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DOI: 10.1002/cmdc.200800076

Structure-Based Discovery and Experimental Verification of Novel AI-2 Quorum Sensing Inhibitors against *Vibrio harveyi*

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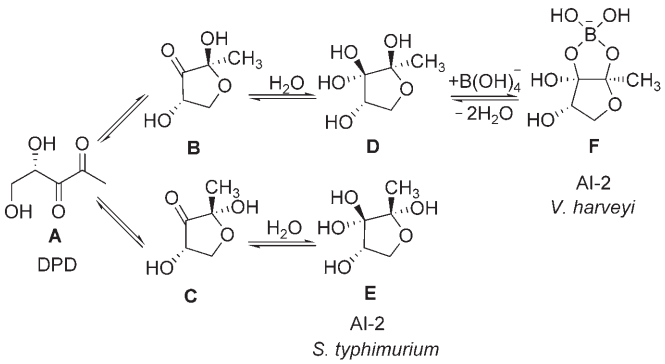
Quorum sensing has been implicated in the control of pathologically relevant bacterial behavior such as secretion of virulence factors, biofilm formation, sporulation, and swarming motility. The AI-2 quorum sensing pathway is found in both Gram-positive and Gram-negative bacteria. Therefore, antagonizing AI-2 quorum sensing is a possible approach to modifying bacterial behaviour. However, efforts in developing inhibitors of AI-2-mediated quorum sensing are especially lacking. High-throughput virtual screening using the V. harveyi *LuxP crystal structure identified two compounds that were found to antagonize AI-2-mediated quorum sensing in* V. harveyi *without cytotoxicity. The sulfone functionality of these inhibitors was identified as critical to their ability to mimic the natural ligand in their interactions with Arg 215 and Arg 310 of the active site.*

Introduction

Bacteria can coordinate gene expression and modulate their behavior in response to cell–cell communications in a process called quorum sensing.[1] This intercellular communication is accomplished through the production, release, and exchange of small signaling molecules called autoinducers (AI).[2] Currently, several major types of small-molecule autoinducers including acyl homoserine lactones (AHLs), autoinducer-2 (AI-2), autoinducer-3 (AI-3), 4-quinolone signal (4Qs) and autoinducing peptides (AIPs) have been identified in various bacteria.[3] Quorum sensing provides a mechanism for the collective regulation of pathologically relevant processes such as biofilm formation, bacterial virulence,[4] and antibiotic production.[5] Therefore, inhibition of quorum sensing is an obvious approach to the development of novel antimicrobial agents.[6–9]

Among all the autoinducers identified, AI-2 alone is functional in both Gram-positive and Gram-negative bacteria. A key compound in the AI-2-mediated quorum sensing pathway is 4,5-dihydroxy-2,3-pentanedione (DPD),[10] which can exist in several forms that are collectively termed as "AI-2" (Scheme 1).



Scheme 1. AI-2 formation from DPD.

Biosynthesis of DPD requires the enzyme LuxS, which is present in over 60 species of bacteria including both Gram-positive and Gram-negative organisms. The list includes Gram-positive bacteria: *B. subtilis*, *B. anthracis*, *B. halodurans*, *B. burgdorferi*, *C. botulinum*, *C. perfringens*, *C. difficile*, *E. faecalis*, *L. monocytogenes*, *M. tuberculosis*, *S. aureus*, *S. pyogenes*, and *S. pneumoniae*;[11] Gram-negative bacteria include: *H. influenzae*, *N. meningitidis*, *V. cholera*, *V. harveyi*, *E. coli*, *S. typhimurium*, *Y. pestis*, *C. jejuni*, and *H. pylori*.[12,13] These species form part of a growing list of bacteria that regulate pathologically relevant cellular processes such as virulence factor production and biofilm formation, in an AI-2-dependent manner.[14] The commonality of LuxS expression and DPD production in different bacteria has led to the proposal that AI-2 plays a role in interspecies communication.[15]

In *V. harveyi*, AI-2-mediated quorum sensing also controls light production through the binding of AI-2 to a receptor protein, LuxP.[16] Interestingly, the biologically active form of AI-2 in *V. harveyi* is a boric acid complex (*S*-THMF-borate, **F**, Scheme 1) in which the boron atom is in an anionic tetrahedral form (Figure 1). In 2002, Bassler and co-workers solved the crystal structure of the *V. harveyi* LuxP–AI-2 complex at 1.5 Å resolution.[17] In this crystal structure, the positively charged side chains of Arg 215 and Arg 310 form multiple hydrogen

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200800076.

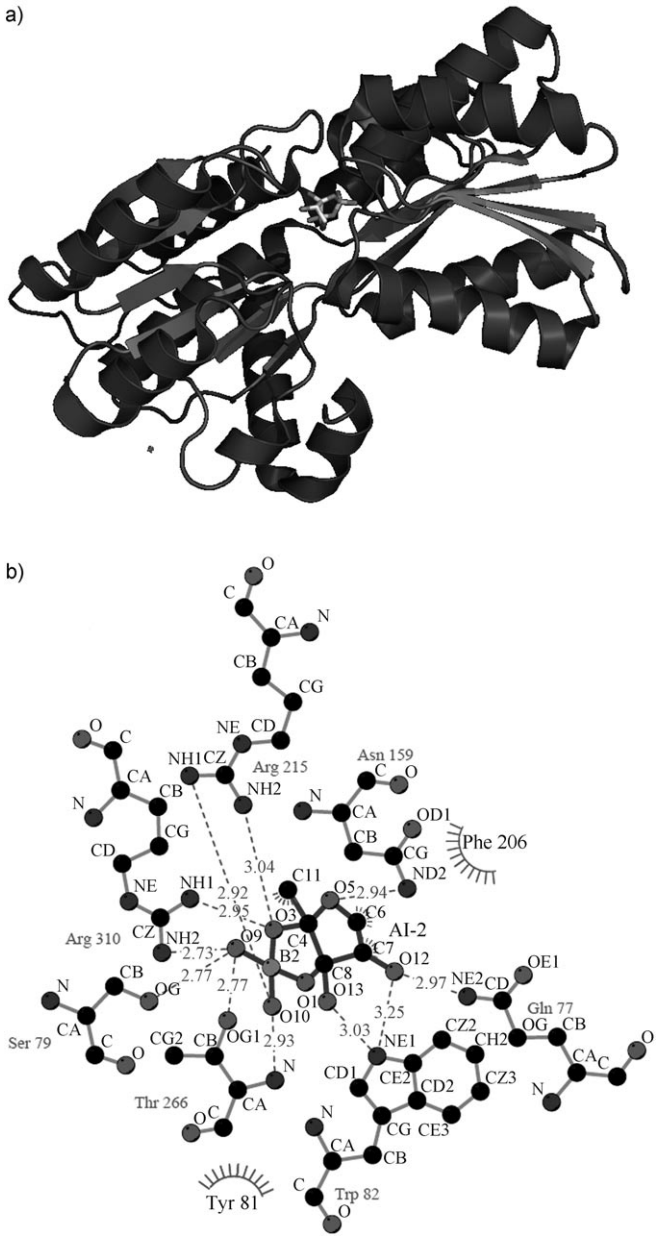


Figure 1. a) Ribbon diagram of LuxP (PDB entry: 1JX6) complexed with ligand AI-2 (*S*-THMF-borate, white sticks); b) a schematic illustration of the interactions of *S*-THMF-borate with LuxP.

bonds with three of the four borate oxygen atoms, and appear to stabilize the negative charge on the borate (Figure 1). The two hydroxy groups of *S*-THMF-borate form three hydrogen bonds with Trp 82 and Gln 77, while the oxygen atom of the furanoyl ring of AI-2 is involved in hydrogen bonding with Asn 159. Previously reported AI-2 analogues developed as potential probes for studying the AI-2 quorum sensing pathways have largely been AI-2 agonists.[18–22] There are several reports of AI-2 antagonists or inhibitors with IC_{50} values in the high micromolar range.[23–28] For example, two AI-2 antagonists ((5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone and cinnamaldehyde) have IC_{50} values of >100 μM.[27,28] Given that these two compounds are Michael acceptors, and the recent report that cinnamaldehyde and its analogues can covalently modify cysteines in protein (cinnamaldehyde EC_{50}: 19 μM),[29] it is unclear whether their antimicrobial activity arises through non-specific protein alkylation or through inhibition of the AI-2-mediated quorum sensing pathway.

We are interested in finding small molecules that can bind to LuxP with high affinity and specificity and can therefore function as AI-2 antagonists. Our previous efforts involved the identification of boronic acids and diol–boric acid complexes as AI-2 antagonists.[30,31] We are also looking for novel structures that allow ready optimization for improved affinity. Because AI-2 analogues seem to possess agonistic effects and other reported antagonists only have weak activities,[27,28] our effort turned to virtual screening using the LuxP–AI-2 *holo*-form crystal structure reported by the Bassler research group (PDB entry: 1JX6).[17] In a structure-based virtual screening effort, a large number of small molecules can be docked into the binding site of a bio-macromolecular target, and scoring functions are used to evaluate their potential complementarity to the binding site.[32–34] Indeed, there are now a number of successful applications of structure-based virtual screening in hit identification and lead optimization.[35,36] In the current study, approximately 1.7 million compounds (Table 1) were screened in silico against LuxP, and 42 hit compounds were obtained through high-throughput molecular docking and consensus scoring. Finally, 27 commercially available compounds were then purchased and tested for their ability to inhibit AI-2-mediated quorum sensing. After biological evaluation, two compounds, **1** (KM-03009) and **2** (SPB-02229) were found to antagonize AI-2-mediated quorum sensing without cytotoxicity in *V. harveyi*.

Table 1. The commercial databases used in virtual screening.

Database	Company Name	Number of Compound
Maybridge	MayBridge	74855
Peakdale	Peakdale Molecular Limited	8188
Asinex Platinum	Asinex Ltd.	130012
Asinex Gold	Asinex Ltd.	232586
Specs	Specs Ltd.	458767
Chemstar	ChemStar Ltd.	60213
Chembridge	ChemBridge Corp.	342958
Aurora	Aurora Fine Chemicals Ltd.	31699
Enamine	Enamine	306496
Focus	Focus Synthesis	849
SynphaBase	SynphaBase AG	193
G&J	G & J Research Chemicals Ltd	1620
KeyOrganics	Key Organics	43410
Toslab	Technology for Organic Synthesis (TOS)	24751
	Total	1716597

Results and Discussion

Bassler's crystal structure (PDB entry: 1JX6)[17] was used in the virtual screening of 14 commercial compound databases, which were combined to give approximately 1.7 million compounds (Table 1). The top 1000 compounds generated were then evaluated by consensus scoring using a combination of ChemScore, PLP, ScreenScore, ChemGauss, and ShapeGauss; this process enhances the ability to discriminate between inactive and active molecules and decrease the number of false positives identified by a single scoring function.[37] Notably, combining multiple scoring functions improves the chance of identifying true positives only if a) each of the individual scoring functions has relatively high performance and b) the individual scoring functions are distinctive.[38] After consensus scoring, the top 10% of these compounds were selected for further assessment, including evaluation of the hydrogen bonding and hydrophobic interactions and comparison of their docking conformations, to generate 42 hit compounds. Due to availability, only 27 compounds were purchased from commercial vendors and evaluated as potential AI-2 mediated quorum sensing inhibitors. Figure 2 shows the docking conformations of these 27 hits overlaid with AI-2 in the binding site of *V. harveyi* LuxP, and Figure 3 depicts their chemical structures. Their consensus scoring results are listed in Table 2.

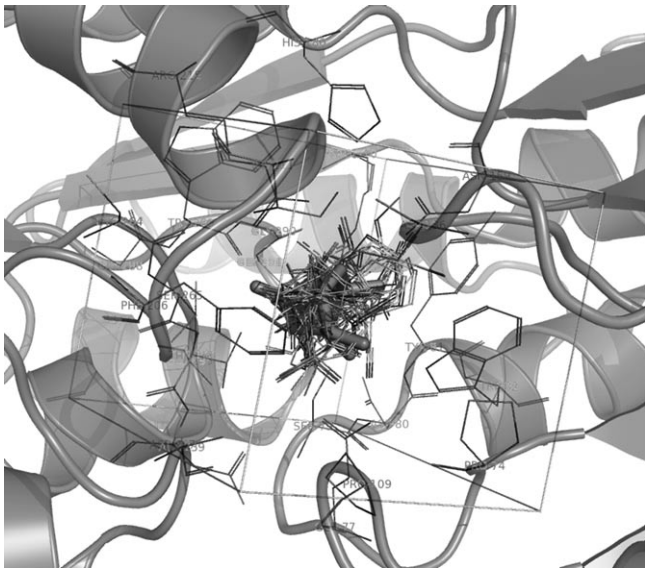


Figure 2. Docking conformations of 27 hits (thin lines) and AI-2 (thick sticks) within the binding site (boxed) of *V. harveyi* LuxP (lines and ribbons).

For the antiquorum sensing assay, the MM32 strain of *V. harveyi* was selected, which lacks the LuxN receptor needed to respond to autoinducer AI-1 and the LuxS enzyme needed to synthesize DPD.[39] Inhibition was measured by observing the amount of luminescence induced by AI-2-mediated quorum sensing in *V. harveyi*.[40] Of the 27 compounds tested, five showed inhibitory activities against AI-2 bioluminescence with IC_{50} values at micromolar concentrations: compound **1** ($IC_{50}=35\pm3$ μM), compound **2** ($IC_{50}=55\pm7$ μM), compound **3** ($IC_{50}=110\pm31$ μM), compound **4** ($IC_{50}=88\pm7$ μM), and compound **5** ($IC_{50}=60\pm40$ μM). Figure 4 shows inhibitory curves reflecting the concentration-dependent luminescence intensity changes observed. In virtual screening, consensus scoring is an estimation of the possibility of meaningful binding and cannot be taken directly or quantitatively.[41] Therefore, one would not expect to see a direct correlation of activity with consensus scores.

Compounds **1**–**5** were also evaluated for their bacterial growth inhibition, as nonspecific toxicity may also lead to a reduction in observed bioluminescence. Compounds **1** and **2** showed no significant growth inhibition that might explain the observed decreases in bioluminescence (Figure 5), identifying these two compounds as antagonists of AI-2-mediated

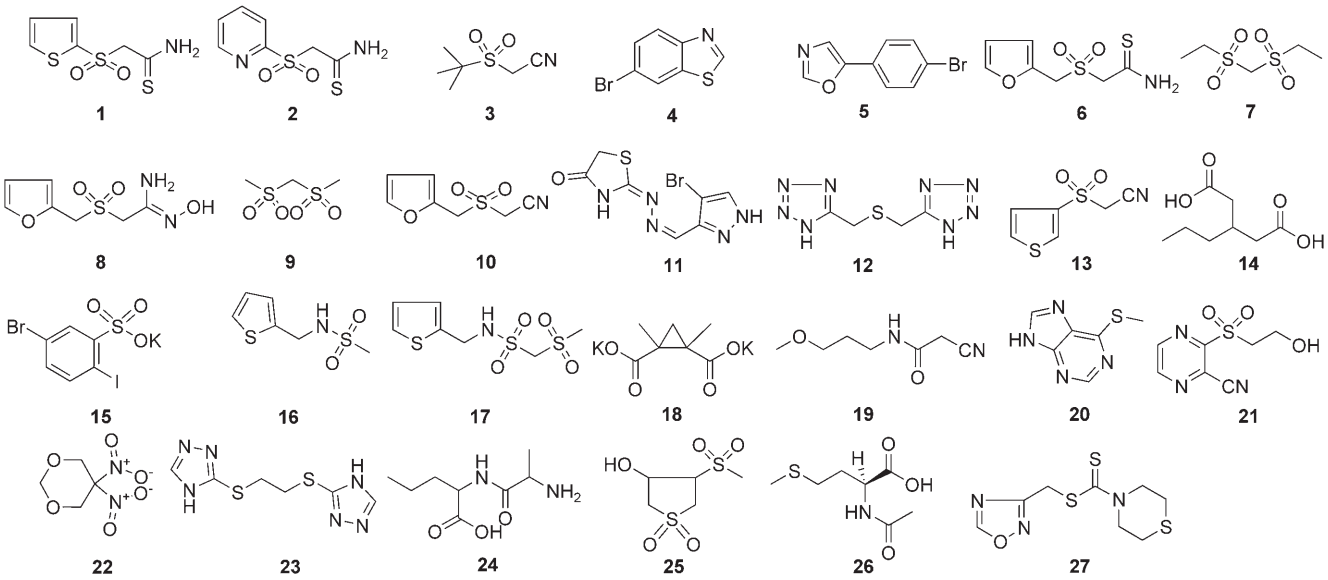


Figure 3. Chemical structures of all compounds tested for AI-2-mediated quorum sensing inhibition.

Table 2. Consensus scores of all 27 hit compounds.

Compd	ChemScore		ChemGauss		PLP		ScreenScore		ShapeGauss		Consensus Score[a]
	Score	Rank	Score	Rank	Score	Rank	Score	Rank	Score	Rank	
1	−62.22	29	−20.69	33	−10.89	36	−64.93	27	−247.8	140	265
2	−55.48	57	−20.63	36	−14.75	30	−61.62	33	−232.07	236	392
3	−73.6	2	−15.16	134	−24.89	11	−83.65	9	−303.93	4	160
4	−63.89	22	−20.93	30	−31.21	6	−90.05	3	−288.53	15	76
5	−68.06	8	−19.45	51	−35.57	4	−88.91	5	−276.62	37	105
6	−67.83	9	−25.79	1	−24.65	12	−73.02	18	−266.27	69	109
7	−64.67	18	−24.93	5	−14.64	31	−64.71	29	−268.52	62	145
8	−64.05	20	−17.77	77	−41.5	2	−92.92	2	−268.27	65	166
9	−61.32	32	−16.28	106	−26.68	7	−83.88	8	−275.59	39	192
10	−66.47	14	−23.69	7	−8.34	42	−52.84	39	−259.04	93	195
11	−55.62	55	−21.58	24	−20.61	20	−72.67	19	−261.14	84	202
12	−58.12	46	−16.72	98	−25.39	10	−75.64	15	−266.53	68	237
13	−62.69	27	−14.54	155	−20.82	18	−78.15	13	−281.72	26	239
14	−66.78	12	−14.06	165	−18.78	23	−73.21	17	−275.48	40	257
15	−60.39	37	−12.61	204	−26.45	8	−82.09	11	−295.2	11	271
16	−66.01	16	−14.93	144	−11.19	35	−66.75	25	−264.71	73	293
17	−67.03	11	−14.45	159	−31.31	5	−89.73	4	−254.85	118	297
18	−48.61	67	−19.95	46	−23.85	14	−75.55	16	−242.85	161	304
19	−55.22	58	−14.45	160	−17.62	25	−71.39	20	−270.28	59	322
20	−61.88	30	−13.19	188	−24.39	13	−80.78	12	−260.6	88	331
21	−61.86	31	-16.5	103	−41.86	1	−98.56	1	−237.12	199	335
22	−68.79	7	−12.57	207	−23.38	15	−84.13	7	−255.66	113	349
23	−57.97	47	−18.06	72	−10.01	39	−56.12	35	−242.28	166	359
24	−60.18	39	−14.48	157	−17.56	26	−62.6	32	−255.79	111	365
25	−60.68	35	−10.23	254	−16.44	28	−67.39	24	−274.21	45	386
26	−66.44	15	−12.27	216	−17.76	24	−65.49	26	−256.37	107	388
27	−54.15	60	−12.52	210	−9.55	41	−63.52	30	−271.1	57	398

[a] Consensus Score = rank sum.

fects, however, at high concentration their cytotoxicity becomes the dominant factor and therefore an overall decrease in luminescence production is observed (Figure 4).

The inhibitory activities of compounds **1** and **2** were also measured against wild-type BB120 *V. harveyi*. Both compounds **1** and **2** inhibited AI-2-derived bioluminescence in a dose-dependent manner, with IC_{50} values at micromolar concentrations (29 ± 10 and 48 ± 9 μM, respectively) similar to those observed against the MM32 strain (Figure 6).

In order to understand the structural features needed for tight binding, we re-docked the structures of **1** and **2** into the binding site of *V. harveyi* LuxP, optimized the complexes using molecular mechanics and molecular dynamics simulation, and examined the possible protein–ligand interactions following similar procedures used in previous studies.[42,43] After refinement, compound **1** (KM-03009) appears to interact with residues Ser 79, Arg 215, Thr 266, and Arg 310 through multiple hydrogen bonds and with residues Tyr 81, Trp 82, Asn 159, Ile 211, Phe 206, and Ser 265 through hydrophobic interactions (Figure 7).

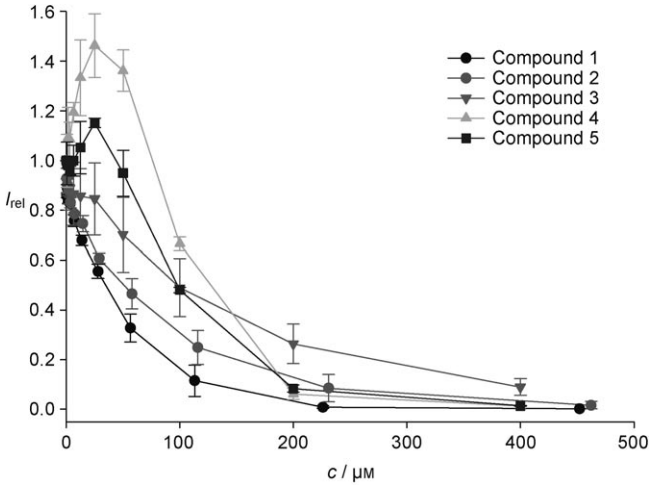


Figure 4. The inhibitory activities of compounds **1**–**5** against *V. harveyi* MM32 quantified by the change in intensity of bioluminescence ($I_{rel} = I/I_0$).

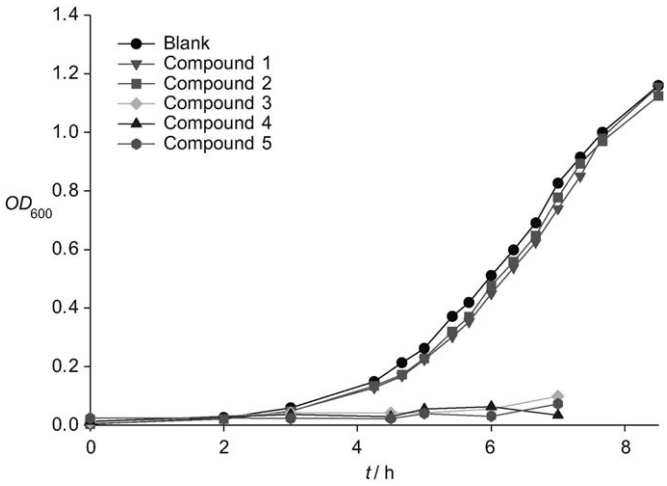


Figure 5. Growth curves for blank (doubling time: 78 min), compound **1** at 68 μM (doubling time: 70 min), **2** at 116 μM (doubling time: 72 min), **3** at 300 μM, **4** at 190 μM, and **5** at 200 μM.

quorum sensing. However, compounds **3**–**5** significantly inhibited bacterial growth in comparison with the control group, which might explain the abnormal results of compounds **4** and **5** in the bioluminescence assay. These two compounds showed an initial agonistic effect at low concentrations (below 40 μM) and an inhibitory effect at high concentrations. It is possible that compounds **4** and **5** have intrinsic agonistic ef-

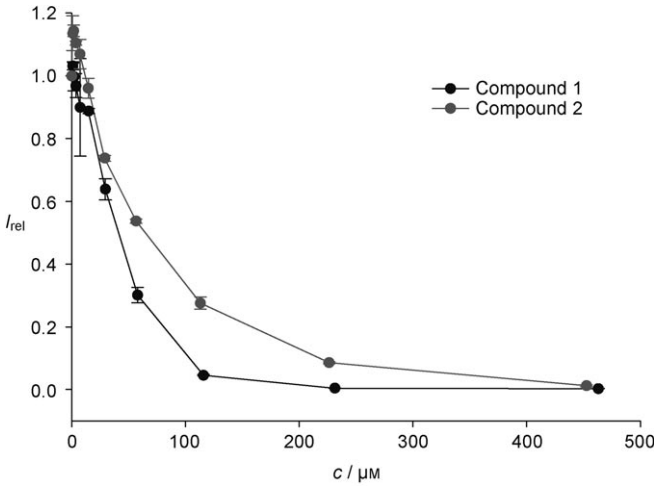
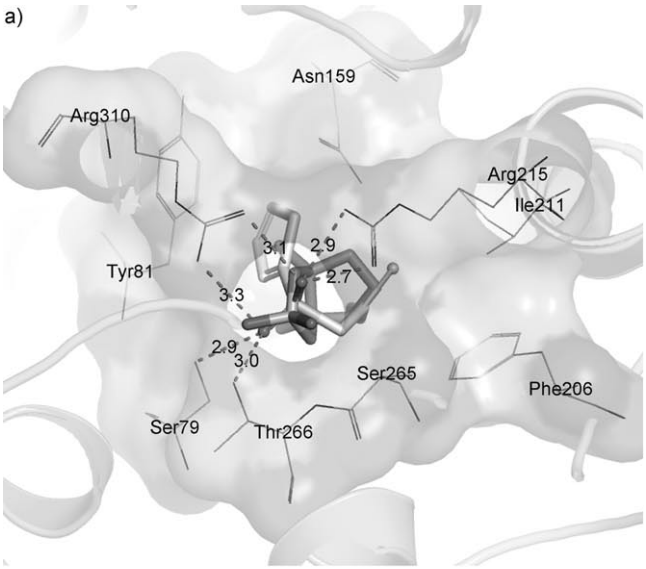


Figure 6. The inhibitory activities of compounds **1**–**2** against wild-type *V. harveyi* BB120 quantified by the change in intensity of bioluminescence ($I_{rel} = I/I_0$).



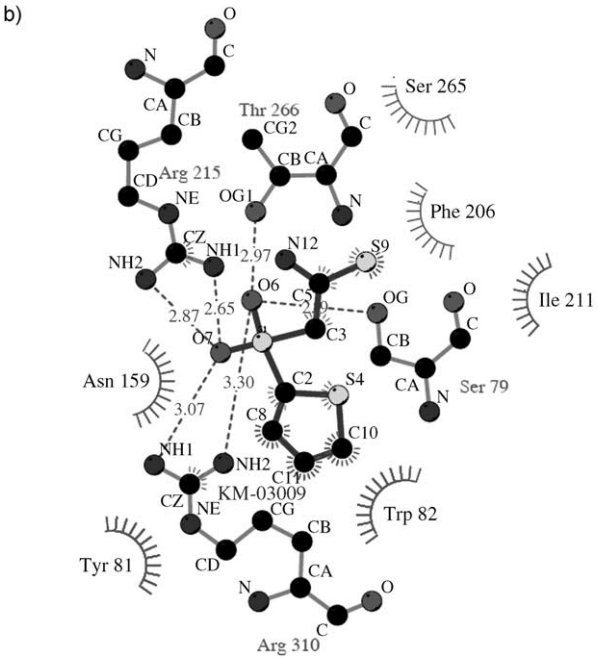


Figure 7. a) Proposed docking conformation of compound **1** (light sticks) and AI-2 (dark sticks) in the *V. harveyi* LuxP binding site and b) proposed interactions of compound **1** with LuxP.

Compound **2** (SPB-02229) has a similar binding mode, forming several hydrogen bonds with residues Asn 159, Arg 215, Thr 266, and Arg 310 and hydrophobic interactions with Tyr 81, Trp 82, Ile 211, Phe 206, and Ser 265 (Figure 8). Notably, both compounds **1** and **2** contain structural moieties, such as a sulfone group, involved in critical interactions with Arg 215 and Arg 310. The sulfone moiety, like the complexed borate group of AI-2, has two oxygen atoms with partial negative charges able to interact favorably with the arginine residues in the binding site, mimicking the natural substrate. More than half of the hits identified by virtual screening contain at least one sulfone group. Comparing the structures of **1** and **2** with the inactive sulfone-containing compounds in the hits pool, it seems that the following features are important for binding: 1) the sulfone group is directly attached to an aryl group and 2) there is a thioamide group separated from the sulfone group by one carbon. However, it is not clear whether the thioamide can be replaced by an ester or an amide and whether the aryl group can be substituted by a phenyl group or other aromatic heterocycles. The initial structure–activity relationship analysis also seems to indicate that a nitrile group (in **12**) or hydroxy group (in **21**) does not work as well as a thioamide. Similar interactions with arginine residues in the active site were also observed for compounds **3**–**5** when docked to the LuxP active site (Supporting Information: figures S1–3).

Compounds **1** and **2** are not considered especially potent when compared with two other AI-2 antagonists recently discovered in our research group (pyrogallol: $IC_{50} = 2$ μM and 2-fluoro-4-methylphenylboronic acid: $IC_{50} = 4$ μM).[30,31] Upon analysis of the structural features of these three classes of compounds, it seems that the boronic acid and the complexed boric acid are good mimics of the DPD–boric acid complex of AI-2 leading to efficient LuxP binding. Although a sulfone group can engage in similar interactions with arginine residues in the binding site, the interactions may not be as strong. This structural insight will be very important for the future design of potent AI-2 antagonists and for further structural optimizations.

Conclusions

Two inhibitors of AI-2-mediated quorum sensing, **1** (KM-03009) and **2** (SPB-02229), were successfully discovered by structure-based virtual screening. These two compounds can antagonize AI-2-mediated quorum sensing with IC_{50} values of 35 and 55 μM, respectively, and do not show cytotoxicity in *V. harveyi*. The structures identified are unique and will be useful as probes for mechanistic studies. More importantly, the structural

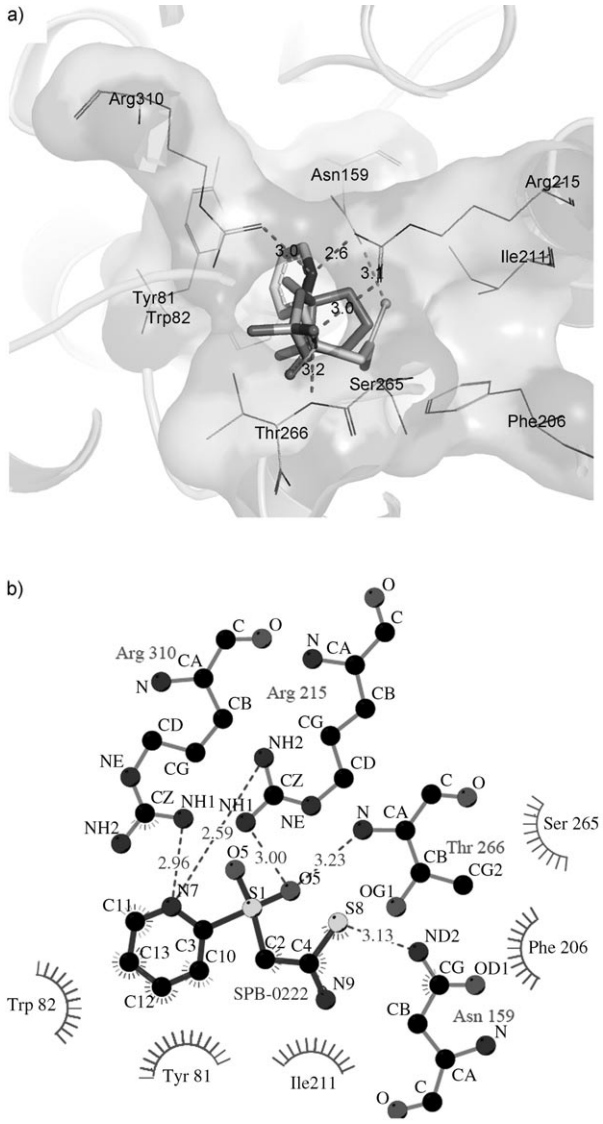


Figure 8. a) Proposed docking conformation of compound **2** (light sticks) and AI-2 (dark sticks) in the *V. harveyi* LuxP binding site and b) proposed interactions of compound **2** with LuxP.

information obtained from the binding of **1** and **2**, and through comparison with earlier boronic acid/boric acid complex-based inhibitors will be very important for further structural optimization.

Experimental Section

Virtual screening and molecular simulation

Virtual screening (DOCK 5.4) and molecular simulation (AMBER 8) were performed using a 40-node Linux-based Biocluster. Visualization (PyMOL 0.99, HBPLUS 3.06, and Ligplot 4.22) was performed on a dual-Xeon Linux graphical workstation. Consensus scoring (FRED 2.2.3) and re-analysis of virtual screening (FILTER 2.0.1 and IDEA 8.8) were performed on a Windows workstation.

Virtual screening

High-throughput virtual screening was conducted as described previously.[44] The combined database, containing approximately 1.7 million compounds from 14 commercial databases (Table 1), was first converted into 3D structures using the CONCORD program[45] and subsequently filtered using drug-like property criteria[46] by the FILTER 2.0.1 software.[47] Before docking, hydrogen atoms were added to the protein structure and all atoms were assigned Kollman all-charges by the SYBYL 7.1 program.[48] Hydrogens were also added to the 3D ligand structures, and Gastiger–Hückel partial charges were assigned. To construct a grid for virtual screening, the active site was defined as all residues within a radius of 6 Å around the center of AI-2: Pro 74, Gln 77, Ser 79, Asp 80, Tyr 81, Trp 82, Pro 109, Asn 159, His 180, Phe 206, Ile 211, Arg 215, Cys 264, Ser 265, Thr 266, Asp 267, Trp 289, Gly 290, Gly 291, Glu 295, and Arg 310. The position and conformation of each compound was first optimized by the anchor fragment orientation method and then by the torsion minimization method implemented in the DOCK 5.4 program.[49,50] Fifty conformations and a maximum of 100 anchor orientations for each compound were generated, and all of the docked conformations were energy minimized by 100 iterations using the same approach as described previously.[50] The docked molecules were ranked based on the sum of the van der Waals and electrostatic energies to obtain the top 1000 compounds.

Compounds selection

The top 1000 hits were evaluated by consensus scoring using a combination of ChemScore,[51,52] PLP,[53] ScreenScore,[54] ChemGauss, and ShapeGauss[55] implemented in the FRED 2.2.3 software.[56] After selecting the top 10% of compounds evaluated by consensus scoring, their hydrogen bond and hydrophobic interaction profiles were also checked by the IDEA 8.8 software[57] to ensure they form at least one hydrogen bond and hydrophobic interaction with the LuxP protein. In the next step, a manual binding orientation and conformation analysis, by comparison with AI-2, was performed to generate 42 hits for biological evaluation. Finally, 27 compounds were selected based on their commercial availability.

Molecular simulation of docked complexes

Molecular simulations were performed as previously described.[42,43] In brief, the docked complexes were solvated by using the TIP3P water model,[58] subjected to 500 steps of molecular mechanics minimization and molecular dynamics simulations at 300 K for 1.5 ns using the SANDER module in AMBER 8 program.[59] The resulting structures were then analyzed using PyMOL 0.99,[60] HBPLUS 3.06,[61] and Ligplot 4.22[62] to identify specific contacts between the ligands and LuxP.

Biology

Quorum sensing assay

The quorum sensing assays were conducted following previously described protocols.[63] Specifically, MM32 (ATCC# BAA-1121) bacteria were streak-seeded on fresh LM plates and then cultured in the presence of kanamycin (50 $\mu g\,mL^{-1}$) and chloramphenicol (10 $\mu g\,mL^{-1}$). Colonies appeared after overnight incubation at 30 °C. A single colony was selected from the LM plate, then this strain was grown for 16 h with aeration (175 rpm) at 30 °C in 2 mL

Autoinducer Bioassay (AB) medium[64] with antibiotics (kanamycin: 50 μg mL^{-1} and chloramphenicol: 10 μg mL^{-1}), and then diluted to OD_{600} 0.7. The pre-inoculum was grown in AB-Fe medium (1.2 mM Fe) with shaking at 175 rpm and at 30 °C for 1–1.5 h to an OD_{600} of 1.0–1.1.[47] The resulting inoculum culture was then diluted 5000-fold in fresh AB medium. Solutions of the test compounds in AB medium were prepared in 96-well plates. To these solutions, freshly synthesized DPD solution (pH 7) was added for a final concentration of 5 μM (DPD was synthesized following a previously reported procedure[65]). Boric acid was added to give a final concentration of 1 mM. The optimal DPD and boric concentrations were determined based on published precedents as well as our own experimental confirmation. After addition of bacteria in AB medium, the microplates were covered with a nontoxic plate sealer and incubated at 30 °C with aeration for 4–6 h. Light production was measured every 30 min using a Perkin Elmer luminescence microplate reader. All experiments were carried out in triplicate. IC_{50} values were defined as the concentration that caused a 50% decrease in bioluminescence. Wild-type BB120 (ATCC# BAA-1116) was also tested in a similar way; however in this instance, DPD and antibiotics were not added and cell-free culture was used as the source of the autoinducers.

Cell growth test

Bacteria were grown for 16 h with aeration (175 rpm) at 30 °C in 2 mL AB medium with antibiotics (kanamycin: 50 μg mL^{-1} and chloramphenicol: 10 μg mL^{-1}). Then this bacterial culture was diluted 100-fold with 20 mL AB medium in a 250-mL flask and incubated at 30 °C (175 rpm). The OD_{600} value was determined every 20 min. The doubling time was calculated based on the OD_{600} value.

Acknowledgements

Financial support from the Molecular Basis of Disease program at GSU, Georgia Research Alliance, and Georgia Cancer Coalition is gratefully acknowledged. We also thank OpenEye Scientific Software for the use of the FRED, FILTER, and QuACPAC programs, and Breadth Technology (http://www.breadth.com.tw) for use of the IDEA package.

Keywords: autoinducer-2 · inhibitors · molecular docking · quorum sensing · *Vibrio harveyi* · virtual screening

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Received: March 6, 2008
Revised: April 25, 2008
Published online on June 9, 2008

