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Inhibition of *Pseudomonas aeruginosa* quorum sensing by AI-2 analogs

Hadas Ganin, Xu Tang [†], Michael M. Meijler [*]

Department of Chemistry, Ben-Gurion University of the Negev, Be'er-Sheva 84105, Israel

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ABSTRACT

Autoinducer-2 (AI-2) has been suggested to serve as a universal interspecies quorum sensing signaling molecule. We have synthesized a set of AI-2 analogs with small incremental changes in alkyl substitution on C-2 and evaluated them for their agonistic and antagonistic potential as quorum sensing (QS) attenuators in two different bacterial species: *Pseudomonas aeruginosa* and *Vibrio harveyi*. Unexpectedly, several of the analogs were found to function as synergistic QS agonists in *V. harveyi*, while two of these analogs inhibit QS in *P. aeruginosa*.

Bacterial life has been seen, until recently, as one marked by silence and asocial behavior. Extensive research efforts during the past two decades have overturned this paradigm and bacteria are increasingly seen as highly communicative organisms. Intercellular communication among bacteria has been uncovered as an important tool to coordinate activity in such manner that was once believed to be restricted to multicellular organisms.[1] The mechanism used by bacteria to coordinate gene expression upon reaching a specific population density is termed quorum sensing (QS).[2,3] As the prevalence of bacterial resistance to antibiotics increases it is becoming increasingly urgent to find new strategies to combat pathogenicity. One of the strategies that has been proposed to attenuate virulence, and thus to decrease harm caused by bacterial infections, is by blocking bacterial QS.[4] This might present an advantage over classical bactericidal strategies, due to a decreased likelihood that resistance against the non-toxic QS inhibitors will develop (Fig. 1).

QS involves the production, detection and response to extracellular signaling molecules known as autoinducers (AIs). As a bacterial population grows, the extracellular concentration of autoinducer increases. When a threshold autoinducer concentration is reached, the bacteria detect the autoinducer through binding to a membrane associated receptor or transcriptional activator and initiate a signal transduction cascade that culminates in a change in gene expression, affecting the behavior of the population.[5]

QS systems have been identified in both Gram-negative and Gram-positive bacteria, and they are used to regulate diverse functions, such as bioluminescence, conjugation, virulence factor expression, biofilm formation, and antibiotic production.[6] Two Gram-negative species in which QS has been studied extensively are *Pseudomonas aeruginosa* and *Vibrio harveyi*.

P. aeruginosa is a major opportunistic human pathogen and immunocompromised individuals (e.g., people with cystic fibrosis (CF) and burn victims) are especially predisposed to contract infections with this organism. It uses several well-studied QS systems (e.g. *las* and *rhl*), in addition to other regulators, and upon reaching a quorum simultaneous expression of several virulence factors is triggered. The primary QS molecule used by *P. aeruginosa* is *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL), that is recognized by the transcriptional activator LasR.[7]

V. harveyi has served as a model for interspecies QS, as one of its QS molecules, autoinducer-2 (AI-2, Fig. 1), has been suggested to

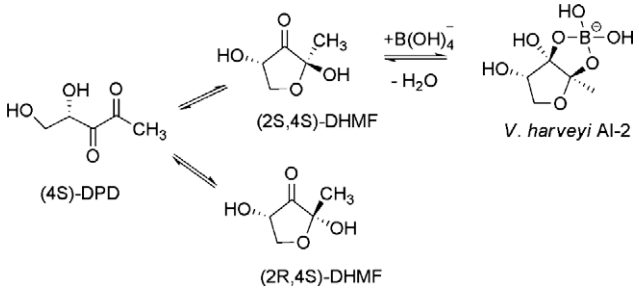

Figure 1. Structure of AI-2 in its furanosyl borate diester form—the *V. harveyi* autoinducer—and its precursor (*4S*)-dihydroxypentanedione (DPD) in equilibrium with two cyclic anomeric forms (*2S*, *4S*)-DHMF and (*2R*,*4S*)-DHMF.

* Corresponding author. Tel.: + 972 8 6472751; fax: +972 8 6472943.
E-mail address: meijler@bgu.ac.il (M.M. Meijler).
† Present address: Third Institute of Oceanography, State Oceanic Administration, Daxue Road 178, Xiamen 361005, China.

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serve as a universal signaling molecule.[8] The enzyme that catalyzes its synthesis, LuxS, has been identified in more than 70 bacterial species. However, the precise role of AI-2 in other bacteria is currently a topic of debate and information is sparse regarding the recognition and signal transduction of this autoinducer in species other than *V. harveyi*.[9] The structure of AI-2 has been solved by X-ray crystallography as a complex with the *V. harveyi* sensor protein LuxP[10] and this receptor is one of only three characterized proteins that bind AI-2.[11,12]

The development of a proper and versatile synthetic route to pure DPD[13,14] together with the verification of DPD as a molecule involved in AI-2-based QS systems,[13,14] has enabled us to approach the important question whether AI-2 is indeed a ubiquitous interspecies QS mediator, by chemical interference with proposed AI-2 based QS systems. Through computational analysis of receptor-ligand interactions between LuxP and AI-2 (Fig. S1), we designed a panel of DPD analogs with small incremental changes in alkyl chain substitution on C-2. These analogs are predicted to fit the receptor binding site, as a cavity in the region near the methyl moiety can be observed and no significant interactions between this carbon (C-1) and residues in close proximity can be observed. Therefore, extending the methyl group to alkyl moieties of increasing length (ethyl up to heptyl, Scheme 1) might lead to altered ligand induced conformational changes, compared to AI-2 induced changes, without significantly altering ligand-receptor affinities.

In order to investigate effects of the analogs on QS in other bacterial species, we focused on *P. aeruginosa*, as this organism has been reported to respond to AI-2—though it is not able to synthesize DPD by itself.[15,16]

The synthesis of all analogs was accomplished following a combination of two previously reported synthetic routes to DPD.[13,14] The key alkylation step was performed according to Shintani et al.[17] yielding the six elongated alkyl substituents on C-2. The compounds were evaluated in *V. harveyi*, using two strains that are widely employed to investigate AI-2 based QS in bacteria, following the protocol reported by Schauder et al.[18] *V. harveyi* strain BB170 lacks the LuxN receptor for AI-1 (the primary QS signal, *N*-3-hydroxybutanoyl homoserine lactone) but does contain the LuxP receptor to sense AI-2 and strain MM30 is a luxS mutant that is unable to synthesize DPD. These mutants show an increase in bioluminescence upon addition of AI-2.

We expected to obtain inhibition of the AI-2 activity upon addition of the synthesized analogs, but the results were unexpectedly different. In assays with strain BB170, all six DPD analogs showed agonist behavior compared to the control (no analog added); the addition of 500 nM of each structural analog resulted in an earlier induction of luminescence (compared to control), with a decrease in activity correlating with increase in alkyl chain length (Fig. 2a).

Each analog was tested over a range of concentrations, and a similar pattern was seen, with EC_{50} values increasing as alkyl chain length increases (Fig. 2b and Table 1). When we tested whether these results can indeed be attributed to typical agonism by the analogs, using strain MM30, we were surprised to find that—except for ethyl-DPD—none of the analogs acted as agonists. However, upon addition of DPD to each of the different analogs, induction of luminescence was restored to levels close to those seen with BB170 (Fig. 3), indicating that DPD and the alkylated analogs act in a synergistic manner. Lowery et al. reported very similar findings in *V. harveyi*, showing synergism between DPD and alkylated DPD analogs in bioluminescence induction.[19] In agreement with our observations, these authors also reported a decrease in activity with increasing alkyl chain length.

We can deduce from these results that in order for the analogs to exhibit an agonistic effect, DPD presence is essential. Two poten-

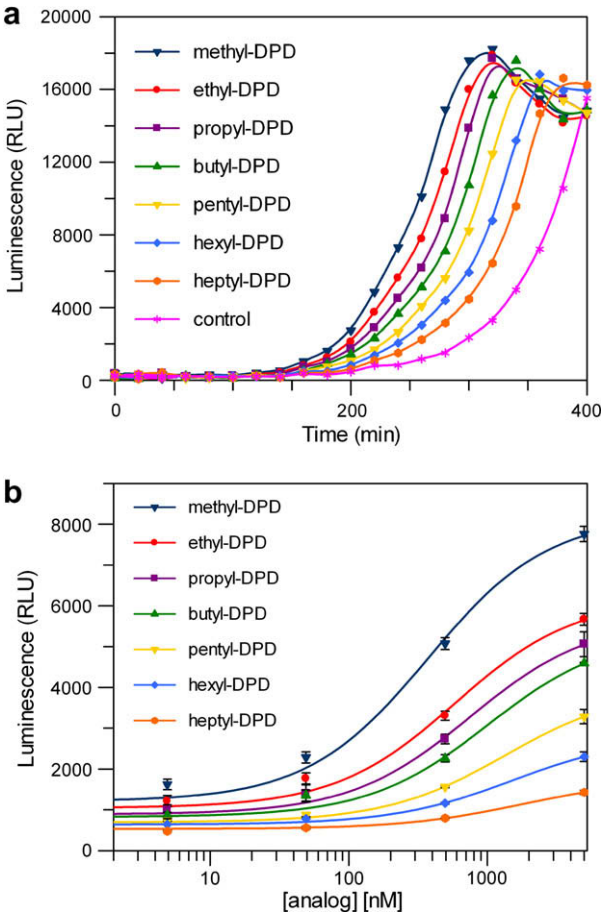
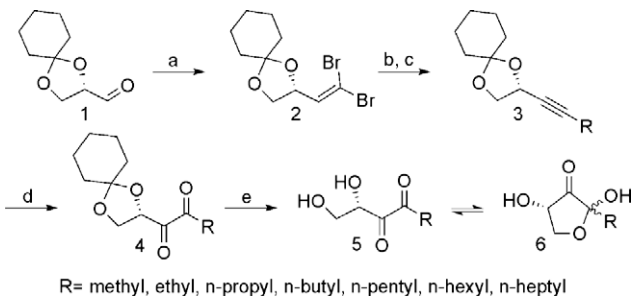


Figure 2. (a) Luminescence in *V. harveyi* strain BB170 versus time, upon incubation of cells in the presence of 500 nM of the synthetic analogs. The control represents luminescence values without the addition of analogs. (b) Luminescence (after 5 h) in *V. harveyi* strain BB170 versus varying concentrations of the tested DPD analogs.



Scheme 1. Synthesis of alkyl-DPD analogs. Reagents and conditions: (a) Ph_3P, CBr_4, CH_2Cl_2, 40%; (b) *n*-BuLi, −78 °C for 45 min, rt for 1.5 h, H_2O, 67%; (c) *n*-BuLi, −78 to −55 °C for 30 min, iodoalkane (R–I), 50 °C for 19 h, 51–83%; (d) cat RuO_2, $NaIO_4$, MeCN, CCl_4, 26–70%; (e) H_2SO_4, pH 1.5.

Table 1
EC_{50} values of alkyl-DPD analogs in the *V. harveyi* BB170 bioassay

Compound	EC_{50} [μM]
Methyl-DPD	0.15 (± 0.12)
Ethyl-DPD	0.58 (± 0.24)
Propyl-DPD	0.75 (± 0.23)
Butyl-DPD	1.01 (± 0.37)
Pentyl-DPD	1.35 (± 0.24)
Hexyl-DPD	1.52 (± 0.30)
Heptyl-DPD	1.81 (± 0.86)

Luminescence was measured after 5 h.

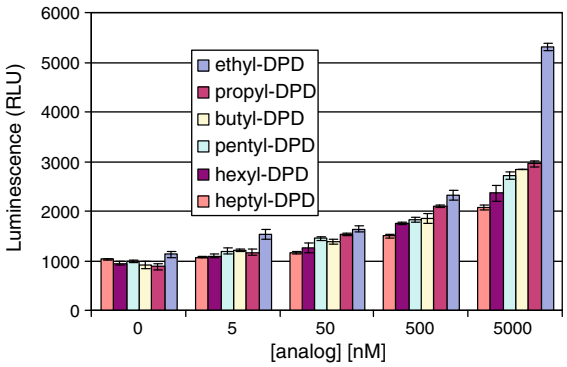


Figure 3. Luminescence (6 h) in *V. harveyi* strain MM30 versus varying concentrations of DPD analogs, in the presence of DPD (200 nM).

tial explanations are (a) these analogs are interacting allosterically with the AI-2 receptor protein LuxP, but only in the presence of DPD; (b) the analogs bind an alternate target protein, resulting either in an increase of DPD induced gene expression, in enhanced DPD synthesis (or decreased degradation), or in both.

Having established that the set of alkylated analogs strongly affect QS in *V. harveyi*, we set out to investigate whether these compounds can interfere with QS in *P. aeruginosa*. Surette and coworkers have reported that AI-2 affects gene expression in this organism,[15] but to date its target is not known. Using the *P. aeruginosa* wild-type strain PAO1, modified with a luminescent reporter gene (*luxCDABE*) cloned downstream of *lasI*, and PAO-JP2-*luxCDABE* (a luminescent reporter strain that lacks the C_4-HSL and 3-oxo-C_{12}-HSL synthetases)[16] we measured interference with 3-oxo-C_{12}-HSL induced luminescence production.

Initial experiments with PAO-JP2-*luxCDABE* revealed that DPD or its alkylated analogs do not function as agonists of the *lasR-lasI* QS system, indicating that the reported induction of virulence factor expression in *P. aeruginosa* by AI-2 is mediated through interaction with other systems. However, when PAO-JP2-*luxCDABE* was incubated with alkylated DPD analogs (50 μM) in the presence of 100 nM 3-oxo-C_{12}-HSL, significant inhibition of luminescence production was observed for butyl- and pentyl-DPD (Fig. S2). This inhibition was concentration dependent (1-200 μM), with a maximal inhibition of ~40% at 200 μM for both analogs (Fig. 4a). A similar effect was seen with wild-type strain PAO1-*luxCDABE*, which was incubated in the presence of alkylated DPD analogs, as significant inhibition was observed for both analogs (butyl- and pentyl-DPD, Fig. 4b). Here too, maximal inhibition reached approximately 40%; it should be noted that at concentrations higher than 200 μM stronger inhibitory effects were observed, but these could not be attributed purely to inhibition of QS, as slight inhibition of growth was observed as well at these concentrations.

We also tested the effects of the DPD-analogs on pyocyanin production in *P. aeruginosa* wild-type strain PAO1, in order to assess inhibitory activities in unmodified bacteria. Pyocyanin is a virulence factor of *P. aeruginosa*, and its production is regulated by QS. At a concentration of both analogs that does not affect growth (100 μM) we observed a strong decrease in pyocyanin production after 19 h. Compared to cultures that were incubated without analogs, addition of butyl-DPD resulted in 59 (±7)% reduction of pyocyanin levels, while addition of pentyl-DPD reduced pyocyanin levels by 53 (±6)%. No inhibition of growth (OD_{600}) was observed at the tested concentrations. Even though the absence of a well characterized target hampered the determination of specific IC_{50} values, we can conclude that moderate concentrations of butyl- and pentyl-DPD inhibit virulence in *P. aeruginosa* by interference with QS. Our combined results suggest that this inhibition most likely occurs through interference with the *las* system, as activation of LasR by 3-oxo-C_{12}-HSL is inhibited in the presence of both butyl- and pentyl-DPD. At this point, however, other targets such as the *rhl* or *qsc*[20] systems, cannot be excluded. Recent studies on autoinducer antagonists, including the halogenated furanones (that share structural similarities with the DPD analogs in their furanosyl conformation), have given rise to several models with respect to the properties and mode of action of these molecules. These include the hypothesis by Givskov and co-workers that inhibitory furanones work by displacing autoinducers from their cognate R-protein and hence prevent activation of this target.[21] Moreover, Manefield et al.[22] have suggested that halogenated furanones induce a rapid degradation of the LuxR protein in *V. fischeri*. These authors showed that the half life of the LuxR protein was reduced as much as 100-fold in the presence of halogenated furanones.

In conclusion, several structural analogs of DPD, the basic chemical entity in the AI-2 family of autoinducers, were identified as attenuators of QS in two different organisms. In *V. harveyi* all analogs displayed agonist activity—which is decreased with increasing alkyl chain length—in the presence of low concentrations of DPD, and could therefore be described as synergistic agonists. Agonist assays suggest that their target is not the AI-2 binding site of LuxP, but rather a yet unidentified binding site. In *P. aeruginosa* two of the analogs acted very differently, showing inhibition of 3-oxo-C_{12}-HSL activated gene expression. Both compounds decrease the production of the virulence factor pyocyanin at moderate concentrations and can therefore be regarded as potential lead compounds in the development of new QS antagonists as inhibitors of bacterial virulence.

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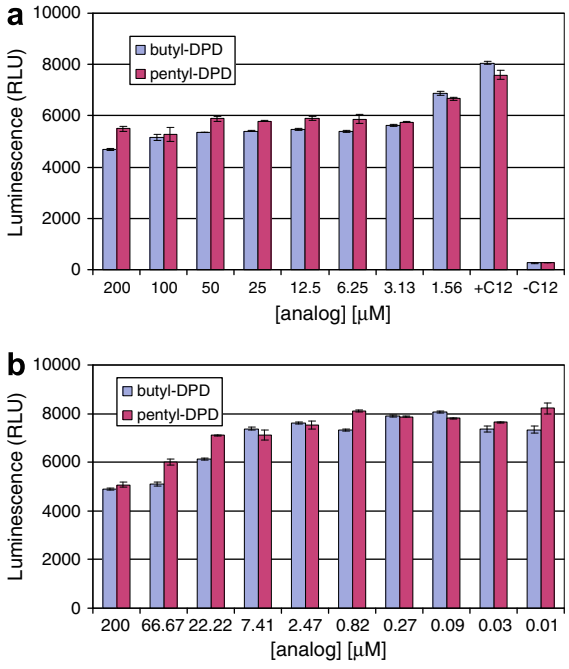

Figure 4. QS inhibition assays: (a) production of luminescence in PAO-JP2-l*uxCDABE* by 100 nM 3-oxo-C12-HSL (C12) in the presence of decreasing (200–1.5 μM) concentrations of butyl- and pentyl-DPD; (b) induction of luminescence in strain PAO1-*luxCDABE*, in the presence of varying concentrations of butyl- and pentyl-DPD.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.163.

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