AHL autoinducers in activating QS-mediated processes.<sup>13,33,34</sup> In this report we show that 3-aminooxazolidinone that lacks a

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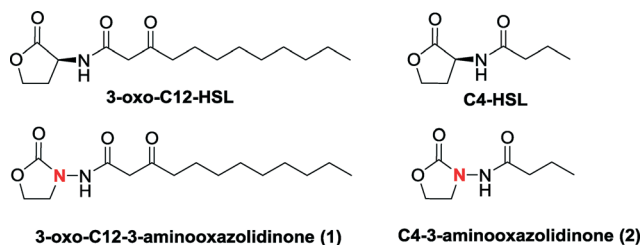


Fig. 1 Structures of oxazolidinone AHL analogs and natural AHL-1.

C3 chirality could still bind to some LuxR-type receptors and is as potent, in binding to LasR, as the native 3-oxo-C12 HSL. As an added advantage, the 3-aminooxazolidinone head group is more resistant to hydrolysis than the AHLs and is therefore a good replacement for the lactone head group in AHL-based QS modulators. 3-Amino-oxazolidinone-based analogs (Fig. 1) can be made from inexpensive materials in a few steps and are drug-like (examples of oxazolidinone drugs are linezolid<sup>35</sup> and rivaroxaban<sup>36</sup>).

## Results and discussion

3-Oxo-HSLs are known to degrade under weakly basic conditions and eventually tautomerize to a tetramic acid derivative *via* a mechanism shown in Scheme 1.<sup>24</sup> To demonstrate that 3-aminooxazolidinone-based analogs (see Scheme 2 for synthetic scheme) are superior to natural AHLs, in terms of chemical stability, we monitored the degradation of 3-oxo-C12-HSL and 3-oxo-C12-3-aminooxazolidinone (1) at pH = 8.0 by monitoring UV absorption at 278 nm, which is an absorption maxima for tetramic acid, as a function of time (Fig. 2). Whereas the UV absorption for the 3-oxo-C12-HSL incubation increased over time, that of the analog 1 remained stable over 3 h. HPLC analysis also revealed that analog 1 remained intact after 3 h of incubation in Tris buffer (pH = 8.0) (see the ESI, Fig. S1†). We therefore concluded that 3-aminooxazolidinone analog (1) is more stable than 3-oxo-C12-HSL to basic hydrolysis.

Recently, Raines revealed that the conformation of free AHLs is different when complexed to LasR.<sup>37</sup> In the free state, the lone pairs of the amide carbonyl form a favorable interaction with the  $\pi^*$  of the lactone carbonyl.<sup>37</sup> This *n* to  $\pi^*$  interaction (about 0.64 kcal mol<sup>-1</sup>) is disrupted upon binding to LasR. Interestingly the substitution of the C3 in 3-oxo-C12-HSL with N3 (aminooxazolidinone-based analogs) did not

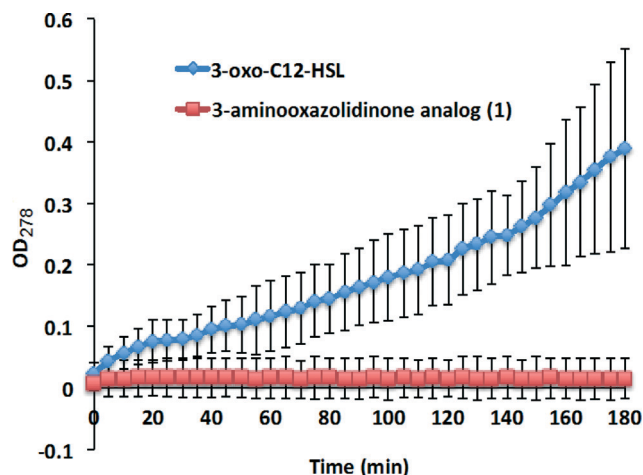
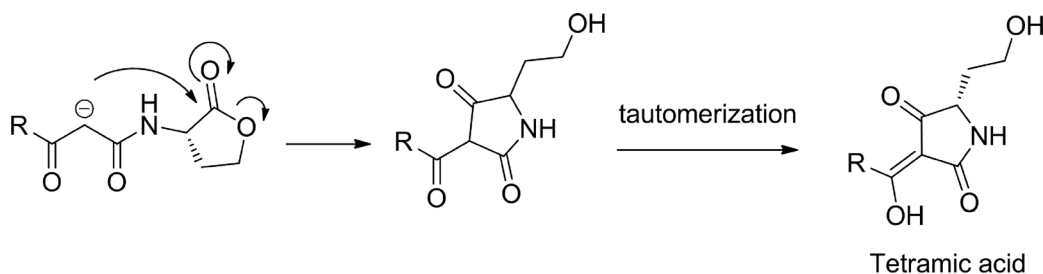


Fig. 2 Stability studies of C12-oxo-HSL and 3-oxo-C12-3-amino-oxazolidinone (1).

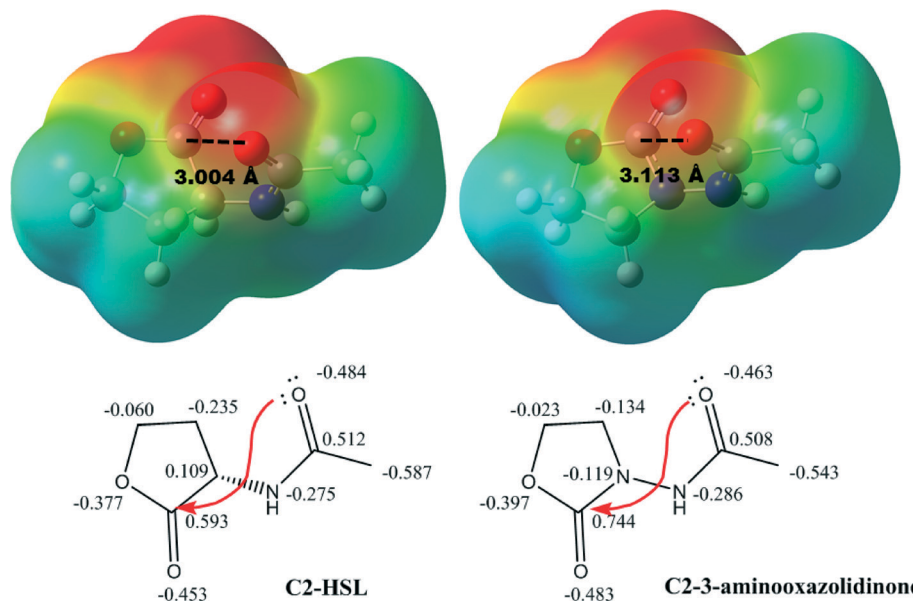
abrogate the *n* to  $\pi^*$  interaction in the free state (see Fig. 3). Also, the C3 to N3 substitution did not drastically change the surface charge potentials of the head group moieties (compare compounds C2-HSL and C2-3-aminooxazolidinone in Fig. 3), implying that our analogs would be able to partake in charge-charge interactions in the 3-oxo-C12-HSL binding site.

In most LuxR-type proteins reported to date, Trp60 is highly conserved.<sup>39–44</sup> Both Suga and Blackwell have shown that this residue determines whether a ligand acts as an agonist or antagonist.<sup>45,46</sup> Ligands that exhibit unfavorable interactions with Trp60 have antagonistic profiles. Recently Blackwell also revealed that the interactions between a ligand and Tyr56 and Ser129 in LasR are also important in determining whether a ligand acts as an antagonist or agonist since these residues bond to the carbonyl of the 3-oxo-C12-HSL ligand to position the lactone head group towards Tyr 60, which is a key residue.<sup>45,47</sup> Docking experiments<sup>48–50</sup> revealed that the docked poses of 3-oxo-C12-HSL and of the 3-amino-oxazolidinone analog (1) are similar, with the exception of the orientation of the 3-oxo group (see Fig. 4). Importantly, the carbonyl head group of both the native ligand and the 3-aminooxazolidinone analog (1) are similarly oriented towards the key Trp60 residue, hinting that the 3-amino-oxazolidinone analog (1) would also act as an agonist.

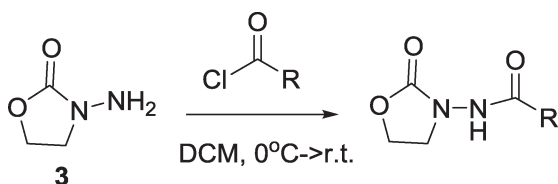


Scheme 1 Degradation of AHL under basic conditions.





**Fig. 3** Surface charge potential on simplified models of AHL and oxazolidinone-based mimic.  $n \rightarrow \pi^*$  Interactions from lone pair ( $n$ ) of the acyl carbonyl group oxygen to the empty  $\pi^*$  on the carbon of carbonyl group in the lactone ring and the distances are highlighted. Computational level: B3LYP/6-311+G(d,2p).<sup>38</sup>

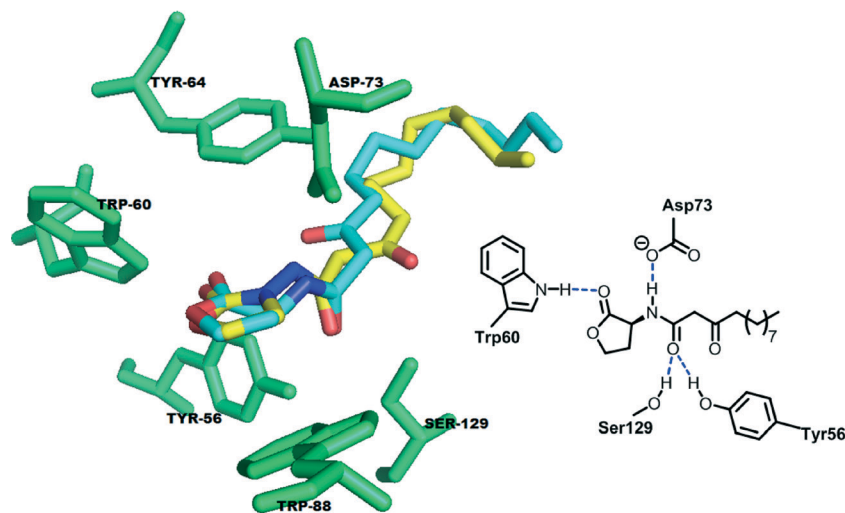


**Scheme 2** Syntheses of the oxazolidinone analogs.

To test whether the 3-aminoxazolidinone analog (**1**) would function similarly to native 3-oxo-C12-HSL, as predicted by the docking experiment (see Fig. 4), we used bacterial reporter strain *E. coli*, pSB1075, (*lasRI::luxCDABE*) to test

for agonism. In the presence of native 3-oxo-C12-HSL, this bacterial strain produced bioluminescence as expected (see Fig. 5). Similarly, the 3-aminoxazolidinone analog (**1**) could also induce bioluminescence in *E. coli* (pSB1075) and the bioluminescence intensities induced by both the native 3-oxo-C12-HSL and the 3-oxo-C12-3-aminoxazolidinone (**1**) were remarkably similar (Fig. 5). The  $EC_{50}$  of 3-oxo-C12-HSL is  $1.5 \pm 0.7$  nM, while the analog **1** gives an  $EC_{50}$  of  $2.1 \pm 0.3$  nM (see Fig. S2†).

Next, we investigated if other LuxR-type proteins would also respond to oxazolidinone analogs. *Chromobacterium violaceum* CV026 is a biosensor strain that does not produce its own AI-1 but can respond to C4 to C8 AHL molecules, via



**Fig. 4** The binding domain (green) in the crystal structure of LasR (PDB code: 2UV0) with native 3-oxo-C12-HSL (cyan) and re-docked 3-aminoxazolidinone analog **1** (yellow).



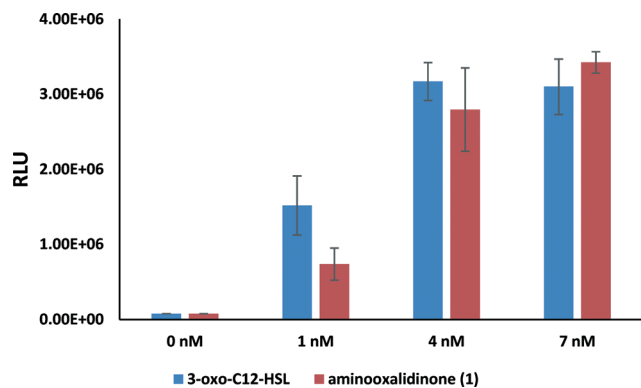


Fig. 5 Bioluminescence induction in *E. coli* pSB1075 after an 8 hour incubation with native 3-oxo-C12-HSL and 3-aminooxalidinone analog 1 at different concentrations.

binding to its LuxR-type QS system CviR, to produce violacein.<sup>51</sup> However, long chain AHLs such as 3-oxo-C12-HSL can inhibit the C4–C8 AHL-induced production of violacein.<sup>51</sup> Addition of 20  $\mu$ M C4-HSL to agar incubated with CV026 led to the production of a dark violet pigment (Fig. 6a).

C4-3-Aminooxalidinone (2) was also able to induce the violacein production. Unlike LasR, CviR preferred the native C4 HSL to C4-3-aminooxalidinone (2). 3-Oxo-C12-HSL can inhibit the C4-HSL-induced violacein production in CV026. In another set of experiment (see Fig. 6b), 3-oxo-C12-3-aminooxalidinone (1) could inhibit C4-HSL-induced violacein production in CV026 but the concentration of 3-oxo-C12-3-aminooxalidinone (1) needed to inhibit the activity of 20  $\mu$ M C4-HSL was higher than the natural 3-oxo-C12-HSL. Whereas 2  $\mu$ M 3-oxo-C12-HSL could completely inhibit 20  $\mu$ M C4-HSL-induced violacein production, it required  $\sim$ 200  $\mu$ M C4-HSL analog 1 to achieve a similar inhibition level (see Fig. 6b).

## Conclusion

In the past decade intensive efforts have been dedicated for the discovery of QS agonists and inhibitors. QS autoinducers have been shown to activate the immune system and hence these molecules and more stable analogs thereof have the potential for use in cancer immunotherapy.<sup>22</sup> Hence the hydrolytically stable 3-oxo-C12-HSL analog 1 described in this manuscript could have anticancer properties and future

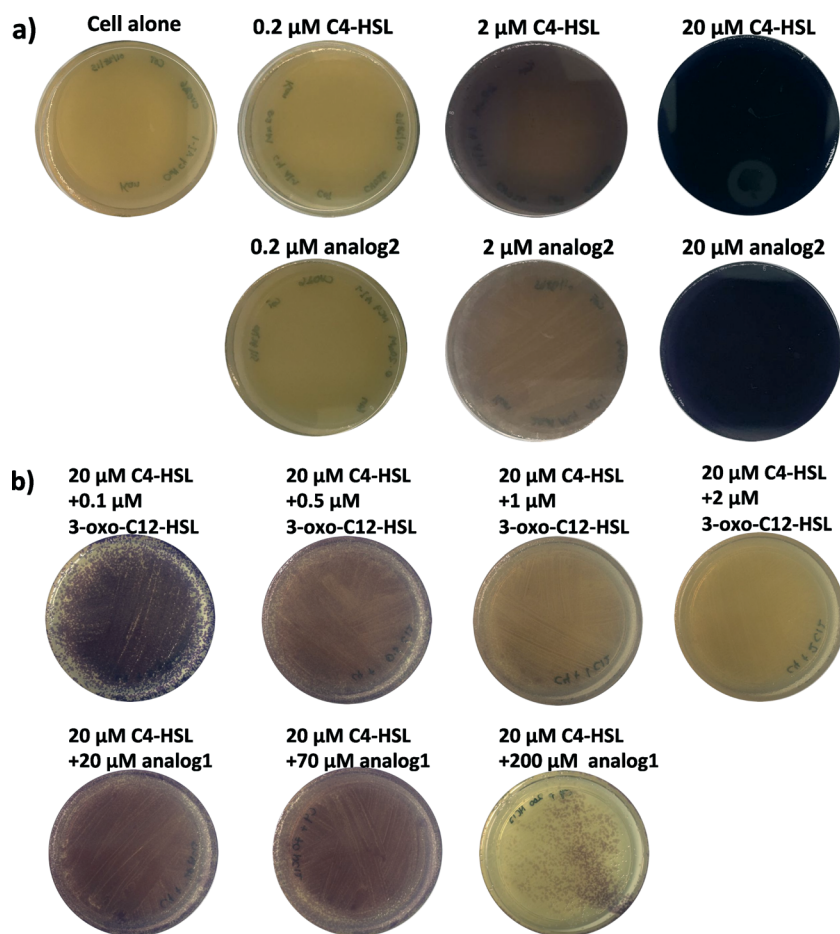


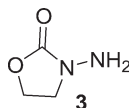
Fig. 6 *Chromobacterium violaceum* CV026 agar plate assay. a) CV026 cultured with different concentrations of C4-HSL and C4-3-aminooxalidinone (2). b) CV026 cultured with different concentrations of 3-oxo-C12-HSL and 3-oxo-C12-3-aminooxalidinone (1) in the presence of 20  $\mu$ M C4-HSL.



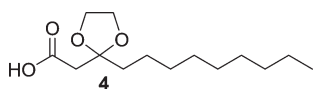
studies along this line are planned. AI-1-based agonists also have the potential for use in synthetic biology applications whereby genetic circuits that are regulated by engineered LuxR-type proteins could be regulated by these molecules.<sup>52,53</sup> In this regard, agonists described in this paper, which are more stable to chemical degradation than the natural autoinducer, could become useful in these applications. LasR receptors could accommodate the C3 to N3 substitution better than CviR protein. LasR is key to the production of various virulence factors during *P. aeruginosa* infection and future study will focus on making side chain variants of the oxazolidinone analogs and test for their activities against *P. aeruginosa*.

## Methods

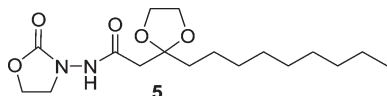
General procedures for preparation of oxazolidinone analogs:



The starting material 3 is commercially available. It is however expensive but can be easily made in the gram scale as follows: a solution of NaOH (0.1 g, 2.5 mmol) in 0.5 ml of methanol was added to a mixture of 2-hydroxyethylhydrazine (2.3 g, 30 mmol) and dimethyl carbonate (4 ml, 48 mmol). The resulting mixture was heated and was stirred at 70 °C for 3 h. Then the reaction was cooled down to room temperature and the unreacted dimethyl carbonate was removed *in vacuo*. The residue was purified by silica column chromatography (methanol:dichloromethane = 1:30, v/v), affording 3 as a white solid (2.01 g, 65%).



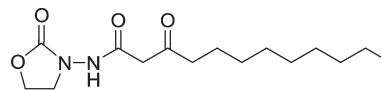
Compound 4 was synthesized according to a procedure in the literature.<sup>54</sup>



**C4-3-aminooxazolidinone (2)**

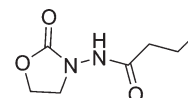
Oxalyl chloride (40 μL, 2.3 equiv.) was added to a solution of 4 (50 mg, 0.19 mmol) in dry dichloromethane at room temperature. The mixture was stirred for 5 h. Then the reaction was concentrated to remove the solvent and excess oxalyl chloride. The residue was re-subjected to dry dichloromethane and the resulting solution was added slowly to a solution of 3 (39 mg, 2 equiv.) in dry dichloromethane at 0 °C. The mixture was allowed to slowly warm up to room temperature and was stirred overnight. Then the reaction was concentrated under vacuum, and the residue was purified by silica column chromatography (methanol:dichloromethane = 1:40, v/v), affording 5 as a white solid (59 mg, 90% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 8.39 (s, 1H), 4.41 (t, *J* = 7.8 Hz, 2H), 4.11–4.02 (m, 2H), 4.02–3.93 (m, 2H), 3.81 (t, *J* = 7.8 Hz, 2H),

2.65 (s, 2H), 1.78–1.67 (m, 2H), 1.44–1.32 (m, 2H), 1.32–1.18 (m, 12H), 0.87 (t, *J* = 6.8 Hz, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) δ 168.8, 157.8, 109.8, 65.6, 62.3, 46.3, 43.5, 38.0, 32.3, 30.1, 29.9, 29.7, 24.0, 23.1, 14.5; HRMS (ESI-TOF) *m/z* calcd. for C<sub>17</sub>H<sub>31</sub>N<sub>2</sub>O<sub>5</sub> [M + 1]<sup>+</sup> 343.2233, found 343.2199.



**3-oxo-C12-3-aminooxazolidinone (1)**

Compound 5 (55 mg, 0.16 mmol) was dissolved in trifluoroacetic acid (0.64 ml) and water (0.16 ml). The mixture was stirred at room temperature overnight. Then the reaction was quenched by saturated NaHCO<sub>3</sub>(aq) until the solution turned neutral. Dichloromethane was used to extract the product three times and the organic phase was dried using anhydrous MgSO<sub>4</sub>. The product was purified by silica column chromatography (methanol:dichloromethane = 1:40, v/v) and afforded 3-oxo-C12-3-aminooxazolidinone (1) as a white solid (34 mg, 71% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 9.08 (s, 1H), 4.44 (t, *J* = 7.8 Hz, 2H), 3.84 (t, *J* = 7.8 Hz, 2H), 3.52 (s, 2H), 2.56 (t, *J* = 7.4 Hz, 2H), 1.64–1.52 (m, 2H), 1.35–1.18 (m, 12H), 0.88 (t, *J* = 6.8 Hz, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) δ 205.8, 165.9, 158.0, 62.5, 48.0, 46.4, 44.1, 32.2, 29.8, 29.6, 29.4, 23.1, 14.5; HRMS (ESI-TOF) *m/z* calcd. for C<sub>15</sub>H<sub>27</sub>N<sub>2</sub>O<sub>4</sub> [M + 1]<sup>+</sup> 299.1971, found 299.1967.



**C4-3-aminooxazolidinone (2)**

Butyryl chloride (50 μL, 0.5 mmol, 1 equiv.) was added to a solution of 3 (102 mg, 1 mmol, 2 equiv.) in anhydrous dichloromethane at 0 °C. The mixture was allowed to warm up to room temperature slowly and was stirred for 3 h. Then the reaction was concentrated under vacuum, and the residue was purified by silica column chromatography (methanol:dichloromethane = 1:30, v/v) and afforded 2 as a 34 mg pale yellow oil (40% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 8.49 (brs, 1H), 4.45 (t, *J* = 8.1 Hz, 2H), 3.84 (t, *J* = 8.1 Hz, 2H), 2.23 (t, *J* = 7.4 Hz, 2H), 1.78–1.61 (m, 2H), 0.98 (t, *J* = 7.4 Hz, 3H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz) δ 172.9, 158.5, 62.5, 46.4, 36.0, 19.0, 14.0; HRMS (ESI-TOF) *m/z* calcd. for C<sub>7</sub>H<sub>13</sub>N<sub>2</sub>O<sub>3</sub> [M + 1]<sup>+</sup> 173.0926, found 173.0903.

## Docking calculations

Docking calculations were performed using Autodock Vina 1.1.1.<sup>48</sup> A large grid box, which is enough to encompass the ligand in the binding pocket, was chosen. The exhaustiveness value was set to 32 in the Autodock calculations and the rest of the parameters were used as default. The ligand PDB files were prepared with ChemDraw. Autodock Tools 1.5.4 was used to convert the PDB files into PDBPT files for the Autodock Vina calculations. The top-ranked conformation



poses were selected for analysis. Results were visualized using PyMOL viewer version 1.3.<sup>49</sup>

### Stability studies

The stability of 3-oxo-C12-HSL and 3-oxo-C12-3-amino-oxazolidinone (**1**) to basic pH was determined *via* HPLC monitoring.<sup>24,55</sup> Briefly, the decomposition of AI-1 or the analog (**1** mM) in 180 mM Tris-HCl, pH 8.0 at 25 °C was monitored by following absorbance changes at 278 nm, using a Jasco V-630 spectrophotometer for 3 hours.

The stability of 3-oxo-C12-3-amino-oxazolidinone (**1**) to basic pH was determined *via* TLC as well (Fig. S1†). 10 mM analog **1** in methanol was mixed with an equivolume of 250 mM Tris-HCl buffer (pH = 8.0) and left at 25 °C for 3 hours. The mixture was spotted on TLC plate and developed using eluent (methanol:dichloromethane = 1:40, v/v). After developing, the TLC plate was air dried and stained using KMnO<sub>4</sub> solution.

### Bioluminescence assay

*E. coli* JM109 (pSB1075) (containing *lasRI::luxCDABE*, a bioluminescent reporter gene) was cultured at 37 °C overnight and diluted 10 times with fresh LB medium. After culture at 37 °C for 7 hours, OD<sub>600</sub> was measured and diluted to OD<sub>600</sub> = 0.01. A cell culture was grown in 37 °C for another hour and diluted to OD<sub>600</sub> = 0.005. 3-Oxo-C12-HSL and analogs were added to the cell cultures and incubated at 37 °C with shaking for 8 hours. Bioluminescence was measured using a Nichols Institute Diagnostics luminometer.

### *C. violaceum* CV026 AHL reporter assay

Agar plate assay:<sup>56</sup> different concentrations of AI-1 and analogs were added into LB agar. *C. violaceum* CV026 culture was diluted to OD<sub>600</sub> of 0.1 and spread onto the agar plates. The plates were incubated at 37 °C overnight and 25 °C for another day.

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