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Letters

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Vibrio cholerae Autoinducer CAI-1 Interferes with *Pseudomonas aeruginosa* Quorum Sensing and Inhibits its Growth

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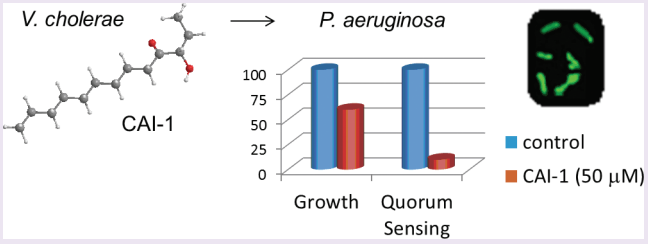
Supporting Information

ABSTRACT: The human pathogen *Vibrio cholerae* uses several small molecules to coordinate gene expression in a process termed quorum sensing (QS), and its main autoinducer is CAI-1. We have examined the activity of this signaling molecule in three other species of bacteria. Interestingly, while showing an inhibitory effect on QS in the opportunistic pathogen *P. aeruginosa* at low micromolar concentrations, it caused also growth inhibition at higher concentrations. In contrast, the two other bacteria were unaffected, and we suggest a possible mechanism for these effects, based on membrane perturbation studies.



As the prevalence of bacterial resistance to antibiotics continues to rise, it has become increasingly important to explore new directions to combat bacterial infections and to further increase our understanding of bacterial pathogenesis. The discovery of a population-density-dependent coordination of gene expression by bacteria, in a process termed quorum sensing (QS), has uncovered a wealth of information on bacterial social behavior and coordinated induction of virulence and pathogenesis.[1,2] In this process, bacteria sense their population density by producing, secreting, and detecting small diffusible signaling molecules termed autoinducers.[2] Quorum sensing controls a variety of processes that are essential for bacterial populations and irrelevant for individual cells, such as light production (bioluminescence), virulence factor expression, and biofilm formation. These cooperative behaviors allow bacterial cultures to compete with (and essentially mimic) multicellular organisms.[3,4]

P. aeruginosa is an opportunistic and ubiquitous human pathogen and is one of the most common Gram-negative bacteria found in nosocomial and life threatening infections of immunocompromised patients.[5] In cystic fibrosis (CF) patients, this pathogen is responsible for the high rate of mortality. QS in *P. aeruginosa* has been widely studied,[6] and several regulators have been identified and characterized. Among these are the *N*-acyl homoserine lactone (AHL) based QS systems *las* and *rhl*. The *lasR-lasI* system consists of the *lasI* gene, which produces the signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) that is detected by a transcriptional regulatory protein LasR, and the *rhlR-rhlI* system in a similar fashion produces and recognizes the signaling molecule *N*-butyryl-L-homoserine lactone (C_4-HSL) that is detected by the transcriptional regulator RhlR. Recent findings show that the primary QS signal in *P. aeruginosa*, 3-oxo-C_{12}-HSL, also affects gene expression in eukaryotes. Most of the examples of this cross-kingdom signaling phenomenon focus on the effect of 3-oxo-C_{12}-HSL on the production of various cytokines by immune cells.[7] Several studies have addressed the mechanism by which 3-oxo-C_{12}-HSL modulates the function of host transcriptional regulators, such as PPARs, and disrupts NF-κB regulating functions in activated mammalian cells.[8]

A different Gram-negative human pathogen, whose QS mechanisms have been investigated extensively in recent years, is *Vibrio cholerae*. This pathogen triggers the disease cholera in humans, which is characterized by acute enteric infection and severe diarrhea and is a major cause of death in developing countries. *V. cholerae* employs QS systems to control its pathogenicity and biofilm formation.[9]

Recently, Higgins *et al.* elucidated the structure of the *V. cholerae* autoinducer, (*S*)-3-hydroxytridecan-4-one (CAI-1, Scheme 1), and demonstrated its control of virulence factor production;[10] another important study by Kelly *et al.* showed that amino-CAI-1 is the precursor of CAI-1 produced by the synthase CqsA.[11] Besides CAI-1, *V. cholerae* also uses the autoinducer AI-2 to control virulence and biofilm formation. CAI-1 and AI-2 operate synergistically to control gene regulation in *V. cholerae*, although CAI-1 was shown to be the dominant signal by Higgins *et al.*[10] While CAI-1 is sensed by the receptor CqsS, AI-2 is synthesized by the enzyme LuxS, and its receptor is the LuxPQ complex.[10]

There is growing evidence that QS signaling molecules can perform important secondary functions, upon encountering

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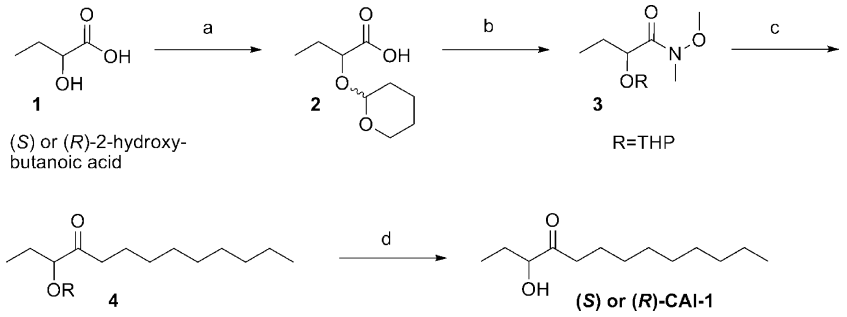
Scheme 1. Synthesis of (*S*)- and (*R*)-CAI-1[a]



[a]Reagents and conditions: (a) DHP (3 equiv), PPTS (cat.), DCM, 18 h, rt, 79%; (b) 1,1-carbonyl-diimidazole, 18 h, rt; imidazole, DMAP, *N*,*O*-dimethyl-hydroxylamine hydrochloride, DCM, 60%; (c) $(CH_2)_8CH_3MgBr$, 0 °C, THF, 74%; (d) PPTS (cat.), ethanol, 55 °C, 3 h, 73%.

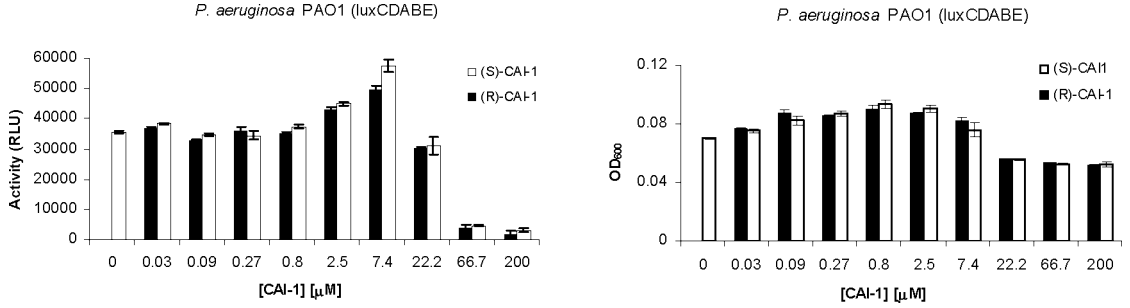


Figure 1. Activation of QS and OD_{600} *versus* different concentrations of (*S*)- and (*R*)-CAI-1 in *P. aeruginosa* PAO1-*luxCDABE* (triplicates, after 5 h of incubation). Activity is shown as relative luminescence units (RLU, luminescence divided by the OD_{600} of the bacteria).

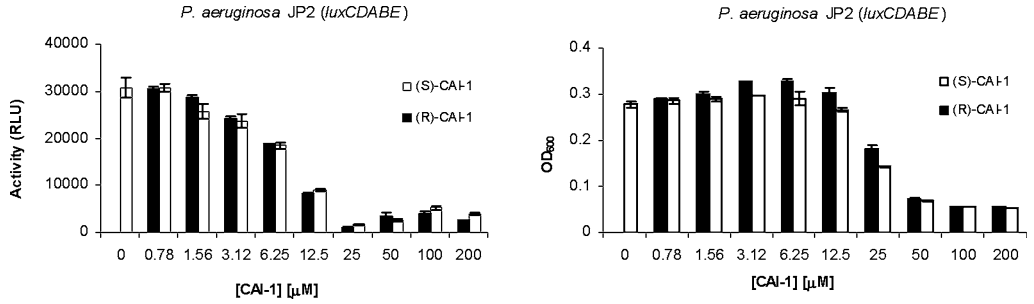


Figure 2. Luminescence and OD_{600} *versus* different concentrations of (*S*)- and (*R*)-CAI-1 in PAO-JP2-*luxCDABE* in the presence of 3-oxo-C_{12}-HSL (100 nM) (triplicates, after 8 h of incubation).

other species, ranging from antibiotic effects or interference with QS in other bacteria, to activation or suppression of eukaryotic defense systems.[12−16] Dual roles for antibiotic agents as growth inhibitors at high concentrations and as signaling molecules at low concentrations have been reported in recent years, and this signaling phenomenon may be explained either as a general stress response or as the result of evolution through co-optation.[17] An example is the production of a third QS molecule by *P. aeruginosa* (3,4-dihydroxy-2-heptylquinolone, PQS), which at higher concentrations displays antibacterial activity against Gram-positive bacteria.[18]

On the basis of structural similarities between CAI-1 and autoinducers employed by other bacteria, we set out to examine whether exogenously added CAI-1 affects signaling in organisms other than *V. cholerae*. While the specificity in binding of its QS molecule is very high for most bacteria, still a certain degree of promiscuity can be found, where closely related structures can effect partial activation of the QS receptor. Examples are the QS systems of *V. fischeri* and *A. tumefaciens*. Whereas the first species shows very high specificity for its autoinducer 3-oxo-C_6-HSL, the latter responds, in addition to its cognate signal 3-oxo-C_8-HSL, also strongly to longer-chain analogues, such as 3-oxo-C_{12}-HSL.[19,20]

We synthesized both enantiomers of CAI-1 (Scheme 1) in order to examine their effects on different bacteria (*P. aeruginosa*, *Staphylococcus aureus*, and *Vibrio harveyi*), by analyzing their ability to interfere with QS and also their potential to inhibit growth. As we encountered difficulties following the published synthesis of CAI-1[10] in purifying the final product, we modified the synthetic route slightly: instead of a *tert*-butyldiphenylsilyl moiety to protect the hydroxyl functionality, we employed a tetrahydropyranyl protection, which after removal significantly facilitated purification of CAI-1.

Both enantiomers of CAI-1 were evaluated for their ability to activate or interfere with the primary QS system (*las*) of *P. aeruginosa*. Using two *P. aeruginosa* reporter assays, based on the luminescent PAO1-*luxCDABE* and PAO-JP2-*luxCDABE* strains, we measured interference with 3-oxo-C_{12}-HSL induced QS activation. PAO1-*luxCDABE* is a *P. aeruginosa* wild-type

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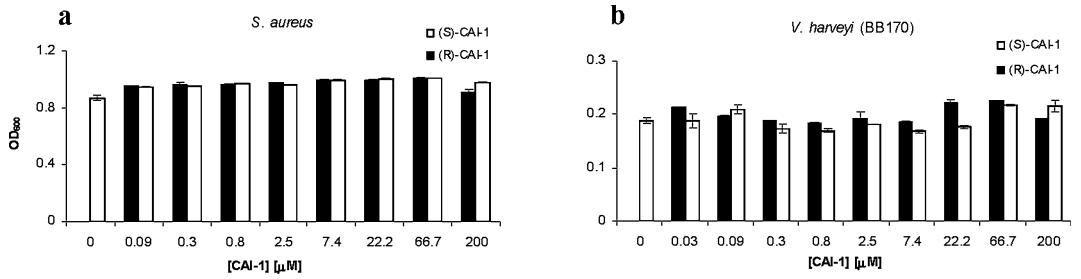

Figure 3. OD_{600} *versus* different concentrations of (*S*)- and (*R*)-CAI-1 in two additional bacteria: (a) *S. aureus* strain (wild-type) after 12 h of incubation and (b) *V. harveyi* (BB170) after 5.2 h of incubation.

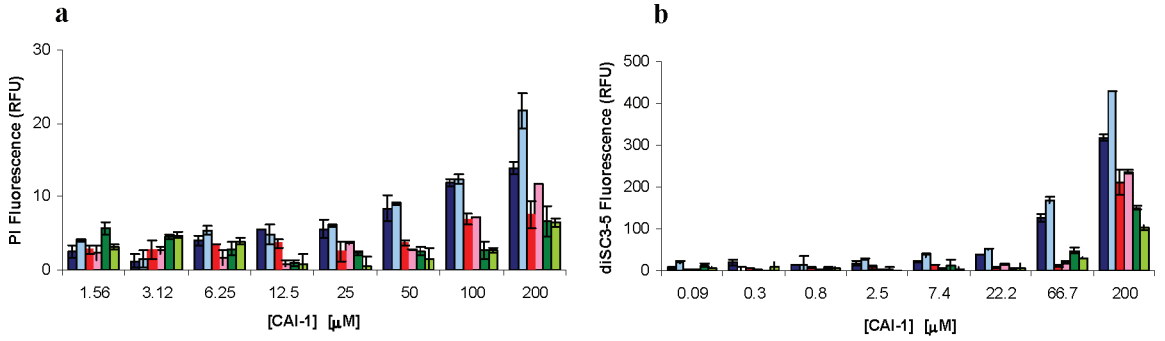

Figure 4. Membrane perturbation assays using two different fluorescent probes. (a) Propidium iodide (PI) fluorescence in relative fluorescence units (RFU) *versus* CAI-1 concentration, after 27 min of incubation with *P. aeruginosa* PAO1 (wild type), *V. harveyi* BB120, and *S. aureus* (wild type strain). (b) diSC3-5 fluorescence (RFU) *versus* CAI-1 concentration, after 27 min of incubation (after reaching a constant fluorescence level) with PAO1 (wild type), *V. harveyi* BB120 and *S. aureus* (wild type strain); *P. aeruginosa* PAO1: (dark blue) (*R*)-CAI-1, (light blue) (*S*)-CAI-1; *S. aureus:* (red) (*R*)-CAI-1, (pink) (*S*)-CAI-1; *V. harveyi*: (dark green) (*R*)-CAI-1, (light green) (*S*)-CAI-1.

strain modified with a luminescent reporter gene *luxCDABE* cloned downstream of the synthase *lasI*, and PAO-JP2-*luxCDABE* is a similar strain but lacks the 3-oxo-C_{12}-HSL and C_4-HSL synthetases. Both strains were incubated in the presence of varying concentrations of CAI-1. The assay with PAO1-*luxCDABE* revealed that while at low concentrations the autoinducer appeared to enhance activation of LasR, at concentrations beyond 22 μM strong inhibition of LasR activation was observed. These effects, however, appeared to correlate with a slight enhancement or inhibition of growth caused by CAI-1 (Figure 1).

Nevertheless, in order to assess whether CAI-1 could specifically mimic or antagonize 3-oxo-C_{12}-HSL, this experiment was repeated with PAO-JP2-*luxCDABE* (a *lasI/rhlI* knockout strain). No activation of LasR was observed, but in the presence of 100 nM 3-oxo-C_{12}-HSL specific and strong antagonism could be seen at concentrations greater than 3 μM, while clear inhibition of growth was observed only at 25 μM and beyond (Figure 2).

While both CAI-1 enantiomers clearly antagonized QS activation by 3-oxo-C_{12}-HSL in *P. aeruginosa*, it was still not clear whether this occurs through competition in binding to LasR. We addressed this question with an *E. coli* β-galactosidase-LasR-based reporter assay. Upon addition of increasing concentrations of 3-oxo-C_{12}-HSL, we measured an increase in the expression levels of β-galactosidase as a result of LasR activation (Supplementary Figure S1, panel b), but no inhibition of activation was observed upon addition of increasing concentrations of CAI-1 (Supplementary Figure S1, panel a). It therefore appears that binding of 3-oxo-C_{12}-HSL to LasR is not affected directly by CAI-1.

In order to study the mechanism of these anti-QS/antibacterial effects and to assess whether other bacteria display similar behavior, we tested the effects of CAI-1 on two additional bacteria. *V. harveyi* a bioluminescent marine bacterium that is closely related to *V. cholerae* and is a pathogen of tiger prawns but nonpathogenic to humans.[27] The Cqs (Cholerae QS) system was discovered a few years ago in *V. harveyi* and acts as a third quorum-sensing system in parallel with the other two systems (the AI-1 and AI-2), and recently the identity of a third autoinducer in *V. harveyi* was confirmed to be (*Z*)-3-aminoundec-2-en-4-one (Ea-C8-CAI-1).[28,29]

In addition we tested the effects of CAI-1 on *Staphylococcus aureus*, which is a Gram-positive bacterium. Gram-positive bacteria lack an outer membrane, while their cell wall contains a thick peptidoglycan layer. *S. aureus* is a human pathogen that is highly resistant to many types of antibiotics, and especially methicillin-resistant *S. aureus* (MRSA) is a major health threat in hospitals.[16,30] In sharp contrast with *P. aeruginosa*, we did not observe any growth inhibition in *V. harveyi* or *S. aureus* upon addition of CAI-1, at concentrations up to 200 μM. (Figure 3).

Interestingly, we did see a certain degree of inhibition of luminescence in *V. harveyi* for high concentrations of (*R*)-CAI-1, which may be caused by competition with *V. harveyi* C8-CAI-1 (Supplementary Figure S2).

We set out to examine the mechanism behind the selective antibacterial behavior of CAI-1. On the basis of recent discoveries that some QS molecules can have secondary functions through perturbation of biological membranes[14] and due to the amphiphilic character of CAI-1 and the similarity in effects between the two CAI-1 enantiomers, we decided to examine whether the antibacterial and anti-QS effect of this autoinducer may be related to disturbance of the

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bacterial membrane. The fluorescent probe propidium iodide (PI), emits a fluorescent signal only upon binding DNA and can enter a bacterial cell only upon formation of pores in the membrane. It is thus used as a probe to determine whether certain antibacterial agents form membranal pores. A second mechanism through which an antibiotic agent can affect membranes is through disruption of the membrane potential gradient ($\Delta\Psi$). The cationic dye diSC3-5 (3,3′-dipropylthiodicarbocyanine iodide) associates with the membrane, and upon aggregation its fluorescence is self-quenched. If $\Delta\Psi$ undergoes a change, due to disturbance and depolarization of the membrane, the dye is released from the cell, resulting in an increase in fluorescent signal.[16,31]

The increase in fluorescence in *P. aeruginosa* PAO1 is probably due to the ability of CAI-1 (beyond 25 μM) to form membranal pores and thus allow DNA binding by the probe PI (Figure 4, panel a). This concentration of increase in fluorescence correlates well with the concentration of growth inhibition and bioluminescence inhibition as a result of cell death (Figure 1). If we compare bacterial growth (OD_{600} measurements) with the membrane potential dissipation ($\Delta\Psi$), caused because of increased membrane permeability as a result of CAI-1 disruption, there is a strong correlation between the CAI concentration in which *P. aeruginosa* growth is inhibited and the concentration in which fluorescence increases due to membrane depolarization (Figure 4, panel b). The rise in fluorescence in the assays performed using diSC3-5 and PI in *V. harveyi* and *S. aureus* at high concentrations (200 μM) of CAI-1 may correlate with the initiation of membrane perturbation by permeabilization of cell membrane and depolarization, which at these concentrations do not yet cause lethality to these two bacteria, although the overall decrease in polarity of the solution (at high concentrations of CAI-1) by itself causes an increase in diSC3-5 fluorescence (Supplementary Figure S3). As negative control DMSO (2%) was added, and the resulting background fluorescence was subtracted from all measurements. As a positive control in both assays we tested the activity of the known antibiotic peptide polymyxin B, which disrupts the membrane structure of Gram-negative bacteria (Supplementary Figure S4).

From the results of the growth inhibition and membrane permeation/depolarization assays in *P. aeruginosa*, *V. harveyi*, and *S. aureus*, there appears to be a strong correlation between the growth inhibitory effects of (*S*)- and (*R*)-CAI-1 and its ability to disturb biological membranes, but thus far it appears to be selective for *P. aeruginosa*. *V. harveyi*, *V. cholerae*, and *P. aeruginosa* are all Gram-negative bacteria, but *V. cholerae* has a different outer membrane lipopolysaccharide (LPS) composition compared to other Gram-negative bacteria.[32] In addition, *V. cholerae*'s outer membrane is relatively permeable to hydrophobic molecules compared to other Gram-negative strains.[33] This may help *V. cholerae* to prevent membrane perturbation by its autoinducer.

Recent findings have shown that several known antibiotics, such as azithromycin and ciprofloxacin, inhibit QS at concentrations that do not inhibit bacterial growth.[34−36] We examined three known antibiotics, in order to compare their effects on QS and growth inhibition in *P. aeruginosa* with the effect observed with synthetic CAI-1. Polymyxin B is a polycationic cyclic lipopeptide antibiotic that disrupts normal permeability to small molecules and acts primarily by disrupting the cytoplasmic membrane, upon binding to the lipid A portion of bacterial LPS, inducing pore formation in bacterial cell walls.[37] Tetracycline and erythromycin act through different modes of action, primarily by inhibiting or interfering with protein synthesis. While the growth-inhibitory IC_{50} values of erythromycin and tetracycline appear to be similar or even lower than their QS IC_{50} values, we observed a strong effect of polymyxin B on both QS and bacterial growth in *P. aeruginosa* (PAO1 and JP2), similar to the effect observed with the CAI-1 enantiomers. Indeed, according to the calculated IC_{50} values of polymyxin B, QS inhibition occurs before the onset of growth inhibition (Table 1, Supplementary Figure S5). We propose

Table 1. IC_{50} Values (μM) for *P. aeruginosa* QS Inhibition (Luminescence) and Growth Inhibition (OD_{600}) of Polymyxin B, Tetracycline, and Erythromycin

	PAO1 (*luxCDABE*)		JP2 (*luxCDABE*)	
IC_{50} [μM]	luminescence	OD_{600}	luminescence	OD_{600}
polymyxin B	0.059 ± 0.004	0.14 ± 0.02	0.032 ± 0.004	0.087 ± 0.017
tetracycline	2.2 ± 1.8	2.2 ± 0.4	4.35 ± 0.57	2.08 ± 0.55
erythromycin	56 ± 25	13.9 ± 4.3	>100	30.3 ± 5.9

that, similar to polymyxin B, CAI-1, destabilizes the outer membrane, resulting in disruption of membranal receptor localization and/or structure, directly affecting QS signaling. This model fits our hypothesis regarding the dual role of CAI-1, showing similarity in anti-QS effects between membrane-perturbing antibiotics and CAI-1.

In order to examine whether *V. cholerae* can actually produce CAI-1 at levels that will affect *P. aeruginosa*, we measured CAI-1 concentrations in *V. cholerae* biofilms. Higgins *et al.* estimated a concentration of CAI-1 in *V. cholerae* cell-free fluids of 1.25 μM; in biofilms, however, this concentration may be substantially higher, as was reported for 3-oxo-C_{12}-HSL concentrations in *P. aeruginosa* biofilms.[21] Biofilms consist of structured sessile communities of bacterial cells surrounded by a self-produced polysaccharide matrix. These microcolonies are attached to solid surfaces and form a three-dimensional architecture that also contains water channels for nutrient and waste passage.[22] As for the physiological role of biofilm, it is generally known to increase bacterial resistance to antibiotics and the host immune response,[22] and in *V. cholerae* it has an additional significance. Zhu *et al.* proposed that as *V. cholerae* must survive the acidic environment of the stomach before reaching the intestine, biofilm formation is likely to assist *V. cholerae* to survive.[23] At higher cell densities biofilm formation is then repressed, enabling *V. cholerae* to leave its host and infect others.[24] We assumed that CAI-1 concentrations in biofilms vary over time, and we set out to quantify its concentration by extraction of CAI-1 from *V. cholerae* O139 biofilms at three time points.

These measurements verify and strengthen our findings regarding CAI-1 inhibitory concentrations for QS activation and growth in *P. aeruginosa* (Figure 5). We found that in one of the biofilm experiments CAI-1 concentrations reached 32 μM (Supplementary Figure S6), and the average CAI-1 concentration detected after 40 h of incubation was 16 μM (Figure 5).

Although *V. cholerae* and *P. aeruginosa* are able to occupy similar habitats (*e.g.*, fresh and saltwater, animals), it is not clear to what extent they compete when they share a certain niche, such as the human intestine.[25,26] Our results, however, indicate that *V. cholerae* at least has the ability to reduce virulence and possibly also inhibit growth of *P. aeruginosa in vivo*. The efficacy

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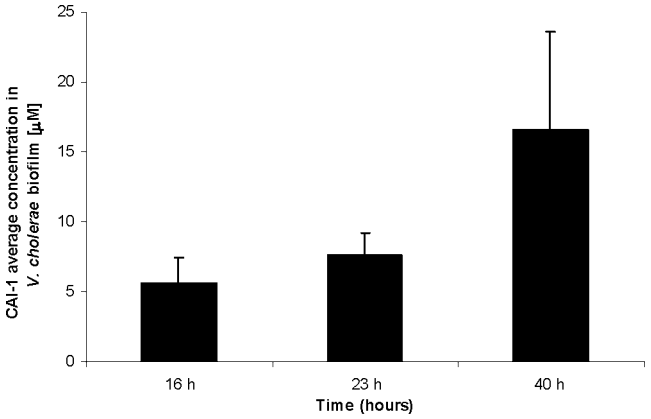

Figure 5. Average CAI-1 concentrations in nine *V. cholerae* O139 biofilm formation experiments at three time points.

of CAI-1 in inhibiting QS in PAO1 is close to values reported for other potent natural and synthetic inhibitors, such as *Delisea pulchra* bromofuranones (IC_{50}: 1−2 μM)[5] and 4-Br-PHL (IC_{50}: 4−16 μM).[20]

In summary, CAI-1 interferes with QS and inhibits growth selectively in *P. aeruginosa*. These two effects appear to be correlated, and while we do not exclude the possibility that CAI-1 binds to a protein that is part of the QS regulon in *P. aeruginosa*, albeit with low selectivity between the two enantiomers, we propose that these effects are related to the ability of CAI-1 to perturb membrane dynamics. At low concentrations this leads to inhibition of QS, possibly through disturbance of QS receptor organization/orientation within the membrane, while at higher concentrations membranal pore formation is initiated, leading to cell death. Whether these previously unknown effects confer a competitive advantage to *V. cholerae* over other bacteria, such as *P. aeruginosa*, in order to exclude these potential rivals from its habitat, remains to be investigated.

METHODS

Bioluminescence Measurements. *BB170 (Ant)agonist Assay.* Both enantiomers of CAI-1 were tested for their (ant)agonistic activity in *V. harveyi* following the protocol reported by Schauder *et al.*[38] as follows: *V. harveyi* strains BB170 (ATCC BAA-1117) were cultured for 18 h at 30 °C in AB medium and then diluted 1:1000 into fresh AB medium. A 96-well microtiter plate was prepared with wells containing test compounds serially diluted into AB medium, and 50 μL of the diluted cells was added to each well. The negative control contained all of the substances besides the autoinducer tested. Boric acid was not added during these assays, as trace amounts are present in the medium. Luminescence generally started to increase after 4 h and was measured every 20 min at 30 °C, using a Microtiter Plate Reader (SpectraMax M2, Molecular Devices).

PAO1-luxCDABE (Ant)agonist Assay. *P. aeruginosa* PAO1 wild type strain, harboring plasmid pKD201 containing a *lasI* promoter coupled to the *luxCDABE* luminescence system (pKD201, obtained from M. Surette[39]) was incubated overnight in LB medium containing 300 μg/mL of trimethoprim. A 96-well black microtiter plate (Greiner) was prepared with the desired concentrations (0.03−200 μM) of (*S*)- and (*R*)-CAI-1, a control well in each row containing medium and 2% DMSO, and bacteria were added to reach a final absorbance density (OD_{600}) of 0.015. The plate was then incubated for a period of 16 h at 37 °C, and during this time luminescence measurements were performed at 20 min intervals using a Microtiter Plate Reader (SpectraMax M2, Molecular Devices). Luminescence values were plotted against the added compound concentration.

PAO-JP2-luxCDABE (Ant)agonist Assay. PAO-JP2, a *lasI-rhlI* double mutant of PAO1 harboring a plasmid pKD201 with a *lasI* promoter coupled to the *luxCDABE* box,[39] was incubated overnight in LB medium containing 300 μg/mL of trimethoprim. A 96-well black microtiter plate (Greiner) was prepared with wells containing test compounds serially diluted into LB medium, and bacteria were added to reach a final absorption density (OD_{600}) of 0.015. For antagonist experiments, a final concentration of 100 nM of the native 3-oxo-C_{12}-HSL was added. The plate was then incubated for 16 h at 37 °C, and during this time luminescence measurements were performed at 20 min intervals. Luminescence values were plotted against the added compound concentration.

Bacterial Growth Inhibition Measurements. The effect of (*S*)- and (*R*)-CAI-1 on the growth of bacterial strains *V. harveyi* (BB170) and *P. aeruginosa* PAO1-*luxCDABE* and PAO-JP2-*luxCDABE* was evaluated as follows. Bacterial strains were grown as described above (except for *S. aureus*, which was grown in CY broth). A 96-well transparent microtiter plate (Greiner) was prepared with wells containing test compounds serially diluted into the appropriate medium, and 50/100 μL of the diluted cells was added to each well. The control contained all of the substances besides the autoinducer tested. The plate was then incubated for 16 h at the appropriate temperature using a Microtiter Plate Reader (SpectraMax M2, Molecular Devices), and during this time OD_{600} values were recorded every 20 min. OD_{600} values were plotted against the added compound concentration. Measurements were performed in triplicates or duplicates, and results are the average of these measurements. Error bars depict the average standard deviation for each measurement.

Membrane Perturbation Studies Using Fluorescent Probes. *Membrane Permeability Assay.* Membrane permeability was assessed using the fluorescent probe propidium iodide (PI) as described previously by Lowery *et al.*[29] The wild type bacterial strains *P. aeruginosa* PAO1, *V. harveyi* BB120 (ATCC BAA-1116), and *S. aureus* (RN4850) were grown overnight, then centrifuged, and washed once with 50 mM potassium phosphate buffer (pH 6.5). PI was dissolved in the same buffer to a concentration suitable for a final concentration of 26 μM, and the cells were resuspended in this solution and diluted to a final OD_{600} of 0.6 (in each well). The cells were incubated with varying concentrations of (*S*)- and (*R*)-CAI-1 (and polymyxin B as a positive control) in a 96-well black microtiter plate (Greiner) and fluorescence values (λ_{ex} 488, λ_{em} 617) were recorded every 20 s for about 1 h using a Microtiter Plate Reader (SpectraMax M2, Molecular Devices).

Membrane Depolarization Assay. Disruption of the membrane potential gradient (ΔΨ) was measured using the membrane-potential-sensitive dye diSC3-5 (3,3′-dipropylthiodicarbocyanine iodide). Bacterial cells (*P. aeruginosa* PAO1, *V. harveyi* BB120, and *S. aureus*) of overnight cultures were centrifuged and washed once with buffer (5 mM HEPES, pH 7.2, containing 5 mM glucose). The cells were resuspended in the same buffer containing diSC3-5 in a concentration suitable for a final concentration of 5 μM and a final OD_{600} of 0.4 (in each well). The cells containing diSC3-5 were incubated for about 30 min at the bacterial growth temperature, until a steady reduction in fluorescence was achieved (due to fluorescence quenching as diSC3-5 becomes concentrated in the cell). Then, the suspension was added into a 96-well black microtiter plate (Greiner) containing varying concentrations of (*S*)- and (*R*)-CAI-1 (and polymyxin B as a positive control) and fluorescence values (λ_{ex} 643, λ_{em} 665) were recorded every 20 s for about 1 h using a Microtiter Plate Reader (SpectraMax M2, Molecular Devices).

Biofilm Assays. *V. cholerae* strain O139-3 *ctxA*(+) (presence of cholerae toxin gene), isolated from India,[40] was grown on TCBS agar plates for 24 h at 37 °C. Colonies were transferred into LB medium and grown overnight with agitation. Biofilms were formed in static cultures, after inoculation (100 μL) with an overnight culture of 10 mL of LB medium at 37 °C (1:100 dilution) in sterile disposable culture flasks (polystyrene, flat flasks, 75 cm^2, 250 mL). At three different time points, 16, 23, and 40 h, the medium was discarded so that the remaining biofilm was nearly dry, and the biofilm was carefully scraped

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off the flask and transferred to a 15 mL Falcon tube, while its volume was measured. Dichloromethane (DCM, 5 mL) was added, and the tube was shaken vigorously for 1 min. Four milliliters of the DCM layer was then transferred to a 20 mL glass vial and evaporated. The concentrate was dissolved in 100 μL of DCM and analyzed for CAI-1 content by GC−MS. Conditions: injection volume: 5 μL, He carrier gas. Inlet split, ratio 25:1, flow 25 mL/min. Heating sequence: 5 min at 50 °C, then 20 °C/min increase to 300 °C, held for 12 min. Total time: 29.5 min.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, spectral data, and biological protocols. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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