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

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Abstract

Synthetic molecules that modulate quorum sensing, QS, in bacteria have great potential to be used 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 proteins active-site residues as 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.

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[1] and communicate with neighbors using diffusible molecules. Bacteria also communicate via contact, using surface associated receptors[2] or connecting nanotubes.[3] Even in the planktonic state, bacteria can still communicate with neighboring self and non-self cells and respond to population density via response to small molecule autoinducers secreted by other bacteria.[4] 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.[5] 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.[6-9]

In the last decade, many small molecules that modulate quorum sensing have been developed.[10-13] These QS modulators have been either agonists or antagonists and have

the potential to be used in diverse applications, ranging from inhibition of bacterial toxin production and biofilm formation (QS antagonists),[14-18] manipulation of bacterial behavior and synthetic biology applications (both agonists and antagonists)[19-21] to the inhibition of cancer (by 3-oxo-C12 HSL of *Pseudomonas aeruginosa*).[22] 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*.[9-15]

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 dramatic reduction of activity.[23] Unfortunately γ-lactones are not chemically stable and can hydrolyze in mild acidic or basic environments.[24] Additionally bacterial, plant or animal lactonases[25-28] and acylases[29-32] 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 as 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.[13, 33, 34] In this report we show that 3-aminooxazolidinone that lack a 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 AHLs and is therefore a good replacement for the lactone head group in AHL-based QS modulators. 3-Aminooxazolidinone-based analogs (Figure 1) can be made from inexpensive materials in a few steps and are drug-like (examples of oxazolidinone drugs are linezolid[35] and rivaroxaban[36]).

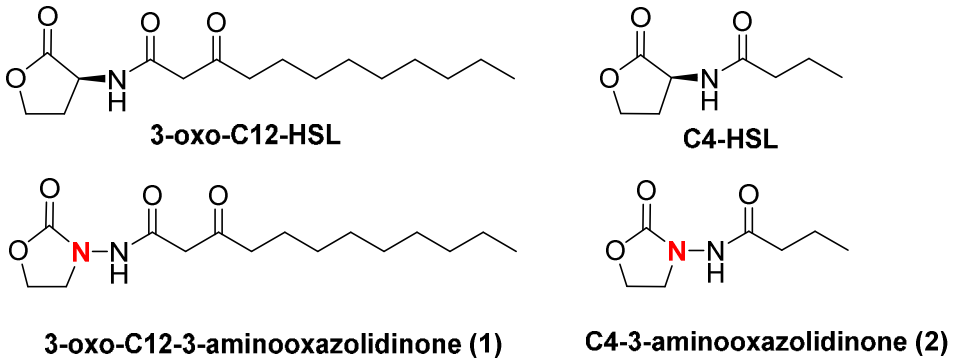
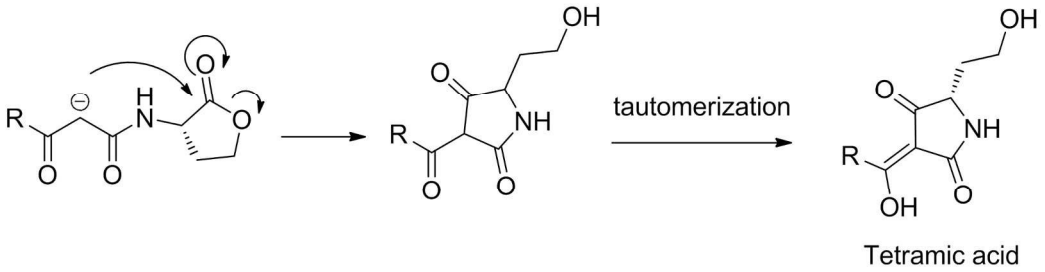


Figure 1. Structures of oxazolidinone AHL analogs and natural AI-1.

Results and Discussion

3-oxo-HSLs are known to degrade under weak, basic conditions and eventually tautomerize to a tetramic acid derivative via a mechanism shown in Scheme 1.[24] To demonstrate that 3-aminooxazolidinone-based analogs 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 (Figure 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 SI (Figure S1). We therefore concluded that 3-aminooxazolidinone analog (**1**) is more stable than 3-oxo-C12-HSL towards basic hydrolysis.

Recently, Raines revealed that the conformation of free AHLs is different from when complexed to LasR.[37] In the free state, the lone pairs of the amide carbonyl forms a favorable interaction with the π* of the lactone carbonyl.[37] This n to π* interaction (worth about 0.64 kcal/mol), is disrupted upon binding to LasR. Interestingly the substitution of the C3 in 3-oxo-C12-HSL with N3 (aminooxazolidinone-based analogs) did not abrogate the n to π* interaction in the free state (see Figure 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 Figure 3), implying that our analogs would be able to partake in charge-charge interactions in the 3-oxo-C12-HSL binding site.



Scheme 1. Degradation of AHL under basic condition.

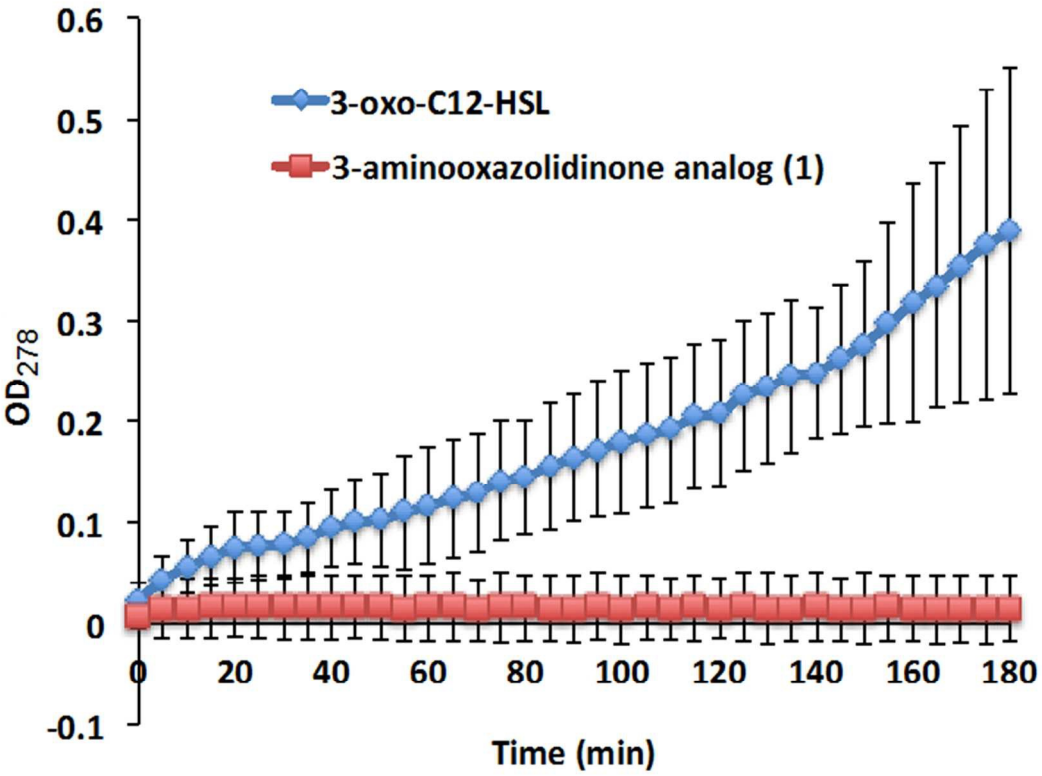


Figure 2. Stability studies of C12-oxo-HSL and 3-oxo-C12-3-aminooxazolidinone (**1**).

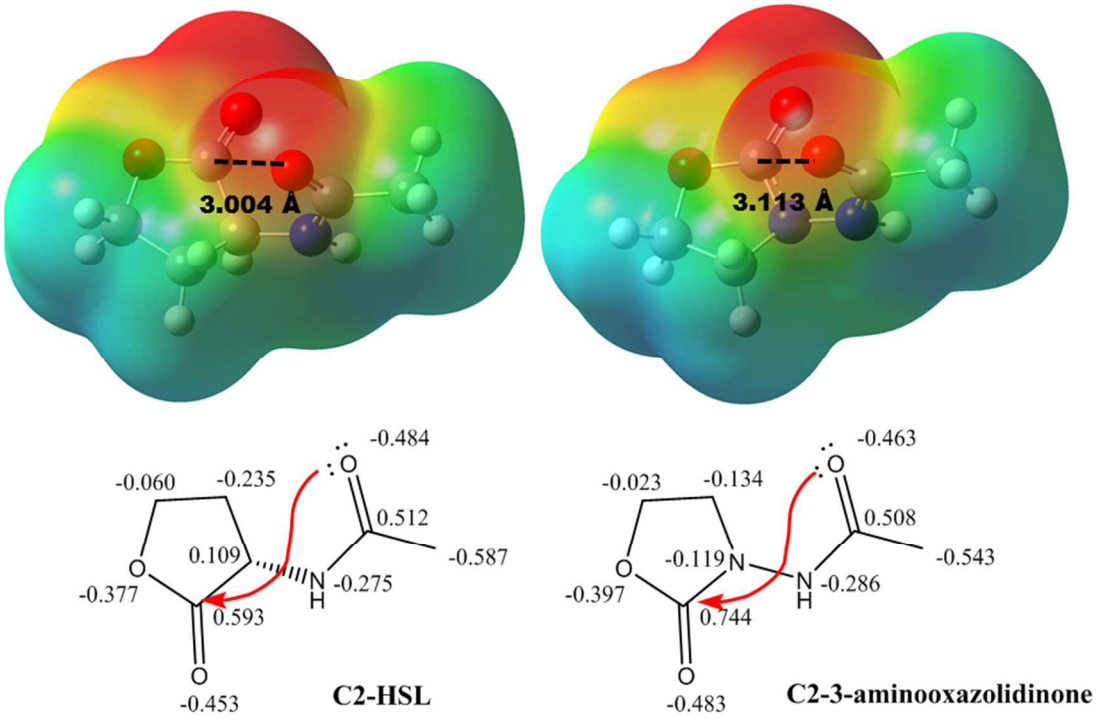


Figure 3. Surface charge potential on simplified models of AHL and oxazolidinone-based mimic. $n \rightarrow \pi^*$ interactions from lone pair (n) of the acyl carbonyl group oxygen to the empty π^* on the carbon of carbonyl group in the lactone ring and the distances are highlighted. Computational level: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

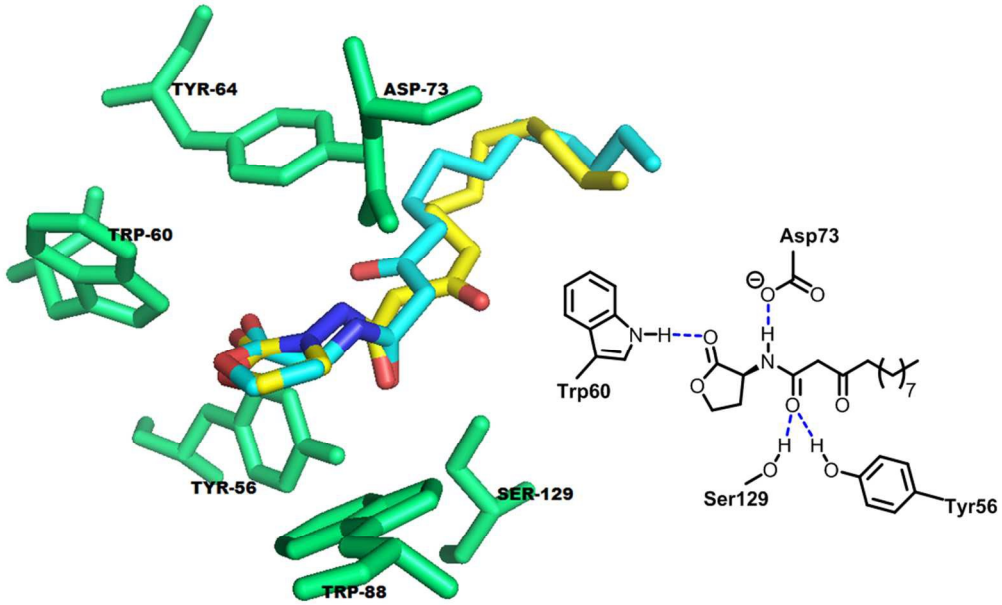


Figure 4. The binding domain (green) in crystal structure of LasR (PDB code: 2UV0) with native 3-oxo-C12-HSL (cyan) and re-docked 3-aminooxazolidinone analog **1** (yellow).

In most LuxR-type proteins reported to date, Trp60 is highly conserved.[39-44] Both Suga and Blackwell have shown that this residue determines whether a ligand acts as an agonist or antagonist.[45, 46] 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.[45, 47] Docking experiments[48-50] revealed that the docked pose of 3-oxo-C12-HSL and 3-aminooxazolidinone analog (**1**) are similar, with the exception of the orientation of the 3-oxo group, see Figure 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 3-aminooxazolidinone analog (**1**) would also act as an agonist.

To test whether 3-aminooxazolidinone analog (**1**) would function similarly as native 3-oxo-C12-HSL, as predicted by the docking experiment (see Figure 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 Figure 5. Similarly, 3-aminooxazolidinone analog (**1**), could also induce bioluminescence in *E. coli* pSB1075 and the bioluminescence intensities induced by both the native 3-oxo-C12-HSL and 3-oxo-C12-3-aminooxazolidinone (**1**) were remarkably similar (Figure 5). The EC_{50} of 3-oxo-C12-HSL is 1.5±0.7 nM, while analog **1** gave an EC_{50} of 2.1±0.3 nM, see Figure S2.

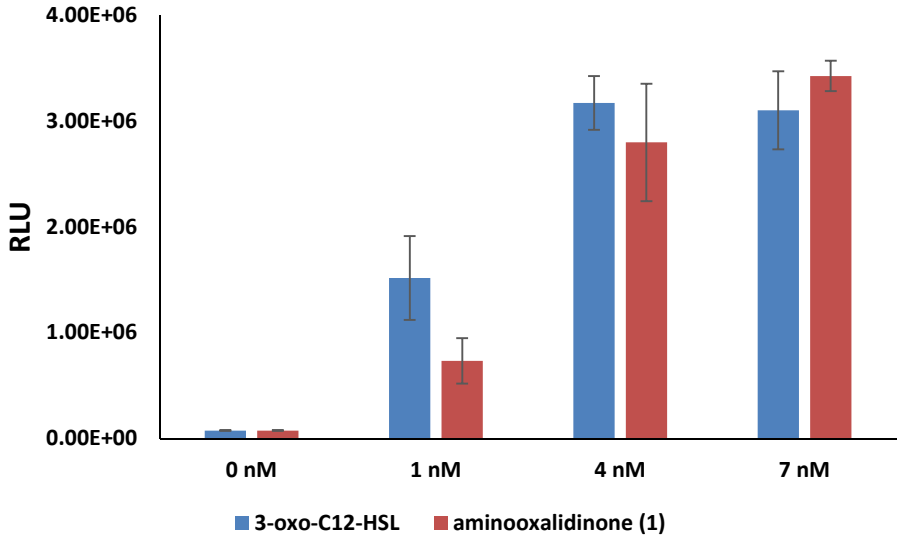


Figure 5. Bioluminescence induction in *E. coli* pSB1075 after 8-hour incubation with native 3-oxo-C12-HSL and 3-aminooxazolidinone analog **1** at different concentrations.

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 binding to its LuxR type QS system CviR, to produce violacein.[51] However, long chain AHLs such as 3-oxo-C12-HSL can inhibit the C4-C8 AHL-induced production of violacein.[51] Addition of 20 μM of C4-HSL to agar incubated with CV026 led to the production of a dark violet pigment (Figure 6a). C4-3-aminooxazolidinone (**2**) was also able to induce the violacein production. Unlike LasR, CviR preferred the native C4 HSL to C4-3-aminooxazolidinone (**2**). 3-oxo-C12-HSL can inhibit the C4-HSL-induced violacein production in CV026. In another set of experiment (see Figure 6b), 3-oxo-C12-3-aminooxazolidinone (**1**) could inhibit C4-HSL-induced violacein production in CV026 but the concentration of 3-oxo-C12-3-aminooxazolidinone (**1**) needed to inhibit the activity of 20 μM C4-HSL was higher than the natural 3-oxo-C12-HSL. Whereas 2 μM of 3-oxo-C12-HSL could completely inhibit 20 μM C4-HSL-induced violacein production, it required ~200 μM of analog **1** to achieve similar inhibition level (see Figure 6b).

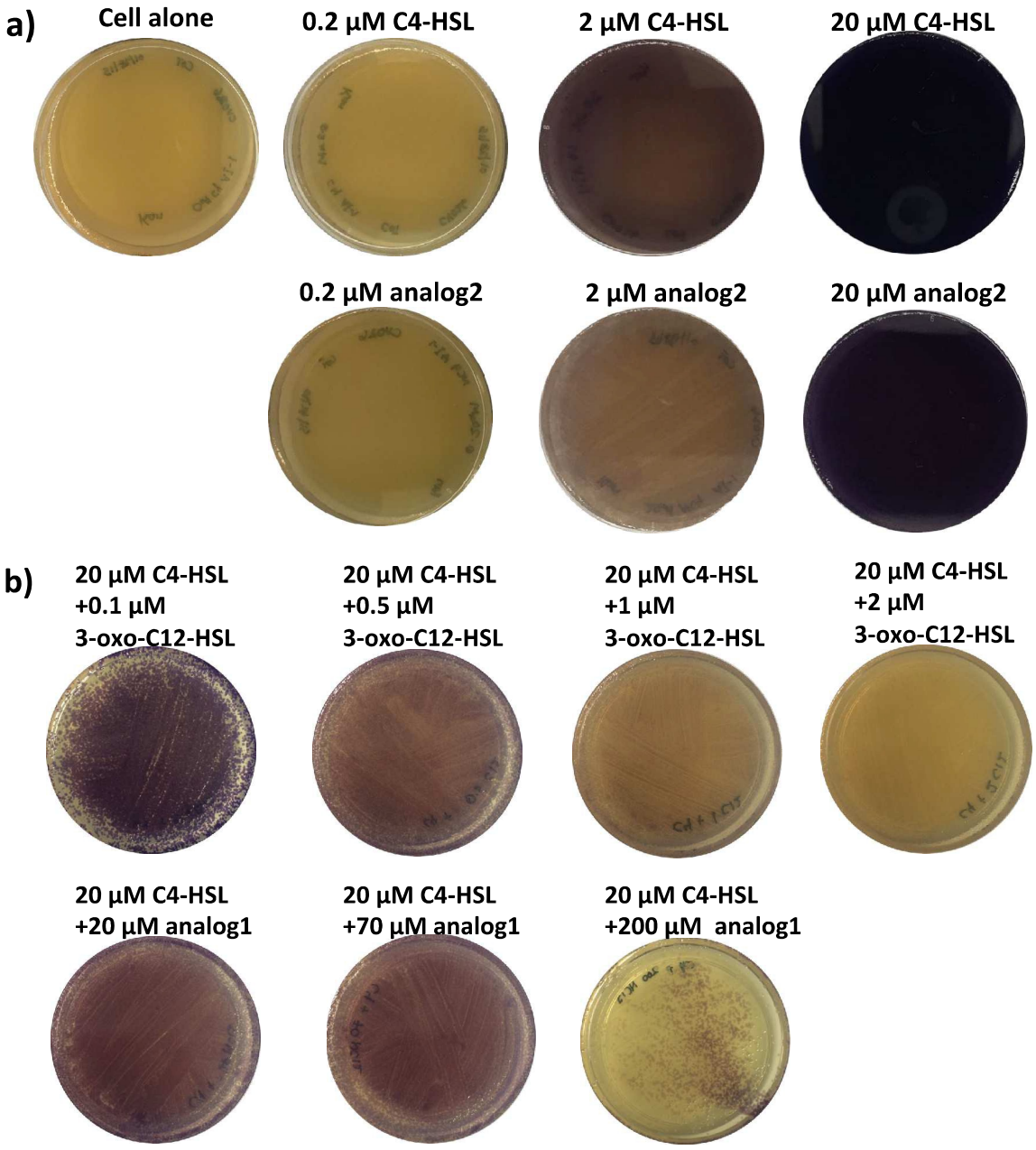


Figure 6. *Chromobacterium violaceum* CV026 agar plate assay. **a)**: CV026 cultured with different concentrations of C4-HSL and C4-3-aminooxazolidinone (**2**). **b)**: CV026 cultured with different concentrations of 3-oxo-C12-HSL and 3-oxo-C12-3-aminooxazolidinone (**1**) in the presence of 20 µM C4-HSL.

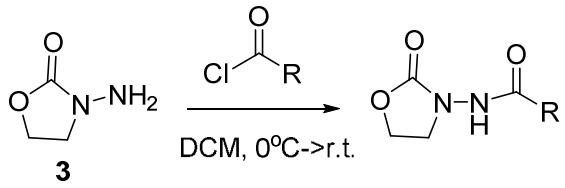
Conclusion

In the past decade intensive efforts have been dedicated to 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 to be used in cancer immunotherapy.[22] Hence hydrolytically stable 3-oxo-C12-HSL analog **1** described

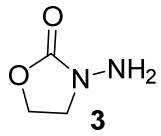
in this manuscript could have anticancer properties and future works along this line are planned. AI-1-based agonists also have the potential to be used in synthetic biology applications whereby genetic circuits that are regulated by engineered LuxR-type proteins could be regulated by these molecules.[52, 53] In this regard, agonists described in this paper, which are more stable towards 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 work will focus on making side chain variants of the oxazolidinone analogs and test for activity against *P. aeruginosa*.

Methods

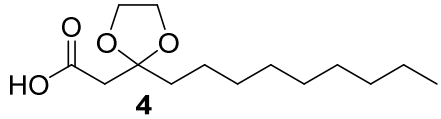
General procedures for preparation of oxazolidinone analogs:



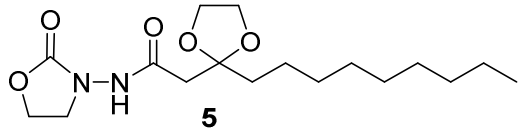
Scheme 1. Synthesis of oxazolidinone analogs



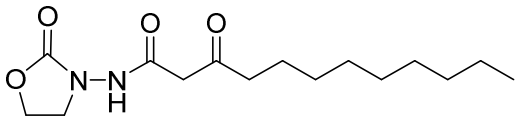
The starting material **3** is commercially available. It is however expensive but can be easily made in gram scale as follows: To a mixture of 2-hydroxylethylhydrazine (2.3g, 30 mmol) and dimethyl carbonate (4ml, 48 mmol) was added a solution of NaOH (0.1g, 2.5 mmol) in 0.5 ml methanol. The resulting mixture was heated and 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*) to afford **3** as a white solid (2.01 g, 65%).



Compound **4** was synthesized according to literature procedure.[54]

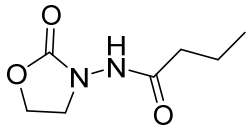


To a solution of **4** (50 mg, 0.19 mmol) in dry dichloromethane was added oxalyl chloride (40 µL, 2.3 equiv) at room temperature. The mixture was allowed to stir for 5 h. Then the reaction was concentrated to remove solvent and excess oxalyl chloride. The residue was re-subjected to dry dichloromethane and the resulting solution was added slowly to a solution of **3** (39 mg, 2 equiv) in dry dichloromethane at 0 ^{o}C. The mixture was allowed to slowly warm up to room temperature and stirred overnight. Then the reaction was concentrated under vacuum, and the residue was purified by silica column chromatography (methanol: dichloromethane = 1: 40, *v/v*) to afford **5** as a white solid (59 mg, 90% yield).^{1}H NMR ($CDCl_3$, 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); ^{13}C NMR ($CDCl_3$, 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 $C_{17}H_{31}N_2O_5$ $[M+1]^+$ 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 $NaHCO_3$ (aq) until the solution turned neutral. Dichloromethane was used to extract the product three times and the organic phase was dried with anhydrous $MgSO_4$. 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). ^{1}H NMR ($CDCl_3$, 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); ^{13}C NMR ($CDCl_3$, 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 $C_{15}H_{27}N_2O_4$ $[M+1]^+$ 299.1971, found 299.1967.



C4-3-aminooxazolidinone (4-2)

To a solution of **3** (102 mg, 1 mmol, 2 equiv) in anhydrous dichloromethane was added butyryl chloride (50 µL, 0.5 mmol, 1 equiv) at 0^{o}C. The mixture was allowed to warm up to room temperature slowly and stir 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 **4-2** as 34 mg pale yellow oil (40% yield).^{1}H NMR ($CDCl_3$, 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),; ^{13}C NMR

($CDCl_3$, 100 MHz) δ 172.9, 158.5, 62.5, 46.4, 36.0, 19.0, 14.0; HRMS (ESI-TOF) *m/z* calcd. for $C_7H_{13}N_2O_3$ $[M+1]^+$ 173.0926, found 173.0903.

Docking calculations:

Docking calculations were performed using Autodock Vina 1.1.1.[48] A large grid box, which is enough to encompass the ligand in the binding pocket was chosen. The exhaustiveness value was set as 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.[49]

Stability studies:

Stability of 3-oxo-C12-HSL and 3-oxo-C12-3-aminooxazolidinone (**1**) towards basic pH was determined via UV monitoring.[24, 55] Briefly, the decomposition of AI-1 or analog (1 mM) in 180 mM Tris-HCl, pH 8.0 at 25 °C was monitored by following absorbance changes at 278 nm, using Jasco V-630 Spectrophotometer for 3 hours.

Stability of 3-oxo-C12-3-aminooxazolidinone (**1**) towards basic pH was determined via TLC, as well (Figure S1). 10 mM of analog **1** in methanol was mixed with equivolume of 250 mM Tris-HCl buffer (pH = 8.0) and left at 25 °C for 3 hours. The mixture along with analog **1** stock solution and Tris-HCl buffer were spotted on TLC plate and developed using eluent (methanol: dichloromethane = 1: 40, *v/v*). After developing, the TLC plate was air dried and stained by $KMnO_4$ solution.

Bioluminescence assay:

E. coli JM109 (pSB1075) (containing *lasRI'::luxCDABE*, bioluminescent reporter,) was cultured at 37 °C overnight and diluted 10 times with fresh LB medium. After culture at 37 °C for 7 hours, OD_{600} was measured and diluted to OD_{600} = 0.01. Cell culture was grown in 37 °C for another hour and diluted to OD_{600} = 0.005. 3-oxo-C12-HSL and analogs were added to cell cultures and incubated at 37 °C with shaking for 8 hours. Bioluminescence was measured with a Nichols Institute Diagnostics luminometer.

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

Agar plate assay[56]: Different concentration of AI-1 and analogs were added into LB agar. *C. violaceum* CV026 culture was diluted to OD_{600} of 0.1 and spread onto the agar plates. Plates were incubated at 37 °C overnight and 25 °C for another day.

Acknowledgements

We are grateful to NSF (CBET 1264509) and Camille Dreyfus Foundation (Teacher-Scholar fellowship to HOS) for funding. YZ is supported by Mehta Graduate Research

Award and Kraybill Biochemistry fellowship. We also thank Professors Shamala Devi Sekaran and Max Teplitski for generous gifts of bacterial strains *Chromobacterium violaceum* CV026 and *E. coli* JM109 (pSB1075) respectively.

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