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Synthesis and evaluation of thiazolidinedione and dioxazaborocane analogues as inhibitors of AI-2 quorum sensing in *Vibrio harveyi*

Gilles Brackman [a,*], Abed Al Aziz Al Quntar [b,c], Claes D. Enk [d], Izet Karalic [e], Hans J. Nelis [a], Serge Van Calenbergh [e], Morris Srebnik [b], Tom Coenye [a]

[a] Laboratory of Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium
[b] Institute of Drug Research, Hebrew University, Jerusalem, Israel
[c] Department of Material Engineering, Al Quds University, Jerusalem, Israel
[d] Department of Dermatology, Hadassah-Hebrew University Medical School, Jerusalem, Israel
[e] Laboratory of Medicinal Chemistry, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium

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We dedicate this article to the memory of our friend and colleague Professor M. Srebnik who passed away in December 2011

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ABSTRACT

Two focused libraries based on two types of compounds, that is, thiazolidinediones and dioxazaborocanes were designed. Structural resemblances can be found between thiazolidinediones and well-known furanone type quorum sensing (QS) inhibitors such as *N*-acylaminofuranones, and/or acyl-homoserine lactone signaling molecules, while dioxazaborocanes structurally resemble previously reported oxazaborolidine derivatives which antagonized autoinducer 2 (AI-2) binding to its receptor. Because of this, we hypothesized that these compounds could affect AI-2 QS in *Vibrio harveyi*. Although all compounds blocked QS, the thiazolidinediones were the most active AI-2 QS inhibitors, with EC_{50} values in the low micromolar range. Their mechanism of inhibition was elucidated by measuring the effect on bioluminescence in a series of *V. harveyi* QS mutants and by DNA-binding assays with purified LuxR protein. The active compounds neither affected bioluminescence as such nor the production of AI-2. Instead, our results indicate that the thiazolidinediones blocked AI-2 QS in *V. harveyi* by decreasing the DNA-binding ability of LuxR. In addition, several dioxazaborocanes were found to block AI-2 QS by targeting LuxPQ.

1. Introduction

Quorum sensing (QS) is a mechanism by which bacteria regulate gene expression in response to population density. This form of signal-dependent communication is present in both Gram-positive and Gram-negative bacteria. At low population density only basal amounts of signal are produced, not provoking an effect. At a certain threshold, signal concentrations will be high enough to result in binding of the signal molecule to the receptor, ultimately leading to altered gene expression.[1] Many bacteria use QS to regulate the production of virulence factors. Hence, QS systems have been proposed as attractive targets for the development of anti-pathogenic compounds.[2,3] Typically, acyl-homoserine lactone (AHL) signal molecules are used by Gram-negative bacteria, while peptides are used as QS molecules in Gram-positive bacteria.[4,5] A mixture of signaling molecules, collectively referred to as autoinducer-2 (AI-2) is thought to function as a universal signal for interspecies communication.[1] LuxS, a key enzyme in the production of AI-2, catalyzes the conversion of *S*-ribosyl-L-homocysteine to 4,5-dihydroxy-2,3-pentanedione (DPD).[6,7] DPD then undergoes several spontaneous cyclization steps to form the AI-2 mixture. The structure of one of these molecules was identified as (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate.[8] In *Vibrio harveyi*, QS is regulated by three synergistically acting signaling molecules, that is, 3-hydroxy-butanoyl-homoserine lactone (3-OH-C_4-HSL), cholera-autoinducer-1 (CAI-1) and AI-2, which are sensed by the LuxN, CqsS and LuxPQ receptors, respectively.[9,10] At low population density, the receptors act as kinases, resulting in the phosphorylation of the downstream response regulator LuxO, through a cascade involving LuxU.[11] The phosphorylation of LuxU results in the production of small RNA's which, together with the chaperone protein Hfq, destabilize mRNA encoding the response regulator LuxR.[12,13] At high cell density, binding of the signals to their cognate receptor leads to a switch from kinase to phophatase activity. LuxO will be dephosphorylated, no small RNA's will be formed, mRNA of LuxR remains stable and will be transcribed.[13] Although AI-2 controls gene expression in different bacteria, QS-regulated bioluminescence in *V. harveyi* serves

* Corresponding author. Tel.: +32 (0) 9 264 80 93; fax: +32 (0) 9 264 81 95.
E-mail address: Gilles.brackman@UGent.be (G. Brackman).

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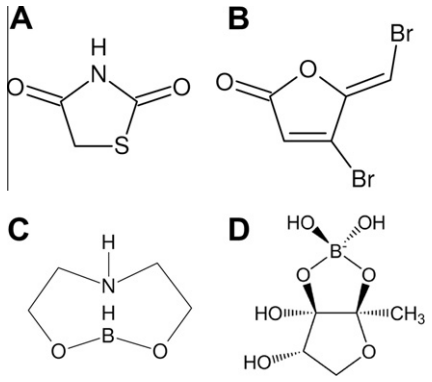

Figure 1. Basic structure of (A) a thiazolidinedione, (B) a synthetic furanone, (C) an dioxazaborocane and (D) AI-2.

as a model to study AI-2 QS inhibition.[14] Several QS inhibitors (QSI) have been described targeting AI-2 QS.[15–18] Structural resemblances can be found between thiazolidinediones (Fig. 1A) and well-known furanone type QSI (Fig. 1B) such as *N*-acylaminofuranones and/or AHL signaling molecules, while dioxazaborocanes (Fig. 1C) structurally resemble previously reported oxazaborolidine derivatives which antagonized AI-2 (Fig. 1D) binding to its receptor.[18–21] For this reason, we hypothesized that thiazolidinedione and dioxazoborocane derivatives could affect QS in *V. harveyi*.

2. Results and discussion

2.1. Library design and synthesis

In the present study two libraries of 5-substituted thiazolidinedione and 2-phenyl-1,3,6,2-dioxazaborocane analogues were investigated. The compounds in the first library (Fig. 2) were designed to investigate the influence of varying the length of the alkylidene chain, saturation of this chain and replacement of the thiazolidine ring by a pyrrolidine ring on AI-2 QS. The dioxazaborocane library (Fig. 3) was designed to evaluate the effect of different substitution patterns on the 2-phenyl-1,3,6,2-dioxazaborocane scaffold. 1,3,6,2-Dioxazaborocanes were previously reported to affect AI-2 QS and 2-phenyl substitution was shown to confer higher activity than alkyl substitution.[15]

The 5-alkylidene substituted thiazolidine-2,4-diones **1**, **2**, **3** and **4** were prepared by treatment of 2,4-thiazolidinedione with the appropriate aliphatic aldehyde in ethanol in the presence of a catalytic amount of piperidine. The desired products were precipitated from the reaction mixture upon addition of 1 M HCl and water and were obtained in analytically pure form after several washing steps. 5-Decylthiazolidine-2,4-dione (**5**) was obtained by catalytic hydrogenation of **2**. The pyrrole-2,5-dione derivative **6** was synthesized via a Wittig reaction between decanal and 3-(triphenylphosphoranylidene)-2,5-pyrrolidinedione, obtained from the addition of triphenylphosphine to maleimide.

The 6-alkyl-2-phenyl-1,3,6,2-dioxazaborocanes **7**–**15** were synthesized by esterification of the appropriate phenyl boronic acid with the suitable 2,2′-(alkylimino)bisethanol, in the presence of molecular sieves, followed by precipitation and recrystallization.

2.2. Compounds do not affect bacterial growth or bioluminescence

The antimicrobial activity of all compounds was evaluated against a number of bacterial strains. Even when used in concentrations up to 1000 μM, the different thiazolidinediones and dioxazaborocane derivatives did not affect growth (data not shown) and in all subsequent experiments, compounds were used in concentrations below the minimal inhibitory concentration. To rule out direct interference with bioluminescence, the direct effect of all compounds on bioluminescence itself was investigated using the constitutively luminescent *Escherichia coli* DH5αpBluelux strain. None of the compounds tested had an effect on bioluminescence (data not shown).

2.3. Effect on AI-2 QS

The effect of all compounds on AI-2 regulated bioluminescence of *V. harveyi* BB170 and MM32 was determined. All thiazolidinediones (**1**–**6**) and dioxazaborocanes (**7**–**15**) interfered with AI-2 QS (Table 1). Compounds **1** and **2** reduced the AI-2 QS signal by more than 99% (*V. harveyi* BB170) or 90% (*V. harveyi* MM32) at 100 μM (Table 1). A decrease in activity was observed when the alkylidene chain length of the thiazolidinedione derivatives was increased from 10 to 12 carbons (compounds **2**, **3** and **4**, respectively) or decreased from 10 to 8 carbons (compounds **2** and **1**, respectively) (Table 1). The pyrrolidinedione compound **6** was less active than its thiazolidinedione counterpart **2**. A loss in activity was also observed when the double bond of the alkylidene chain was saturated (EC_{50} of 21.9 and 37.9 μM for compound **5** in *V. harveyi* BB170 and MM32, respectively). In general, these results indicate that a thiazolidinedione with a 10 carbon-alkylidene chain without N-substitution yields the highest QS-inhibitory activity. Although the other compounds were still active, alterations in the alkylidene chain, saturation of the double bond and replacement of the thiazolidine ring by a pyrrolidine ring led to a decrease in QS-inhibitory activity.

An increase in activity was observed when the phenyl moiety of the 2-phenyl-1,3,6,2-dioxazaborocanes was substituted with a hydroxy, amino or a carboxyl group (EC_{50} of 12.2 μM, 29.2 and 46.9 μM for compounds **13**, **8** and **7** respectively in *V. harveyi* BB170). Compound **11** resulted in a decrease of 65.4 ± 8.4% in bioluminescence signal (Table 1). In addition, a decrease in activity was observed when the carboxyl function was moved from *para* to *meta* position (EC_{50} 38.8 μM and 46.9 μM for compounds **9** and **7**, respectively in *V. harveyi* BB170) and when the amino group was substituted with hydroxyalkyl moieties (EC_{50} of 35.7 and 41.3 μM for compounds **14** and **15**, respectively in *V. harveyi* BB170) (Table 1). Strong AI-2 QS inhibitory activity was observed for compound 10 (decrease of 95.8 ± 0.2% and 84.2 ± 7.3% in bioluminescence signal of *V. harveyi* BB170 and MM32, respectively, compared to the control).

2.4. Identification of the molecular target in the *V. harveyi* QS system

To identify the molecular target of the different QSI we measured the effect of selected compounds on bioluminescence production in different *V. harveyi* QS-mutants (Table 2). The signal synthase mutants *V. harveyi* BB152 (does not produce AHL but produces AI-2 and CAI-1) and MM30 and MM32 (does not produce AI-2 but responds to exogenously added AI-2 from *E. coli* K12 or to synthetic DPD) and the signal receptor mutants *V. harveyi* BB170 (does not respond to 3-OH-C_4-HSL), BB886 (does not respond to AI-2) and JMH597 (does not respond to 3-OH-C_4-HSL and CAI-1) were used. Compound **13** did not block bioluminescence in a mutant which does not produce AI-2 (*V. harveyi* MM30) or lacks the AI-2 receptor LuxPQ (*V. harveyi* BB886) (Fig. 4; *Supplementary* data Fig. S16). Although the supernatants of *E. coli* K12 treated with the compounds revealed no difference in AI-2 activity compared with the control (data not shown), differences in inhibitory activity were observed for the *V. harveyi* MM30 mutant (Fig. 4). Compounds **1** and **2** blocked bioluminescence in *V. harveyi* MM30, while no inhibitory effects were observed with compound **13** in

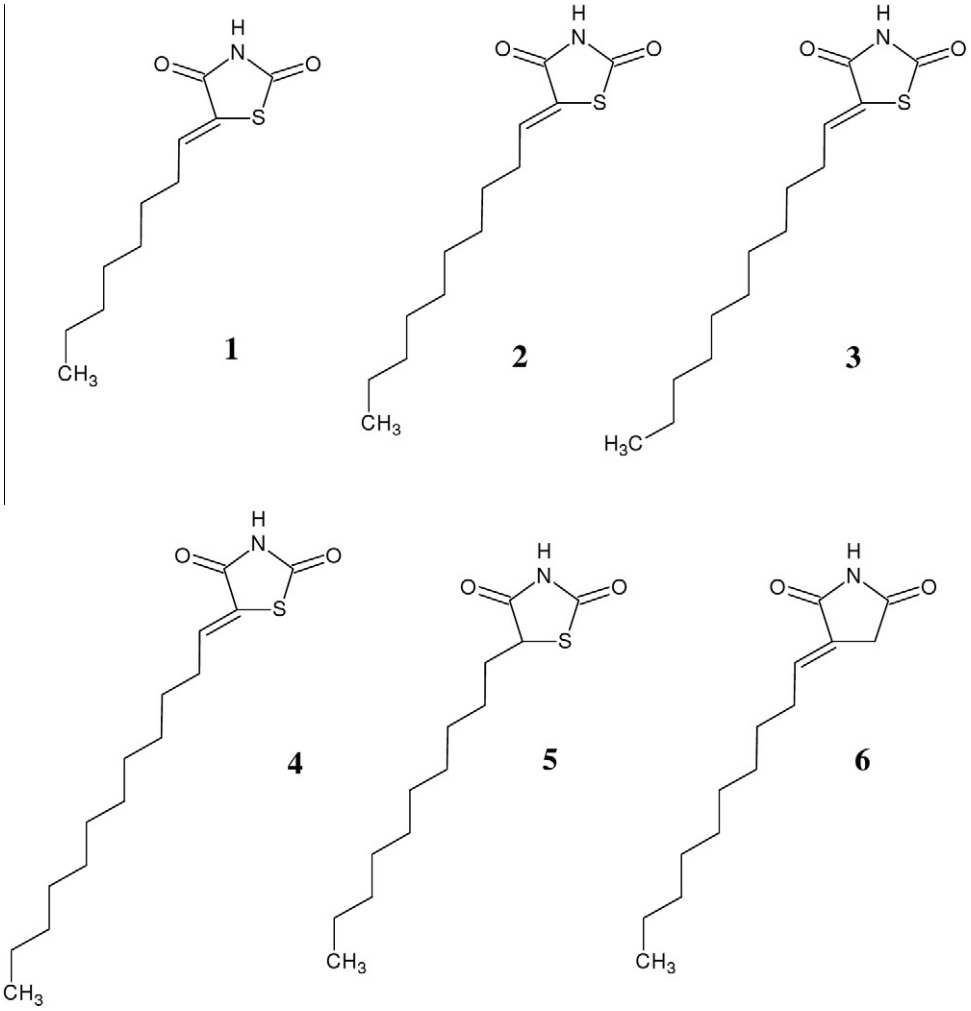

Figure 2. Thiazolidinediones and a pyrroledinedione.

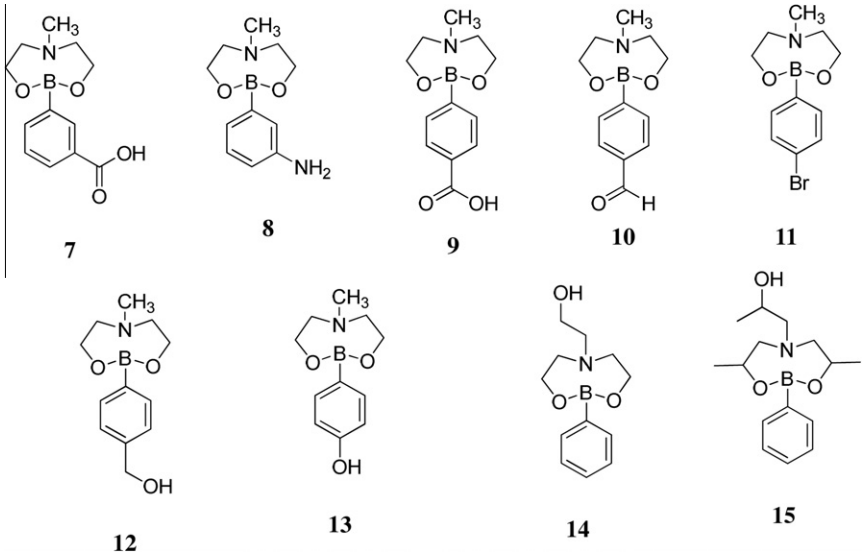

Figure 3. Dioxazaborocanes.

the absence of exogenous AI-2 (Fig. 4). However, compound **13** reduced bioluminescence when supernatants containing AI-2 or synthetic DPD was added to *V. harveyi* MM30, indicating that these compounds exert their activity at the level of the AI-2 receptor (data not shown). Although the boronated-ring in AI-2 does not contain an aromatic substituent or nitrogen, a structural relation-

Table 1
Inhibition of QS-regulated bioluminescence in *V. harveyi* BB170 or *V. harveyi* MM32 (activity is expressed as the % inhibition of the bioluminescence signal of the untreated control ± standard deviation; $n \geqslant 30$)

Compound code	*V. harveyi* BB170			*V. harveyi* MM32		
	Reduction in bioluminescence (%± SD)		EC_{50}	Reduction in bioluminescence (%± SD)		EC_{50}
	50 μM	100 μM	μM	50 μM	100 μM	μM
Thiazolidinediones						
1	96.1 ± 3.6	99.6 ± 0.3	8.2	79.1 ± 1.9	93.5 ± 0.5	16.8
2	98.3 ± 1.0	99.4 ± 0.5	2.1	92.3 ± 0.3	97.5 ± 9.8	9.8
3	91.1 ± 7.5	97.6 ± 1.7	17.0	72.6 ± 8.3	96.9 ± 0.9	23.7
4	75.0 ± 18.8	88.3 ± 10.9	29.4	62.6 ± 7.6	77.5 ± 11.2	34.6
5	65.5 ± 5.2	84.6 ± 5.3	21.9	56.7 ± 13.9	75.6 ± 11.4	37.9
6	58.9 ± 15.2	79.2 ± 10.6	24.5	52.1 ± 8.8	67.6 ± 3.4	39.8
Dioxazaborocanes						
7	52.3 ± 3.0	73.7 ± 7.3	46.9	39.5 ± 5.4	70.1 ± 16.1	54.1
8	64.6 ± 13.7	75.4 ± 12.3	29.2	51.8 ± 13.5	61.8 ± 4.1	47.1
9	51.6 ± 7.0	74.7 ± 10.7	38.8	53.4 ± 15.9	68.4 ± 9.5	49.1
10	95.8 ± 0.2	99.2 ± 0.9	10.7	84.2 ± 7.3	95.5 ± 1.3	15.9
11	65.4 ± 8.4	92.5 ± 1.7	35.4	67.5 ± 5.4	93.5 ± 0.6	29.2
12	51.8 ± 18.3	71.9 ± 14.9	39.0	41.1 ± 9.3	67.7 ± 6.3	61.2
13	98.1 ± 2.6	99.8 ± 0.1	12.2	87.3 ± 8.1	96.9 ± 0.9	12.6
14	51.7 ± 20.3	71.9 ± 17.5	35.7	51.2 ± 9.8	71.2 ± 2.7	49.3
15	50.1 ± 16.6	67.8 ± 16.6	41.3	47.3 ± 7.9	68.2 ± 10.6	57.4

Table 2
Strains used in this study

Strain	Characteristics	References
V. harveyi BB120	Wild-type	27
V. harveyi BB152	*luxM*::Tn5	12
V. harveyi MM30	*luxS*::Tn5	28
V. harveyi BB170	*luxN*::Tn5	29
V. harveyi BB886	*luxPQ*::Tn5	12
V. harveyi MM32	*luxN*::Cm^R *luxS*::Tn5	28
V. harveyi JAF375	*luxN*::Cm^R *luxQ*::Kan^R	11
V. harveyi JMH597	*luxN*::Tn5 *cqsS*::Cm^R	30
V. harveyi JMH612	*luxPQ*::Tn5 *cqsS*::Cm^R	30
V. harveyi JAF553	*luxU* H58A linked to Kan^R	11
V. harveyi JAF483	*luxO* D47A linked to Kan^R	13
V. harveyi BNL258	*hfq*::Tn5*lacZ*	31
E. coli DH5αpBluelux	Strain (not producing AI-2) containing pBluelux polylinker and *luxCDABE* genes	17
E. coli BL21 pGET-1	Strain containing the *gst-luxR overexpression construct*	26
E. coli K12	AI-2 producing strain	32

ship of compound **13** with known AI-2 QSI is evident.[15] The dioxazaborocane compounds contain a negatively charged boron in a hydrated heterocyclic ring-structure. In addition, a phenyl substituted dioxazaborocane was previously shown to induce AI-2 regulated bioluminescence in *V. harveyi*,[15] while phenyl substituted dioxazaborocanes in the present study were capable of blocking AI-2 QS. This strongly suggests that the phenyl substituent can interact with the AI-2 binding site of LuxPQ, leading to either activation or inhibition of AI-2 signal transduction. Only few specific inhibitors of the *V. harveyi* AI-2 receptor have been described. Several *para*-substituted phenylboronic acids (EC_{50} in low micromolar range), pyrogallol derivatives (EC_{50} below 10 μM) and sulfonyl compounds (EC_{50} below 40 μM) were reported to block AI-2 QS in a way similar to the compounds reported in this study.[22–24] In addition, LMC-21, an adenosine derivative with a *p*-methoxyphenylpropionamide moiety at C-3′ blocked AI-2 QS (EC_{50} of 20 μM). Although the available evidence suggests that this compound interferes with the LuxPQ receptor, the molecular interaction of LMC-21 with this receptor remains to be determined.[18]

In contrast, compounds **1** and **2** blocked QS-controlled bioluminescence in all mutants, indicating that they block all three channels of the QS system and act downstream at the level of signal transduction. To reveal their target, the effect of the compounds on bioluminescence production was evaluated in three signal transduction mutants. The *V. harveyi* JAF553 and JAF483 mutants contain a point mutation in the *luxU* and *luxO* genes, respectively, thereby abolishing their phosphorelay capacity.[11,13] *V. harveyi* BNL258 has a Tn5 insertion in the *hfq* gene, resulting in a non-functional Hfq protein.[31] *V. harveyi* strains JAF553, JAF483 and BNL258 are all constitutively luminescent, hence a decrease in bioluminescence signal would indicate that the compounds act downstream of the mutation. Compounds **1** and **2** blocked QS-controlled bioluminescence in all mutants, including *V. harveyi* JAF553, JAF483 and BNL258, which suggests that they possibly act downstream of the AI-2 QS signal transduction pathway at the level of LuxR. To further investigate their effect on LuxR, a fluorescently labeled fragment of a *V. harveyi* consensus LuxR binding sequence was incubated together with purified LuxR protein in the presence and absence of compounds **1** and **2**. Incubation of LuxR with this DNA fragment in the absence of compounds resulted in a significant increase in anisotropy (Fig. 5). When LuxR was incubated with this DNA fragment in the presence of compounds **1** or **2**, binding to DNA was strongly inhibited (Fig. 5), indicating that these compounds inhibit AI-2 mediated QS by decreasing the DNA-binding ability of LuxR. However, in order to gather additional support for this proposed mechanism and in order to identify the LuxR site(s) that interact with compounds **1** and **2**, the protein–inhibitor complex should be investigated in more detail. In the signal transduction mutants no effects on bioluminescence (Fig. 5) and on the LuxR DNA-binding ability (data not shown) were observed for

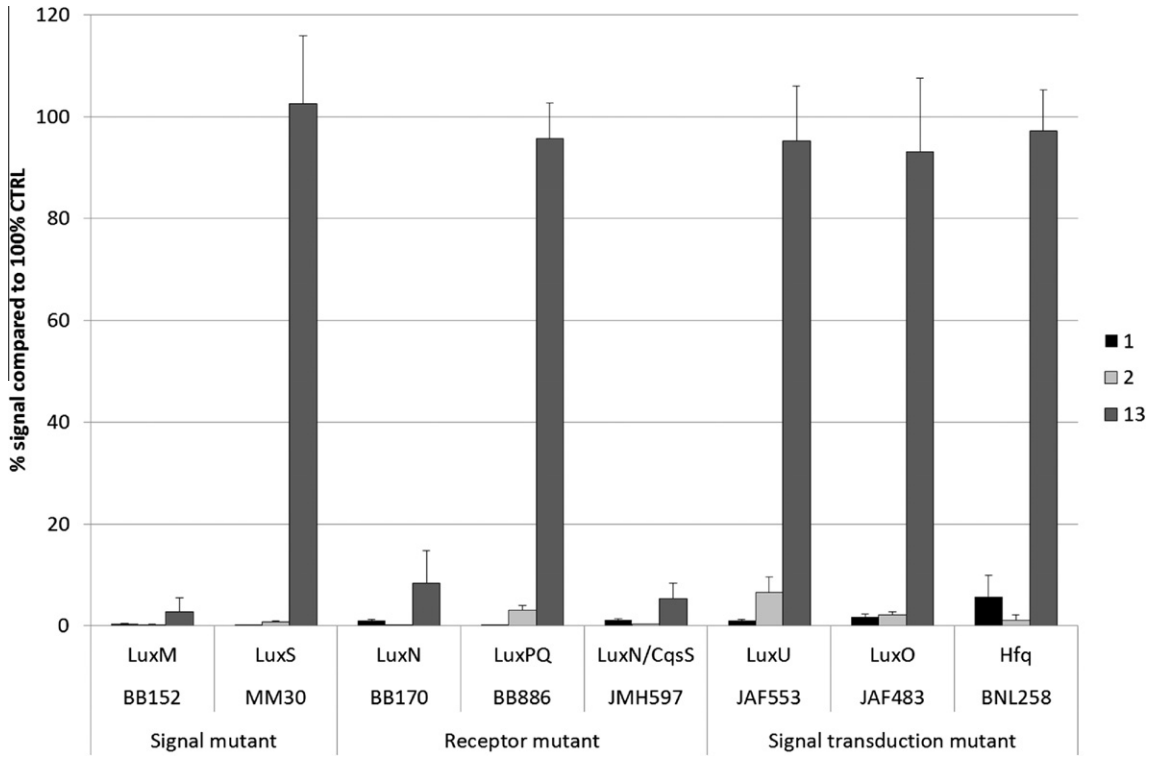

Figure 4. Effect of compounds on the bioluminescence of the different *Vibrio harveyi* QS mutants. The percentage of bioluminescence of the *Vibrio harveyi* with 50 μM of compound **1**, **2** and **13** are presented. Measurements were performed 6 h after the addition of the compounds. Bioluminescence of the control (without addition of compound) was set at 100% and the response for the other samples was normalized accordingly. The error bars represent the standard deviation.

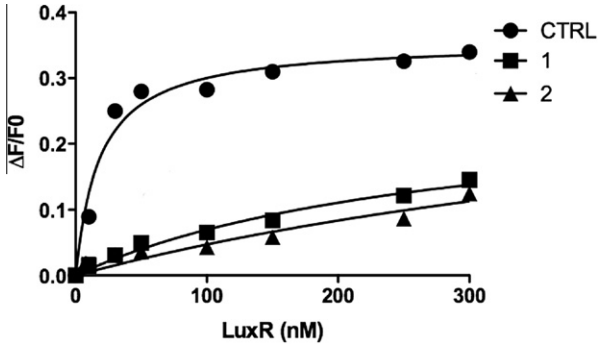

Figure 5. DNA binding of LuxR in the absence and presence of inhibitors (10 μM). The fractional change in anisotropy, $\Delta F/F_o$, is plotted against the concentration of LuxR (nM).

compound **13**. Cinnamaldehyde and cinnamaldehyde derivatives were previously reported to act in a similar manner by decreasing the DNA-binding ability of LuxR.[17,25] However, compounds **1** and **2** are much more active inhibitors of AI-2 QS than cinnamaldehyde, with concentrations of 8.2 and 2.1 μM exhibiting similar QS-inhibitory activity as 100 μM of cinnamaldehyde.[17,25]

3. Conclusion

Thiazolidinedione and dioxazaborocane compounds were synthesized and their effect on AI-2 QS was evaluated. Although all compounds blocked QS, the thiazolidinediones were the most active AI-2 QS inhibitors, with EC_{50} values in the low micromolar range, making them among the most active AI-2 QS inhibitors reported to date. Our results indicate that the thiazolidinedione compounds blocked AI-2 QS in *V. harveyi* by decreasing the DNA-binding ability of LuxR. In addition, several dioxazaborocanes were found to block AI-2 QS by targeting LuxPQ.

4. Materials and methods

4.1. Synthesis of the thiazolidinediones

4.1.1. Synthesis of thiazolidinediones—general procedure

2,4-Thiazolidinedione (1 equiv) is added to a solution of a long chain aldehyde in ethanol in the presence of a catalytic amount of piperidine at room temperature. The reaction is kept at 80–90 °C for 24 h and then cooled to 0 °C in an ice bath. Thereafter 1 M HCl and water are added and the precipitate is filtered over a glass sintered filter, washed with water and petroleum ether and dried in vacuum to obtain an analytically pure thiazolidinedione derivative.

4.1.2. (*Z*)-5-octylidenethiazolidine-2,4-dione (1)

(*Z*)-5-octylidenethiazolidine-2,4-dione (**1**) was prepared following the general procedure, using 2,4-thiazolidinedione and octanal as reactants (55%). ^{1}H NMR (500 MHz): δ 0.90 (t, 3H, J_{HH} 6.0 Hz), 1.29–1.34 (m, 8H), 1.53–1.57 (m, 2H), 2.21–2.25 (q, 2H, J_{HH} = 7.5 Hz), 7.06 (t, 1H, J_{HH} = 8 Hz) ppm; ^{13}C NMR (75.9 MHz, $CDCl_3$): δ 14.33, 22.87, 28.04, 29.43, 29.46, 29.50, 29.64, 32.08, 126.48, 140.03, 166.03, 167.78 ppm. *m*/*z*: 227.0980 (100%), 228.1014 (11.9%), 229.0938 (4.5%). elemental analysis: C,58.12; H, 7.54; N 6.16; O, 14.08; S, 14.11. ($M+H+NH_4$), calcd: 226.0902; found: 226.0907 (Supplementary data file 1).

4.1.3. (*Z*)-5-decylidenethiazolidine-2,4-dione (2)

(*Z*)-5-decylidenethiazolidine-2,4-dione (**2**) was prepared following the general procedure, using 2,4-thiazolidinedione and decenal as reactants (57%). ^{1}H NMR f δ 0.85 (t, J_{HH} = 6.6 Hz, 3H), 1.25 (s, 12H), 1.56 (m, 2H), 2.20 (q, J_{HH} = 7.2 HZ, 2H), 7.05 (t, J_{HH} = 7.5 HZ,

1H) ppm; ^{13}C NMR (75.9 MHz, $CDCl_3$): δ 14.33, 22.87, 28.04, 29.43, 29.46, 29.50, 29.64, 32.08, 126.48, 140.03, 166.03, 167.78 ppm. *m*/*z*: 255.1293 (100%), 256.1327 (14.1%), 257.1251 (4.5%). Elemental analysis: C, 61.14; H, 8.29; N 5.48; O, 12.53; S, 12.56. ($M+H+NH_4$), calcd: 254.1220; found: 254.1224 (Supplementary data file 1).

4.1.4. (*Z*)-5-undecylidenethiazolidine-2,4-dione (3)

(*Z*)-5-undecylidenethiazolidine-2,4-dione (**3**) was prepared following the general procedure, using 2,4-thiazolidinedione and undecanal as reactants (60%). ^{1}H NMR (300 MHz): δ 0.88 (t, 3H, J_{HH} 6.9 Hz), 1.26 (s, 14H), 1.45–1.55 (m, 2H), 2.19–2.25 (q, 2H, J_{HH} = 7.5 Hz), 7.04 (t, 1H, J_{HH} = 7.8 Hz) ppm; ^{13}C NMR (75.9 MHz, $CDCl_3$): δ 14.44, 22.92, 23.11, 28.12, 29.46, 29.55, 29.56, 29.59, 32.11, 126.54, 139.95, 165.97, 167.82 ppm. *m*/*z*: 269.1449 (100%), 270.1483 (15.1%), 271.1407 (4.5%), 271.1517 (1.1%). Elemental analysis: C, 62.42; H, 8.61; N 5.20; O, 11.88; S, 11.90. ($M+H+NH_4$), calcd: 268.1377; found: 268.1381 (Supplementary data file 1).

4.1.5. (*Z*)-5-dodecylidenethiazolidine-2,4-dione (4)

(*Z*)-5-dodecylidenethiazolidine-2,4-dione (**4**) was prepared following the general procedure, using 2,4-thiazolidinedione and dodecanal as reactants (52%). ^{1}H NMR (300 MHz): δ 0.88 (t, 3H, J_{HH} 6.6 Hz), 1.26 (s, 16H), 1.49–1.56 (m, 2H), 2.17–2.25 (q, 2H, J_{HH} = 7.0 Hz), 7.04 (t, 1H, J_{HH} = 7.8 Hz) ppm; ^{13}C NMR (75.9 MHz, $CDCl_3$): δ 14.36, 22.84, 22,92, 23.15, 28.00, 29.48, 29.47, 29.60, 29.57, 32.00, 126.40, 140.11, 166.13, 167.68 ppm. *m*/*z*: 283.1606 (100%), 284.1640 (16.2%), 285.1564 (4.5%), 285.1673 (1.2%). Elemental analysis: C, 63.56; H, 8.89; N, 4.94; O, 11.29; S, 11.31. ($M+H+NH_4$), calcd: 282.1533; found: 282.1543 (Supplementary data file 1).

4.1.6. 5-Decylthiazolidine-2,4-dione (5)

5-Decylthiazolidine-2,4-dione (**5**) was prepared following general procedure, using 2,4-tiazoldinedione and decenal as reactants. Then, the product was hydrogenated in methanol under a suspension of Pd/C at 29 psi (48%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.85 (t, J_{HH} = 6.6 Hz, 3H), 1.25 (overlap, 14H), 1.56 (m, 2H), 2.20 (q, J_{HH} = 7.2 Hz, 2H), 3.72 (m, 1H); ^{13}C NMR (75.9 MHz, $CDCl_3$): δ 14.31, 22.88, 24.80, 28.01, 29.48, 29.43, 29.54, 29.69, 32.11, 55.00, 166.00, 167.82 ppm. *m*/*z*: 257.1449 (100%), 258.1483 (14.1%), 259.1407 (4.5%). Elemental analysis: C, 60.66; H, 9.01; N, 5.44; O, 12.43; S, 12.46. ($M+H+NH_4$), calcd: 256.1377; found: 256.1364 (Supplementary data file 1).

4.2. Synthesis of (*E*)-3-decylidenepyrrolidine-2,5-dione (6)

Compound **6** was prepared by mixing maleimide (0.1994 g, 2 mmol) and triphenyl phosphine (0.5232 g, 1.994 mmol), followed by reflux for 1 h in acetone (10 ml). A precipitate was obtained which was washed with several portions of acetone and then dried under reduced pressure to yield 100%. This product (2 mmol) and decanal (0.376 ml, 2 mmol) are mixed in methanol (10 ml) and refluxed for 12 h. Thereafter the reaction mixture was cooled and HCl (1 M, 0.03 ml and water 10 ml) was added. The precipitate (45%) was filtered and washed with petroleum ether and purified by flash chromatography (silica, ethyl acetate: petroleum ether, 30:70) to yield pure **6**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, 3H, J_{HH} = 6 Hz), 1.29 (s, 10H), 1.46–1.51 (o, 2H), 2.13–2.25 (o, 2H), 3.25 (s, 2H), 6.80 (t, 1H, J_{HH} = 7.8 Hz), 8.00 (s, 1H) ppm; ^{13}C NMR (75.0 MHz, $CDCl_3$): δ 13.60, 22.21, 23.33, 25.58, 27.69, 28.63, 28.82, 29.48, 31.30, 32.51, 126.43, 138.89, 170.71, 175.32 ppm. *m*/*z*: 237.1729 (100%), 238.1762 (15.1%), 239.1796 (1.1%). Elemental analysis: C, 70.85; H, 9.77; N, 5.90; O, 13.48. ($M+H+NH_4$), calcd: 236.1656; found: 236.1646 (Supplementary data file 1).

4.3. Synthesis of the dioxazaborocanes

4.3.1. Synthesis of dioxazaborocanes—general procedure

To a solution of phenyl boronic acid (7.14 mmol) in dry toluene (30 ml) stuffed with 5 g of molecular sieves, was added the appropriate 2,2′-azanediyldiethanol (7.14 mmol) at room temperature. After stirring for 5 h at room temperature, the solvent was evaporated and the solid was recrystallized from ether/petroleum ether.

4.3.2. 3-(6-Methyl-1,3,6,2-dioxazaborocan-2-yl)benzoic acid (7)

Identical to the procedure used in the general procedure for the synthesis of oxazoborolines using 3-boromobenzoic acid and *N*-methyl diethanol amine (58%). ^{1}H NMR (300 MHz, DMSO-d) δ: 2.23(s, 3 H), 3.05 (m, 2H), 3.22 (m, 2H), 4.19 (m, 4H), 7.20–8.10 (overlap, 4H); ^{13}C NMR (75.9 MHz, DMSO-*d*) δ: 47.0, 60.5, 62.2, 128.5, 131.2, 133.5, 135.3, 140.1, 168.8; ^{11}B NMR (125 MHz, DMSO-*d*) δ: 12.0.*m*/*z*: 249.1172 (100%), 248.1209 (24.8%), 250.1206 (13.0%), 249.1242 (3.2%). Elemental analysis: C, 57.87; H, 6.47; B, 4.34; N, 5.62; O, 25.69. ($M+H+NH_4$), calcd: 267.1511; found: 267.1414 (Supplementary data file 1).

4.3.3. 3-(6-Methyl-1,3,6,2-dioxazaborocan-2-yl)aniline (8)

Identical to the procedure used in the general procedure for the synthesis of oxazoborolines using 3-aminophenylboronic acid and *N*-methyl diethanol amine (52%). ^{1}H NMR (300 MHz, DMSO-*d*) δ: 2.25 (s, 3 H), 3.01 (m, 2H), 3.15 (m, 2H), 4.15 (m, 4H), 6.82–7.25 (overlap, 4H); ^{13}C NMR (75.9 MHz, DMSO-*d*) δ: 47.8, 60.0, 62.0, 115.4, 123.2, 129.2, 131.9, 146.4; ^{11}B NMR (125 MHz, DMSO-*d*) δ: 11.4.*m*/*z*: 220.1383 (100%), 219.1419 (24.8%), 221.1417 (11.9%), 220.1453 (3.0%). Elemental analysis: C, 60.03; H, 7.79; B, 4.91; N, 12.73; O, 14.54. ($M+H+NH_4$), calcd: 238.1721; found: 238.1747 (Supplementary data file 1).

4.3.4. 4-(6-Methyl-1,3,6,2-dioxazaborocan-2-yl)benzoic acid (9)

Identical to the procedure used in the general procedure for the synthesis of oxazoborolines using 4-boronobenzoic acid and *N*-methyl diethanol amine (45%). ^{1}H NMR (300 MHz, DMSO-*d*) δ: 2.22(s, 3 H), 3.01 (m, 2H), 3.15 (m, 2H), 4.18 (m, 4H), 6.92 (dt, 2H), 7.63 (dt, 2H), 10.5; ^{13}C NMR (75.9 MHz, DMSO-*d*) δ: 47.2, 60.2, 62.0, 112.9, 113.8, 134.2, 134.5, 168.2; ^{11}B NMR (125 MHz, DMSO-*d*) δ: 10.7. *m*/*z*: 249.1172 (100%), 248.1209 (24.8%), 250.1206 (13.0%), 249.1242 (3.2%). Elemental analysis: C, 57.87; H, 6.47; B, 4.34; N, 5.62; O, 25.69. ($M+H+NH_4$), calcd: 267.1516; found: 267.1551 (Supplementary data file 1).

4.3.5. 4-(6-Methyl-1,3,6,2-dioxazaborocan-2-yl)benzaldehyde (10)

Identical to the procedure used in the general procedure for the synthesis of oxazoborolines using 4-formylphenylboronic acid and N-methyl diethanol amine (43%). ^{1}H NMR (300 MHz, DMSO-*d*) δ: 2.23(s, 3 H), 3.00 (m, 2H), 3.15 (m, 2H), 4.20 (m, 4H), 7.02 (dt, 2H), 7.80 (dt, 2H), 8.50 (s, 1H); ^{13}C NMR (75.9 MHz, DMSO-*d*) δ: 47.8, 59.9, 61.6, 114.2, 114.9, 135.4, 135.2, 189.3; ^{11}B NMR (125 MHz, DMSO-*d*) δ: 11.4. *m*/*z*: 233.1223 (100%), 232.1260 (24.8%), 234.1257 (13.0%), 233.1293 (3.2%). Elemental analysis: C, 61.84; H, 6.92; B, 4.64; N, 6.01; O, 20.59. ($M+H+NH_4$), calcd: 251.1567; found: 251.1452 (Supplementary data file 1).

4.3.6. 2-(4-Bromophenyl)-6-methyl-1,3,6,2-dioxazaborocane (11)

Identical to the procedure used in the general procedure for the synthesis of oxazoborolines using 4-bromophenylboronic acid and *N*-methyl diethanol amine (44%). ^{1}H NMR (300 MHz, DMSO-*d*) δ: 2.25(s, 3 H), 3.00 (m, 2H), 3.20 (m, 2H), 4.18 (m, 4H), 6.90 (dt, 2H), 7.60 (dt, 2H). ^{13}C NMR (75.9 MHz, DMSO-*d*) δ: 47.4, 60.2, 62.0, 113.8, 114.0, 134.6, 134.7; ^{11}B NMR (125 MHz, DMSO-*d*) δ:

10.7. *m*/*z*: 283.0379 (100%), 285.0359 (97.3%), 282.0416 (24.8%), 284.0395 (24.2%), 284.0413 (11.9%), 286.0392 (11.6%), 283.0449 (3.0%), 285.0429 (2.9%). Elemental analysis: C, 46.53; H, 5.32; B, 3.81; Br, 28.14; N, 4.93; O, 11.27. ($M+H+NH_4$), calcd: 301.0717; found: 301.0732 (Supplementary data file 1).

4.3.7. 4-(6-Methyl-1,3,6,2-dioxazaborocan-2-yl)phenyl)methanol (12)

Identical to the procedure used in the general procedure for the synthesis of oxazoborolines using 4-(hydroxymethyl)phenylboronic acid and *N*-methyl diethanol amine (45%). ^{1}H NMR (300 MHz, DMSO-*d*) δ: 2.26 (s, 3H), 3.09 (m, 2H), 3.27 (m, 2H), 4.21 (m, 4H), 4.50 (s, 2H), 6.91 (dt, 2H), 7.62 (dt, 2H). ^{13}C NMR (75.9 MHz, DMSO-*d*) δ: 47.2, 60.2, 62.5, 65.0, 113.2, 114.3, 134.7, 134.3; ^{11}B NMR (125 MHz, DMSO-*d*) δ: 11.0. *m*/*z*: 235.1380 (100%), 234.1416 (24.8%), 236.1413 (13.0%), 235.1450 (3.2%). Elemental analysis: C, 61.31; H, 7.72; B, 4.60; N, 5.96; O, 20.42. ($M+H+NH_4$), calcd: 253.1723; found: 253.1713 (Supplementary data file 1).

4.3.8. 4-(6-Methyl-1,3,6,2-dioxazaborocan-2-yl)phenol (13)

Identical to the procedure used in the general procedure for the synthesis of oxazoborolines using 4-hydroxyphenylboronic acid and *N*-methyl diethanol amine (55%). ^{1}H NMR (300 MHz, DMSO-*d*) δ: 2.26 (s, 3H), 3.05 (m, 2H), 3.28 (m, 2H), 4.19 (m, 4H), 6.91 (dt, 2H), 7.62 (dt, 2H); ^{13}C NMR (75.9 MHz, DMSO-*d*) δ: 47.0, 60.3, 62.5, 113.7, 114.4, 134.5, 134.5; ^{11}B NMR (125 MHz, DMSO-*d*) δ: 11.2. *m*/*z*: 221.1223 (100%), 220.1260 (24.8%), 222.1257 (11.9%), 221.1293 (3.0%). Elemental analysis: C, 59.77; H, 7.30; B, 4.89; N, 6.34; O, 21.71. ($M+H+NH_4$), calcd: 239.1567; found: 239.1454 (Supplementary data file 1).

4.3.9. 2-(2-Phenyl-1,3,6,2-dioxazaborocan-6-yl)ethanol (14)

Identical to the procedure used in the general procedure for the synthesis of oxazoborolines using phenylboronic acid and triethanol amine (60%). ^{1}H NMR (300 MHz, DMSO-*d*) δ: 2.23 (broad, 2H), 2.80–3.15 (overlap, 2H), 3.50 (broad, 2H), 3.90 (broad, 4H), 7.15–7.60 (overlap, 5H); ^{13}C NMR (75.9 MHz, DMSO-*d*) δ: 57.2, 57.7, 60.7, 60.3, 127.3, 127.7, 133.3; ^{11}B NMR (125 MHz, DMSO-*d*) δ: 13.2. *m*/*z*: 235.1380 (100%), 234.1416 (24.8%), 236.1413 (13.0%), 235.1450 (3.2%). Elemental analysis: C, 61.31; H, 7.72; B, 4.60; N, 5.96; O, 20.42. ($M+H+NH_4$), calcd: 253.1216; found: 253.1493 (Supplementary data file 1).

4.3.10. 1-(4,8-Dimethyl-2-phenyl-1,3,6,2-dioxazaborocan-6-yl)propan-2-ol (15)

Identical to the procedure used in the general procedure for the synthesis of oxazoborolines using phenylboronic acid and triisopropyl amine (60%). ^{1}H NMR (300 MHz, DMSO-*d*) δ: 1.05 (d, 3H), 1.09 (d, 3H), 2.86 (m, 2H), 3.10 (m, 2H), 3.60 (m, 1H), 4.15 (overlap, 2H), 6.95–7.70 (overlap, 5H); ^{13}C NMR (75.9 MHz, DMSO-*d*) δ: 20.5, 20.90, 62.2, 62.4, 64.3, 64.5, 65.1, 66.00, 113.6, 113.8, 134.2, 134.5; ^{11}B NMR (125 MHz, DMSO-*d*) δ: 10.7. *m*/*z*: 277.1849 (100%), 276.1886 (24.8%), 278.1863 (16.2%), 277.1919 (4.0%), 279.1916 (1.2%). Elemental analysis: C, 65.00; H, 8.73; B, 3.90; N, 5.05; O, 17.32. ($M+H+NH_4$), calcd: 295.2193; found: 295.2079 (Supplementary data file 1).

4.4. Strains and culture conditions

All bacterial strains used in this study are listed in Table 2. *V. harveyi* strains were cultured in Marine-Broth (MB) (BD) in the presence of antibiotics at 30 °C with shaking. *E. coli* BL21 pGET-1 (containing the *gst-luxR* overexpression construct) and *E. coli* DH5α pBlueLux (containing pBluelux polylinker and *luxCDABE* genes) were grown in Luria-Bertani broth with aeration at 37 °C in the presence of ampicillin (100 μg/ml). *E. coli* K12 was routinely cultured in TSB at 37 °C.

4.5. Determination of the MIC

MICs of the compounds were determined in triplicate according to the EUCAST broth microdilution protocol, using flat-bottom 96-well microtiter plates (TPP, Trasadingen, Switzerland). The inoculum was standardized to approximately 5×10^5 CFU/ml. The plates were incubated at 37 °C for 20 h, and the optical density at 590 nm was determined by using a multilabel microtiter plate reader (Envision; Perkin–Elmer LAS, Waltham, MA). The lowest concentration of compound for which the optical density was not different from that in the uninoculated control wells was recorded as the MIC.

4.6. Determination of the QS inhibitory effect

The bioluminescence assay with *E. coli* DH5αpBlueLux and the AI-2 QS inhibition assay using *V. harveyi* BB170 and *E. coli* K12 supernatans were conducted as described previously.[18] The AI-2 QS inhibition assay using *V. harveyi* MM32 was conducted in a similar way using synthetic DPD (5 μM; OMM Scientific, US). Each compound was tested at least six times in triplicate ($n \geqslant 18$).

4.7. Identification of the molecular target of the QS inhibitors

The bioassay for LuxS inhibition (using *V. harveyi* MM30 and MM32) and assays to determine the molecular target of the compounds tested (using *V. harveyi* BB120, BB152, BB170, BB886, JAF375, JMH597, JMH612, JAF553, JAF483 and BNL258) were conducted as described previously.[17,18] Each compound was tested at least ten times in triplicate ($n \geqslant 30$).

4.8. LuxR-DNA binding assay

Induction of GST-LuxR overexpression and protein purification were conducted as previously described.[26] Fluorescence polarization measurements in the presence and absence of QS inhibitors (10 μM) were conducted as described previously.[26]

4.9. Statistics

The normal distribution of the data was checked using the Shapiro–Wilk test. Normally distributed data were analyzed using an independent sample *T*-test. Statistical analyses were carried out using SPSS software, version 19.0 (SPSS, Chicago, IL, USA).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.055. These data include MOL files and InChiKeys of the most important compounds described in this article.

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