Al-2 quorum-sensing inhibitors affect the starvation response and reduce virulence in several *Vibrio* species, most likely by interfering with LuxPQ

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The increase of disease outbreaks caused by *Vibrio* species in aquatic organisms as well as in humans, together with the emergence of antibiotic resistance in *Vibrio* species, has led to a growing interest in alternative disease control measures. Quorum sensing (QS) is a mechanism for regulating microbial gene expression in a cell density-dependent way. While there is good evidence for the involvement of auto-inducer 2 (AI-2)-based interspecies QS in the control of virulence in multiple *Vibrio* species, only few inhibitors of this system are known. From the screening of a small panel of nucleoside analogues for their ability to disturb AI-2-based QS, an adenosine derivative with a p-methoxyphenylpropionamide moiety at C-3’ emerged as a promising hit. Its mechanism of inhibition was elucidated by measuring the effect on bioluminescence in a series of *Vibrio harveyi* AI-2 QS mutants. Our results indicate that this compound, as well as a truncated analogue lacking the adenine base, block AI-2-based QS without interfering with bacterial growth. The active compounds affected neither the bioluminescence system as such nor the production of AI-2, but most likely interfered with the signal transduction pathway at the level of LuxPQ in *V. harveyi*. The most active nucleoside analogue (designated LMC-21) was found to reduce the *Vibrio* species starvation response, to affect biofilm formation in *Vibrio anguillarum*, *Vibrio vulnificus* and *Vibrio cholerae*, to reduce pigment and protease production in *V. harveyi*, and to protect gnotobiotic *Artemia* from *V. harveyi*-induced mortality.

INTRODUCTION

*Vibrio* species are ubiquitous in marine environments worldwide (Igbinosa & Okoh, 2008). As opportunistic pathogens they can cause mild to severe infections in humans and marine animals. *Vibriosis* is one of the most prevalent fish diseases, mainly caused by *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio harveyi* and *Vibrio campbellii* (Garcia et al., 1997; Austin & Zhang, 2006). Other *Vibrio* species are pathogenic for humans. *Vibrio vulnificus* is associated with gastrointestinal infections primarily following the consumption of raw and undercooked seafood, but it can also cause wound or soft-tissue infections (Bross et al., 2007). In addition, systemic *V. vulnificus* infections are notorious for their high mortality rate (Chiang & Chuang, 2003). *Vibrio cholerae* is responsible for pandemic and epidemic outbreaks of cholera (Griffith et al., 2006). *V. cholerae* serotype O1 causes the majority of the outbreaks worldwide, while the O139 serotype has only been detected in South-East and East Asia (Sack et al., 2004; Griffith et al., 2006). Cell-cell communication (quorum sensing; QS) in *Vibrio* species plays an important role in virulence. QS in *Vibrio* species involves three types of signal molecules. N-Acylhomoserine lactones (AHLs) are used in the LuxM/N QS system, cholera auto-inducer 1 (CAI-1) in the CqsA/S system and auto-inducer 2 (AI-2) in the LuxS/PQ QS system (Bassler et al., 1993, 1997; Higgins et al., 2007; Ryan & Dow, 2008). AI-2 is synthesized starting from S-adenosylmethionine, which (through a series of enzymatic reactions, including the reaction catalysed by LuxS) is converted to 4,5-dihydroxy-2,3-pentanedione (DPD) (Surette et al., 1999; Winzer et al., 2002). The spontaneous

**Abbreviations:** AI-2, auto-inducer 2; CV, crystal violet; DPD, 4,5-dihydroxy-2,3-pentanedione; MCPBA, 4-methoxycarbonyl-phenylboronic acid; QS, quorum sensing; OSI, QS inhibiting.

A set of supplementary methods, three supplementary figures and four supplementary tables are available with this online version of this paper.
cyclization of DPD followed by esterification with a tetrahydroxyborate anion results in the formation of AI-2 (Miller et al., 2004). In Vibrio species, sensing of extracellular AI-2 involves two proteins, LuxP and LuxQ (Chen et al., 2002). At a low AI-2 concentration, LuxQ will be autophosphorylated, resulting in the transfer of a phosphate group to LuxO via LuxU (Freeman & Bassler, 1999a, b). Phosphorylation of LuxO leads to its activation and the production of small regulatory RNAs. These small RNAs, together with the chaperone protein Hfq, destabilize mRNA of the response regulator LuxR. In the absence of AI-2, LuxR is not produced and LuxR-dependent genes are not transcribed. Binding of AI-2 to the LuxPQ complex initiates a switch from kinase to phosphatase activity, which results in the dephosphorylation of the downstream proteins LuxU and LuxO. Dephosphorylated LuxO is inactive and does not induce the production of small regulatory RNAs. Hence, the response regulator LuxR is produced and initiates transcription of target genes, including several virulence genes. Therefore, QS inhibitors are promising antipathogenic agents. Due to the presence of the luxS gene in diverse bacterial species, AI-2 is considered to be a signal for interspecies communication (Xavier & Bassler, 2003). However, the LuxPQ signal transduction system is restricted to Vibionales (Sun et al., 2004; Rezzonico & Duffy, 2008). The increase of Vibrio disease outbreaks in aquatic organisms as well as in humans (Harvell et al., 2002; Boyd et al., 2008; Kapp, 2009), together with the emergence of antibiotic resistance in Vibrio species (Karunasagar et al., 1994; Scrascia et al., 2006), has resulted in a growing interest in alternative disease control measures (Lynch & Wiener-Kronisch, 2008). A novel approach consists of interfering with bacterial communication (Ni et al., 2009). Several cinna- maldehyde and furanone derivatives disrupt AI-2-based QS in Vibrio species by decreasing the DNA-binding activity of the response regulator LuxR, and are active both in vitro and in vivo (Defoirdt et al., 2006, 2007; Brackman et al., 2008). Other compounds, including S-anhydroribosyl-L-homocysteine and S-homoribosyl-L-cysteine, block the production of AI-2 by inhibiting the key enzyme LuxS (Alfaro et al., 2004; Shen et al., 2006). Based on the concept of molecular mimicry and through virtual screenings using the crystal structure of LuxP, new AI-2 QS inhibitors have been discovered (Li et al., 2008; Ni et al., 2008a, b). However, although these compounds affect bioluminescence in V. harveyi, they were evaluated neither for their effect on QS-regulated virulence factors nor for their activity in vivo. The goal of the present study was to test whether previously described AI-2 QS inhibitors targeting LuxPQ and various compounds from our collection have the ability to block the production of QS-regulated virulence factors in Vibrio species.

**METHODS**

**Bacterial strains and growth conditions.** All bacterial strains used in this study are listed in Table 1. They were cultured in Marine-Broth (MB) (BD) in the presence of appropriate antibiotics at 30 °C with shaking, except for Escherichia coli DH5α and E. coli K-12, which were grown in Luria–Bertani broth (LB) (BD) at 30 °C and 37 °C, respectively, without shaking.

**Compound library.** The compounds used in the present study consisted of a selected set of known AI-2 QS inhibitors, supplemented with a series of nucleoside (mainly adenosine) analogues (Fig. 1). 3′-Azido-3′-O-[N-(3′-O-acetyl)-N,N,N′,N′-tetramethyl-1H-benzotriazole-1-yl]uronium hexafluorophosphate (HTCu) as the coupling agent in a mixture of dimethylformamide (DMF) and dichloromethane (see Supplementary Methods and Supplementary Fig. S1). The synthetic route followed for the synthesis of the 3′-branched-chain analogue SC-23, differing from that for LMC-21 by the insertion of a CH2 group between C-3′ of the ribofuranose ring and the amide moiety started from the previously described intermediate 1 (Kim et al., 2003) (see Supplementary Methods and Supplementary Fig. S3). The 2′-modified adenosine analogues LMC-29, LMC-30 and LMC-35 have been recently synthesized and found to be potent adenosine A3 receptor antagonists/partial agonists (Cosyn et al., 2006), while PVR-121 is an agonist for the same receptor (Ohno et al., 2004). The amides derived from 3′-(4-methoxyphenyl)propanoic acid (i.e. SC-1, SC-2 and SC-3) were prepared by ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-mediated coupling of the parent carboxylic acid with the appropriate amine in the presence of N,N′-diisopropylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC) and N-hydroxysuccinimide (NHS) as coupling agents in a mixture of dimethylformamide (DMF) and dichloromethane (see Supplementary Methods and Supplementary Fig. S3) that was converted to methyl glycoside (intermediate 5) upon reaction with SnCl4 and dry methanol (Moradei et al., 1991). Remarkably, during this reaction, a larger amount of intermediate 6 was formed. The reaction mixture could be efficiently separated by flash chromatography, and 5 and 6 were separately deprotected upon treatment with NH3 in methanol, thereby yielding 7.1 and 7.2. NMR analysis revealed that 7.1 and 7.2 differ only at the anomeric position (α- or β-keto group). Although the configuration of each anomer remains uncertain, we anticipate that 7.1 represents the β-anomer, since it was formed from 5, which still possessed the participating acetate group at C-2. Subsequently, we continued with azide 7.2 (presumably the α-anomer), which was reduced through a Staudinger reaction. Finally, the resulting amine was coupled to 3′-(4-methoxyphenyl)propanoic acid using N,N,N′,N′-tetramethyl-O-(6-chloro-1H-benzotriazol-1-yl)uronium hexafluorophosphate (HTCu) as the coupling agent. All synthesized compounds were structurally confirmed using 1H and 13C NMR spectroscopy and exact mass measurements (see Supplementary Methods) and were shown to possess a purity of at least 95% by combustion analysis. The previously described AI-2 QS inhibitor 2′-(2-thienylsulfonyl)ethanethioamide (KM-03009) (Li et al., 2008) was purchased from Acros Organics, while pyrogallol (Ni et al., 2008a) and 4-methoxycarbonyl-phenylboronic acid (MCPBA) (Ni et al., 2008b) were purchased from Sigma-Aldrich. If necessary, compounds were diluted in DMSO (final concentration 0.5 %, v/v). The stock solutions were stored at −20 °C. Control solutions contained the same amount of DMSO.

**Determination of MICs.** MICs were determined for each compound by using a microdilution assay, as previously described (Brackman et al., 2009). MB and LB media were used for all Vibrio species and both E. coli strains, respectively. The plates were incubated and the
Table 1. Strains used in this study

BCCM/LMG, Belgian Co-ordinated Collections/Laboratory of Microbiology collection (Ghent University, Belgium); HPACC, Health Protection Agency Culture collection.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Additional information</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. harveyi</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB120</td>
<td>Wild-type from which strains BB170, BB886, MM30, JAF553, JAF483, BNL258, JAF375 and JMH597 are derived</td>
<td>Bassler et al. (1997)</td>
</tr>
<tr>
<td>BB170</td>
<td>luxN::Tn5</td>
<td>Bassler et al. (1993)</td>
</tr>
<tr>
<td>BB886</td>
<td>luxPQ::Tn5 KanR</td>
<td>Bassler et al. (1994)</td>
</tr>
<tr>
<td>MM30</td>
<td>luxS::Tn5</td>
<td>Surette et al. (1999)</td>
</tr>
<tr>
<td>JAF553</td>
<td>luxU H58A linked to KanR</td>
<td>Freeman &amp; Bassler (1999a)</td>
</tr>
<tr>
<td>JAF483</td>
<td>luxO D47A linked to KanR</td>
<td>Freeman &amp; Bassler (1999b)</td>
</tr>
<tr>
<td>BNL258</td>
<td>hflq::Tn5luxZ</td>
<td>Lenz et al. (2004)</td>
</tr>
<tr>
<td>JAF375</td>
<td>luxN::CmR luxQ::KanR</td>
<td>Freeman &amp; Bassler (1999b)</td>
</tr>
<tr>
<td>JMH597</td>
<td>luxN::Tn5 qps::CmR</td>
<td>Defoirdt et al. (2006)</td>
</tr>
<tr>
<td><em>V. anguillarum</em> strain</td>
<td>LMG4411 Isolated from young sea trout (<em>Salmo trutta</em>)</td>
<td>BCCM/LMG</td>
</tr>
<tr>
<td><em>V. campbellii</em> strain</td>
<td>LMG21363 Isolated from <em>Penaeus monodon</em> juvenile, lymphoid organ</td>
<td>BCCM/LMG</td>
</tr>
<tr>
<td><em>V. cholerae</em> strain</td>
<td>NCTC8457 Isolated from human, biotype El Tor</td>
<td>HPACC</td>
</tr>
<tr>
<td><em>V. vulnificus</em> strain</td>
<td>LMG16867 Isolated from tank water on eel farm</td>
<td>BCCM/LMG</td>
</tr>
<tr>
<td><em>E. coli</em> strains</td>
<td>DH5+pBlueLux Strain (not producing AI-2) containing pBlueLux polylinker and luxCDABE genes</td>
<td>Brackman et al. (2008)</td>
</tr>
<tr>
<td>K12</td>
<td>AI-2-producing strain</td>
<td>Ren et al. (2004)</td>
</tr>
</tbody>
</table>

OD<sub>590</sub> was measured after 24 h using a Victor Wallac<sup>2</sup> multilabel counter (Perkin Elmer Life and Analytical Sciences).

**Identification of the molecular target of the QS inhibitors.** The assay for the effect on constitutively expressed bioluminescence (using *E. coli* DH5<sup>5</sup> containing the pBlueLux plasmid), the bioassay for LuxS inhibition (using *V. harveyi* MM30) and assays to determine the molecular target of the compounds tested (using *V. harveyi* BB120, BB170, BB886, JAF375, JAF553, JAF483, JMH597 and BNL258) were conducted as described previously (Brackman et al., 2008). Each compound was tested at least six times in triplicate (*n*≥18).

**Effect on QS-regulated virulence phenotypes in vitro.** The effect of AI-2 QS inhibitors on pigment production and protease activity in *V. anguillarum* LMG 4411 was determined as described previously (Croxatto et al., 2002). Each compound was tested at least twice in triplicate (*n*≥6). Biofilms were grown according to Brackman et al. (2008). In brief, the *Vibrio* strains were grown overnight in MB and approximately 10<sup>6</sup> c.f.u. ml<sup>-1</sup> was added to the wells of a 96-well microtitre plate in the presence or absence of QS inhibiting (QSI) compounds. Bacteria were allowed to adhere and grow without agitation for 4 h at 30 °C. After 4 h, plates were emptied and rinsed with sterile physiological saline (PS). After this rinsing step, fresh MB (with or without compounds) was added and the plate was incubated for 20 h at 30 °C.

Biofilm biomass was quantified by crystal violet (CV) staining (Peeters et al., 2008). The control signal corresponded to an A<sub>590</sub> of 0.604±0.108 and 0.639±0.129 for *V. anguillarum* LMG4411 and *V. vulnificus* LMG16867, respectively. For quantification of the number of metabolically active (i.e. living) cells in the biofilm, a resazurin assay was used (Peeters et al., 2008). Each compound was tested at least six times in triplicate (*n*≥18).

**Effect on QS-regulated stress responses in vitro.** *Vibrio* species were grown overnight in MB, and cells were collected by centrifugation and resuspended in artificial seawater (ASW) (Bang et al., 2007). A 1 ml sample of the bacterial suspension was transferred to 100 ml glass bottles containing 19 ml ASW (with and without test compound). These suspensions were incubated at 30 °C without shaking. After 48 h, 1 ml samples were taken and the number of culturable cells was determined by plating serial dilutions on tryptic soy agar (TSA) (Oxoid) plates containing 2 % (w/v) NaCl. Results were expressed as numbers of viable cells present after 48 h. Each assay was repeated at least three times. A change in MIC was considered relevant if there was a shift of more than two doubling dilutions in either direction.

**Artemia challenge tests.** All experiments were performed with high-quality hatching cysts of *Artemia franciscana* shrimps (EG type, batch 6940, INVE Aquaculture). Cysts (200 mg) were hydrated in 18 ml tap water over 1 h. The procedure of Marques et al. (2004) was used to obtain sterile decapsulated cysts and nauplii. Challenge tests (in triplicate) were performed as described previously (Brackman et al., 2008). Each assay was repeated at least three times. A change in MIC was considered relevant if there was a shift of more than two doubling dilutions in either direction.

**Cytostatic activity assay.** The murine (L1210) and human (CEM, HeLa) cells were seeded at a concentration of 5.0–7.5×10<sup>4</sup> cells per 200 μl in wells of a 96-well microtitre plate in the presence of serial (fivefold) dilutions of the test compound, using RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 0.075 % (w/v) NaHCO₃, and 10 % (w/v) fetal bovine serum. After 48 h (L1210) or 72 h (CEM, HeLa), the cell numbers were determined using a Coulter Counter (Analys).
Statistics. The normal distribution of the data was checked using the Shapiro–Wilk test. Normally and non-normally distributed data were analysed using an independent samples t test and the Mann–Whitney U test, respectively. Statistics were performed using SPSS software, version 17.0.

RESULTS

Inhibition of AI-2 controlled bioluminescence

The antimicrobial activity of all compounds was evaluated against all strains used in the present study, and MICs were found to be higher than 320 μM (160 μM for pyrogallol). Unless otherwise mentioned, the compounds were used at a concentration of 40 μM, which is well below the MIC for all strains tested. Bioluminescence in a constitutively bioluminescent strain E. coli DH5αpBluelux was not inhibited by any of the compounds tested (see Supplementary Table S1). The effect on AI-2 QS was assessed using V. harveyi BB170. LMC-21 was the most active adenosine derivative and a concentration-dependent inhibitory effect was observed (Fig. 2). Its isomer LMC-28, which only differed in the substitution site of the methoxy group, and SC-20, a truncated ribofuranosyl analogue, also inhibited AI-2 QS (Fig. 2), but proved significantly weaker than LMC-21. SC-23 yielded a significant inhibition of QS in the V. harveyi BB170 reporter strain only when tested at a concentration above 40 μM (Fig. 2). In addition, MCPBA, KM-03009 and pyrogallol were able to block the AI-2 QS system (Fig. 2). None of the other compounds resulted in a reduction in bioluminescence, even when used in higher concentrations (up to 160 μM).

Molecular target of the phenylpropionamidofuranosyl derivatives

To identify the molecular target of the 3′-deoxy-3′-(4-methoxymethylpropionamido)ribofuranosyl derivatives, bioluminescence assays were conducted using several AI-2 QS mutants. No inhibitory effects were observed using the V. harveyi JAF375 and V. harveyi BB886 mutants, while inhibition was observed using the V. harveyi JMH597 mutant, suggesting an effect on AI-2 QS. The supernatants of E. coli K-12 treated with the compounds revealed no difference in AI-2 activity compared with the control. Furthermore, LMC-21 blocked bioluminescence in V. harveyi MM30, but not in V. harveyi JAF553, JAF483 or BNL258, suggesting that the target is located upstream of the mutations in the AI-2 signal transduction pathway and most likely is the LuxPQ complex in V. harveyi. Similar
results were obtained with SC-23, LMC-28, MCPBA and KM03009, suggesting that these molecules also target LuxPQ.

**Effect on protease activity and pigment production**

LMC-21 significantly decreased pigment production by *V. anguillarum* LMG4411 after 48 h of growth, but none of the other compounds tested was able to significantly alter pigment production (Table 2). Addition of LMC-21, MCPBA or pyrogallol resulted in a significantly decreased *V. anguillarum* LMG4411 protease activity (Table 2).

**Effect on in vitro-grown biofilms**

The effect of the AI-2 QS inhibitors on the number of metabolically active cells in the biofilms of several *Vibrio* strains was evaluated using a resazurin assay. This assay revealed no significant decrease in the number of metabolically active cells in the biofilms of the different *Vibrio* strains following treatment (see Supplementary Fig. 2).

![Bioluminescence in V. harveyi BB170 in the absence (control) and presence of QS inhibitors. Bioluminescence measurements were performed 6 h after the addition of the compounds. Bioluminescence of the control (without addition of compound) was set at 100% and the responses for other samples were normalized accordingly. Error bars, SD. Bioluminescence was significantly lower than that of the untreated control for all compounds (P<0.05).](image)

**Table 2. Effect of the AI-2 QS inhibitors (40 μM) on QS-regulated phenotypes**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Protease activity,*</th>
<th>Pigment production,†</th>
<th>Biofilm formation‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>V. anguillarum LMG4411</em></td>
<td><em>V. anguillarum LMG4411</em></td>
<td><em>V. anguillarum LMG4411</em></td>
</tr>
<tr>
<td>LMC-21</td>
<td>23 ± 3 %§</td>
<td>19 ± 10 %§</td>
<td>35 ± 11 %§</td>
</tr>
<tr>
<td>KM-03009</td>
<td>5 ± 12 %</td>
<td>2 ± 13 %</td>
<td>2 ± 22 %</td>
</tr>
<tr>
<td>MCPBA</td>
<td>20 ± 2 %§</td>
<td>5 ± 16 %</td>
<td>36 ± 8 %§</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>18 ± 5 %§</td>
<td>10 ± 22 %</td>
<td>10 ± 10 %</td>
</tr>
</tbody>
</table>

*Percentage reduction in protease activity compared with an untreated control (A$_{590}$ 1.230 ± 0.132) (mean ± SD).
†Percentage reduction in pigment production compared with an untreated control (A$_{405}$ 0.480 ± 0.090) (mean ± SD).
‡Percentage reduction in biofilm biomass (CV staining) compared with an untreated control (mean ± SD).
§Significantly different compared with an untreated control (P<0.05; independent samples t test).
Table S2). In contrast, several compounds decreased the CV staining of *V. anguillarum* LMG 4411 and *V. vulnificus* LMG16867 biofilms (Table 2). However, no significant anti-biofilm effects were observed for *V. harveyi* BB120 and *V. campbellii* LMG21363. In addition, the use of LMC-21 yielded a minor but significant increase in the CV signal for *V. cholerae* El Tor NCTC8457 (15 ± 8% compared with the untreated control).

**Effect on susceptibility of Vibrio species to stress**

The effect of the different compounds on the starvation response and on the antimicrobial susceptibility of the different *Vibrio* species was investigated. Upon treatment with LMC-21, cell numbers significantly decreased in all *Vibrio* species (Table 3). Treatment with MCPBA, pyrogallol and KM-03009 reduced the number of culturable cells in some *Vibrio* species only (Table 3). There were no significant differences in the MICs for all *Vibrio* species only (Table 3). There were no significant differences in the MICs for all *Vibrio* strains tested for chloramphenicol and doxycycline when used alone or in combination with a QS inhibitor (see Supplementary Tables S3 and S4).

**Effect on virulence in vivo and cytotoxicity**

High mortality rates were observed when *Artemia* shrimps were exposed to *V. harveyi* BB120, but LMC-21 was able to completely protect *Artemia* during bacterial challenge (Fig. 3). LMC-21 alone had an effect neither on *Artemia* (Fig. 3) nor on *V. harveyi* BB120 (data not shown). In addition, LMC-21 was found to have IC₅₀ values ≥250 μM (L1210 cells) or ≥125 μM (CEM and HeLa cells).

**DISCUSSION**

QS is an important regulator of bacterial virulence in some bacterial species. Accordingly, QS inhibition is gaining interest as a potential alternative strategy for the treatment of bacterial infections. Although LuxS appears to be omnipresent in the bacterial world, the LuxPQ signal transduction system is restricted to Vibrionales (Sun et al., 2004; Rezzonico & Duffy, 2008). This makes the AI-2 receptor complex of Vibrionales an interesting target for the selective control of *Vibrio* species QS-regulated virulence.

In this study, we not only confirmed the QS inhibitory activity of several established AI-2 QS inhibitors but also discovered several new inhibitors. To identify their molecular target, we evaluated the effect of the most active compound (LMC-21) on different *V. harveyi* QS mutants. Although we originally anticipated that certain adenosine analogues might disturb the biosynthesis of DPD, due to their structural similarity to S-adenosylmethionine, our data indicate that LMC-21 exerts its effect at the level of the AI-2 transduction system rather than that of AI-2 production. For these experiments, several *V. harveyi* QS mutants with mutations in the AI-2 signal transduction system were used. *V. harveyi* JAF553 and JAF483 contain a point mutation in the luxU and luxO genes, respectively, thereby preventing the phosphorelay capacity of LuxU and LuxO. *V. harveyi* BNL258 has a Tn5 insertion in the *hfq* gene, resulting in a non-functional Hfq protein. Since *V. harveyi* strains JAF553, JAF483 and BNL258 are all constitutively luminescent, a lack of inhibition of bioluminescence in one of these strains indicates that the inhibitor acts upstream of the mutated protein. Our compound proved incapable of blocking bioluminescence in these three QS mutants. This suggests that the target of the 3-(methoxyphenylpropionamido)ribofuranosyl derivatives is the upstream component of the AI-2 signalling transduction pathway, LuxPQ. In addition, no effect was observed when testing the compound in *V. harveyi* BB886, a mutant which lacks the LuxP receptor required for the AI-2 response, and in *V. harveyi* JAF375, a mutant which lacks LuxQ. Although several compounds inhibit the AI-2 QS system, there are few reports of QS inhibitors targeting LuxPQ. Phenylboronic acids, pyrogallol derivatives and 2-(2-thienylsulfonyl)ethanethioamide, previously reported to block the AI-2 QS system at the level of LuxPQ (Li et al., 2008; Ni et al., 2008a, b), were at best as active as LMC-21.

**Table 3. Effect of the AI-2 QS inhibitors (40 μM) on the QS-regulated starvation response**

Data are presented as numbers of viable cells (×10⁸) present after 48 h.

<table>
<thead>
<tr>
<th>Compounds</th>
<th><em>V. anguillarum</em> LMG4411</th>
<th><em>V. campbellii</em> LMG21363</th>
<th><em>V. cholerae</em> NCTC8457</th>
<th><em>V. harveyi</em> BB120</th>
<th><em>V. vulnificus</em> LMG16867</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial number of cells</td>
<td>1.05 ± 0.30</td>
<td>1.00 ± 0.23</td>
<td>1.16 ± 0.11</td>
<td>1.15 ± 0.14</td>
<td>1.11 ± 0.21</td>
</tr>
<tr>
<td>Control</td>
<td>0.77 ± 0.25</td>
<td>0.91 ± 0.18</td>
<td>1.10 ± 0.07</td>
<td>1.19 ± 0.45</td>
<td>1.09 ± 0.21</td>
</tr>
<tr>
<td>LMC-21</td>
<td>0.08 ± 0.07*</td>
<td>0.47 ± 0.09*</td>
<td>0.86 ± 0.12*</td>
<td>0.53 ± 0.43*</td>
<td>0.67 ± 0.01*</td>
</tr>
<tr>
<td>KM-03009</td>
<td>0.58 ± 0.07</td>
<td>0.93 ± 0.46</td>
<td>0.94 ± 0.45</td>
<td>0.85 ± 0.16*</td>
<td>1.11 ± 0.17</td>
</tr>
<tr>
<td>MCPBA</td>
<td>0.32 ± 0.17*</td>
<td>1.02 ± 0.32</td>
<td>1.24 ± 0.66</td>
<td>1.17 ± 0.37</td>
<td>0.93 ± 0.11</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>0.21 ± 0.04*</td>
<td>0.56 ± 0.15*</td>
<td>0.91 ± 0.13</td>
<td>1.22 ± 0.67</td>
<td>1.03 ± 0.05</td>
</tr>
</tbody>
</table>

*Significantly different from the number of cells present after 48 h in the control (P<0.05; Mann–Whitney U test).
None of these compounds has been previously evaluated for its effect on AI-2-related virulence. One molecule from each group of LuxPQ inhibitors was selected for further experiments. LMC-21 was able not only to reduce pigment production in *V. anguillarum* LMG4411 but also to decrease protease activity in this strain. In contrast, none of the established QS inhibitors targeting LuxPQ was able to block pigment production or to reduce protease more than did LMC-21. In addition, LMC-21 decreased the biofilm biomass of *V. anguillarum* and *V. vulnificus*, without reducing the number of viable cells present in the biofilms. Pyrogallol only decreased biofilm biomass in *V. vulnificus*, but to a higher extent than LMC-21. These data confirm the finding that pigment and protease production in *V. anguillarum* and biofilm formation in *V. anguillarum*, *V. vulnificus* and *V. cholerae* are (at least partially) controlled by the AI-2 QS system (Croxatto *et al.*, 2002; Zhu *et al.*, 2002; Hammer & Bassler, 2003; Lee *et al.*, 2007; Brackman *et al.*, 2008). Mutations in the LuxR homologues of *V. anguillarum* (VanT) and *V. vulnificus* (SmcR) have been shown to reduce biofilm formation in these species, indicating that AI-2 QS promotes biofilm formation in these species (Croxatto *et al.*, 2002; Lee *et al.*, 2007). In contrast, *V. cholerae* HapR represses the expression of vps genes (involved in the production of exopolysaccharides) and biofilm formation (Zhu *et al.*, 2002; Hammer & Bassler, 2003), indicating that AI-2 QS negatively influences biofilm formation in this species. However, the main QS signalling molecule in *V. cholerae* is CAI-1, and this may explain the limited effect of AI-2 QS inhibitors on *V. cholerae* biofilm formation. Whether the increase in *V. cholerae* biomass, due to LMC-21, would impose problems in *in vivo* situations remains to be determined. In addition, *Vibrio* species are also known to regulate stress adaptation by means of their QS system. AI-2 is capable of regulating different stress responses, including starvation in *V. cholerae*, *V. vulnificus*, *V. anguillarum* and *Vibrio angustum* (McDougal *et al.*, 2001, 2003; Larsen *et al.*, 2004; Joelsson *et al.*, 2007; Lee *et al.*, 2007; Weber *et al.*, 2008). Our data indicate that LMC-21 suppresses the QS-regulated starvation response in all *Vibrio* species examined, while the other compounds increase susceptibility to starvation-associated stress conditions in some *Vibrio* species only, and to a lesser extent than LMC-21. However, our results indicate that AI-2 inhibition in five *Vibrio* species did not change their antimicrobial susceptibility. Of all the compounds tested, LMC-21 was the most interesting, since it was clearly at least as active in inhibiting *in vitro* virulence as the other active compounds tested in this study. Although a decrease in virulence *in vitro* is not always linked to a decrease in virulence *in vivo*, LMC-21 was shown to be a potent suppressor of *V. harveyi* BB120 virulence *in vivo*. LMC-21 alone had no effect on *Artemia* survival, and its lack of cytotoxicity, when used at 40 μM, was confirmed using murine and human cell lines. It is interesting to note that halogenated furanones, well-documented QS inhibitors, have toxic side-effects in concentrations comparable to those used in the present study (Defoirdt *et al.*, 2006; Janssens, 2008).

![Fig. 3. Effect of LMC-21 on the survival of Artemia.](image-url)
In a preliminary search for the active pharmacophore of LMC-21, we synthesized two compounds based on the phenylpropionamidofuranosyl backbone. Based on their effect on AI-2-regulated bioluminescence in V. harveyi BB120, we identified the most important structural elements required for achieving QS inhibition. Minor changes, e.g. removing the methoxy group from the para position (LMC-21) to the meta position (LMC-28), or the insertion of an extra CH₂ group between the phenylpropionamido substituent and the ribose moiety (SC-23), resulted in a decreased activity. Other molecules strongly resembling LMC-21, e.g. LMC-20 (longer side chain), LMC-23 (lacking the methoxy-substituted aromatic ring), LMC-27 (lacking the methoxy substitution on the aromatic ring) and IK-1, failed to inhibit the AI-2 QS system, and together point toward a specific (receptor-mediated) effect. We also investigated the importance of the adenine moiety present in LMC-21 by evaluating the effect of SC-1, SC-2, SC-3 and SC-20. Only SC-20 inhibited AI-2 QS, clearly showing that the ribofuranose moiety is required for activity. In addition, these results show that although an adenine group is not essential for activity, its presence results in more active compounds. However, the molecular interaction of these compounds with LuxPQ remains to be determined.

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