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# Revisiting AI-2 quorum sensing inhibitors: Direct comparison of alkyl-DPD analogs and a natural product fimbrolide

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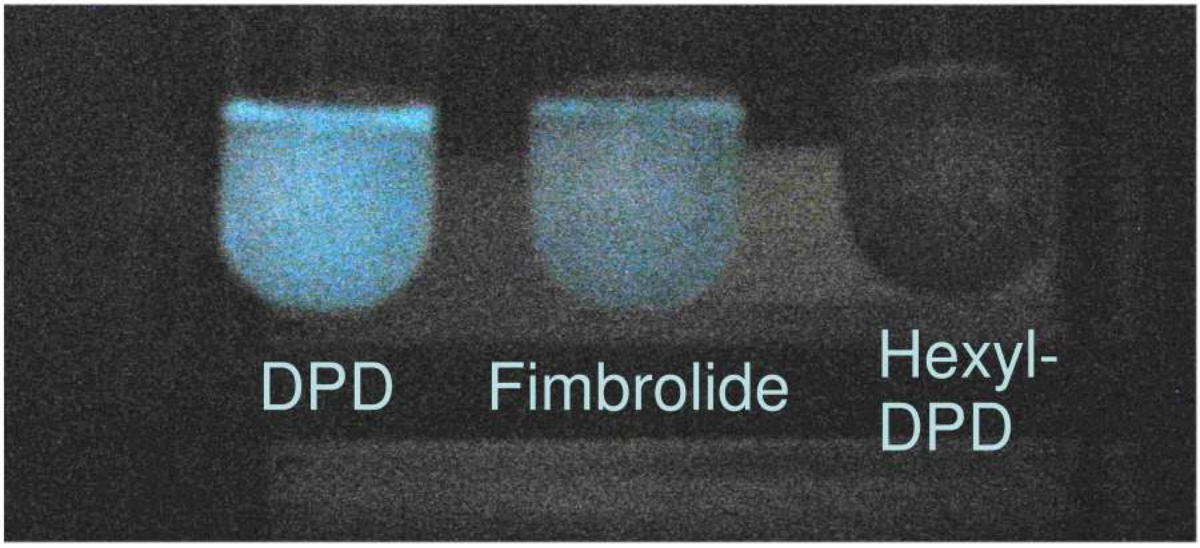
## Abstract




Quorum sensing (QS) systems have been proposed in a wide variety of bacteria. The AI-2-based QS system represents the most studied of these proposed interspecies systems, and has been shown to regulate diverse functions such as bioluminescence, expression of virulence factors, and biofilm formation. As such, the development of modulatory compounds, both agonists and antagonists, is of great interest for the study of unknown AI-2 based QS systems and the potential treatment of bacterial infections. The fimbrolide class of natural products has exhibited excellent inhibitory activity against AI-2-based QS, and as such may be considered the "gold-standard" of AI-2 inhibitors. Thus, we sought to include a fimbrolide as a control compound for our recently developed alkyl-DPD panel of AI-2 modulators. Herein, we present a revised synthesis of a commonly studied fimbrolide, as well as a direct comparison between the fimbrolide and alkyl-DPD analogs. We demonstrate that our alkyl-DPD analogs are more potent inhibitors of QS in both *Vibrio harveyi* and *Salmonella*

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**Supporting Information Available:** Experimental procedures, spectral data, and biological protocols are available free of charge via the Internet at http://pubs.acs.org.

*typhimurium*, the two organisms with defined AI-2 systems, and in doing so, call into question the widely accepted use of fimbrolide-derived compounds as the "gold standard" of AI-2 inhibition.

Because of the sheer number of cells in bacterial populations, coordinated efforts of individual cells would enable these colonies to effectively compete with higher multicellular organisms. This is indeed the case in a process known as "quorum sensing" (QS). In this process, bacteria secrete and respond to small diffusible chemical signals, or autoinducers, in a cell density-dependent process.[1] As the number of cells, and thus autoinducer concentrations, increase, bacteria coordinate their gene expression to behave as a unified group. These concerted efforts are beneficial to the bacterial population, but often come at the expense of human health, as QS has been shown to regulate such functions as biofilm formation and the expression of virulence factors. Consequently, the modulation of QS has emerged as a therapeutic target of considerable interest.[2]

The AI-2 family of autoinducers, derived from the common precursor 4,5-dihydroxy-2,3-pentanedione (DPD), is of particular interest as the gene encoding the DPD synthase, LuxS, has been identified in a plethora of bacterial species.[3] This has led to the hypothesis of AI-2 as an interspecies signaling molecule. However, the investigations of this hypothesis have been subject to criticism based on the necessity to create luxS mutants to study AI-2-controlled phenotypes, which may result in metabolic defects and subsequently growth impairment. Additionally, a lack of structural evidence of the AI-2 signal and AI-2 signaling pathways in bacteria, other than *Vibrio harveyi* or *Salmonella typhimurium*, with putative AI-2 systems has hindered the study of AI-2 QS systems. We have reported a panel of DPD analogs active in the two species with established AI-2 QS pathways.[4] These compounds, derived from the DPD signal itself, inhibit the QS of *S. typhimurium* and exhibit a synergistic effect on the QS of *V. harveyi*.[4,5]

The fimbrolide natural product (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone **1** has been reported to be a potent antagonist of both AHL and AI-2-based QS in several organisms.[6] Several reports have detailed the activity of furanone **1** as a QS inhibitor in *V. harveyi* and have even shown protective effects for shrimp against *V. harveyi* infection.[7–9] Indeed, fimbrolides are the only general AI-2 inhibitor, and can be considered the "gold standard" with regards to antagonists of AI-2-based QS. Consequently, we sought to incorporate **1** as a control in QS assays with our panel of alkyl-DPDs (**2–5**).

Several syntheses of **1** have been reported, and based on the short sequence we elected to pursue the route developed by Beechan and Sims and reinvestigated by Manny et al.[10,11] This route relies on the acid-catalyzed oxidative cyclodehydration of the acid precursor **9** to assemble the furanone heterocycle. Using the route described, compound **1** was synthesized according to Scheme 1.[11] Unfortunately, the final cyclization step, performed in refluxing sulfuric acid as previously described in the literature,[11] proved to be untenable as we were only able to obtain diminishing yields due to both polymerization and decomposition. Furthermore, in our hands, we also experienced an explosion of the reaction contents upon scale-up (5 g scale) of this final step. To solve this dilemma, several alternative conditions were examined for the acid catalyzed cyclization, including $HNO_3$ (0% yield), $HCl/Et_2O$ (0%), AcOH (0%), $CF_3CO_2H$ (10%), $H_3PO_4$ (0%), $AlCl_3/CH_2Cl_2$ (0%), and $BF_3{\bullet}Et_2O$ (0%) but were largely unsuccessful. Gratifyingly, stirring in the presence of fluorosulfuric acid at 0°C for 1 h, followed by heating to 50°C for 2 h, provided compound **1** in 33% yield over the final two steps.

Fimbrolide compounds have exhibited potent activity when added to cultures of *V. harveyi* that have already initiated QS.[7] Thus, bacterial cultures were grown to an $OD_{600}$ of about 1, and then diluted 1:1 into fresh medium containing the test compounds and incubated for 30 minutes. BB170 cells (ATCC BAA-1121, Δ*luxN*), a strain capable of producing luminescence

through the AI-2 pathway but not through the acyl homoserine lactone (AHL) pathway, was investigated because the fimbrolides have been shown to also inhibit bioluminescence produced by AHL signaling, and we wished to focus on AI-2 based QS. Under these conditions, furanone **1** was found to inhibit the bioluminescence of *V. harveyi* BB170 cells with an $EC_{50}$ of 33.9 ± 5.75 μM, which is in excellent agreement with previous reports (Figure 2).[7] We included our set of alkyl-DPD analogs in these assays, and as a general trend, we observed that increasing the length of the carbon chain corresponded to an increase in inhibitory activity (Figure S1). In fact, hexyl-DPD **5** was the most potent inhibitor identified, with an $EC_{50}$ value of 9.65 ± 0.86 μM. Similar effects were also observed in MM32 cells (ATCC BAA-1121 Δ*luxN*, Δ*luxS*), a strain that is incapable of producing its own DPD. This strain was examined to insure the inhibitory effects were exerted on the AI-2 response system, rather than DPD production. Thus, using MM32 cells in the presence of 1 μM synthetic DPD, furanone **1** had an $EC_{50}$ of 38.8 ± 6.4 μM, compared to the $EC_{50}$ of hexyl-DPD **5**, 6.92 ± 1.82 μM. In each case, hexyl-DPD was about 4-fold more active than furanone **1**. It is important to note that in most reported assays involving **1**, including those reported herein, the bacterial culture was not grown in the presence of fimbrolide **1** but rather only incubated for a brief time,[7,9] as this effectively avoids detrimental effects on bacterial growth that we observed with **1** under certain culture conditions (Figure S4).

The inhibition of luminescence as described above was measured after a 30 min treatment with the furanone or hexyl-DPD; however, after 2 h incubation time, *V. harveyi* BB170 was able to overcome the effects of hexyl-DPD while the inhibitory effects of furanone **1** remained (Figure 3). To determine if the luminescence could be "turned off" again by hexyl-DPD, furanone **1** and hexyl-DPD were added to the cultures twice: once at the start of the experiment and again immediately after the 2 h measurement. Readings were taken 30 min. after each addition, and, after 2.5 h, hexyl-DPD reduced luminescence to the level seen after the initial 30 min. treatment (Figure 3), an observation suggestive of a difference in mechanism of action between furanone **1** and hexyl-DPD. It has been demonstrated that furanone **1** interacts with the QS master regulator protein LuxR to prevent induction of the target genes and covalently modifies the DPD synthase, LuxS.[12] In this light, it is evident that there is some covalent interaction between the furanone and its target proteins, which is in accord with the observed activity reported herein. *In contrast, our data is suggestive of a non-covalent mechanism, thus allowing for on-off control of QS using hexyl-DPD and rendering it an effective probe for the temporal study of AI-2 QS.*

We also measured the effects of furanone **1** on the QS of *S. typhimurium* in parallel with our alkyl-DPD analogs. However, compound **1**, at 10 μM, exerted no effects on the AI-2-dependent β-galactosidase activity, nor did it inhibit bacterial growth at this concentration. These results agree with literature reports detailing a lack of activity of **1** against the QS of *S. typhimurium*, although it has been reported to possess significant activity against biofilm formation by *S. typhimurium.*[13] *In this light, and in combination with our previous reports,*[4] *the alkyl-DPD analogs represent, to the best of our knowledge, the only reported compounds effective against both known AI-2-QS systems.*

We have previously demonstrated the absence of toxicity of DPD and the corresponding C1-substituted DPD analogs against mammalian cells to explore the suitability of these compounds for *in vivo* applications.[4] A similar analysis of furanone **1** against a mouse leukemic monocyte macrophage cell line (RAW 264.7) revealed that **1**, at 50 μM, resulted in only 16% cell viability, as compared to hexyl-DPD **5** which exhibited no toxic effects (Table S1). This is in agreement with a recent report by Kuehl et al, who reported the toxicity of a series of furanone compounds against L929 fibroblasts.[14] Although *in vivo* studies have not been performed using the alkyl-DPD analogs, it is important to consider toxicity effects in the development of novel QS

modulatory compounds so that they may serve as viable compounds in *in vivo*, environmental, or even clinical settings.

In conclusion, we report a revised synthesis of the most commonly studied fimbrolide and report on its cytotoxic effects against mammalian cells. We also present a direct comparison between the naturally occurring fimbrolide and our recently developed panel of DPD analogs. We show that not only are our DPD analogs more potent than the fimbrolide against the QS of *V. harveyi*, but they are also active against the AI-2 QS of *S. typhimurium*. In doing so, we present a viable alternative to the widely accepted use of fimbrolide-derived compounds as "gold standard" antagonists of AI-2 based QS, and we also show that our panel of analogs represents the only known compounds active against the two well-defined AI-2 based QS systems.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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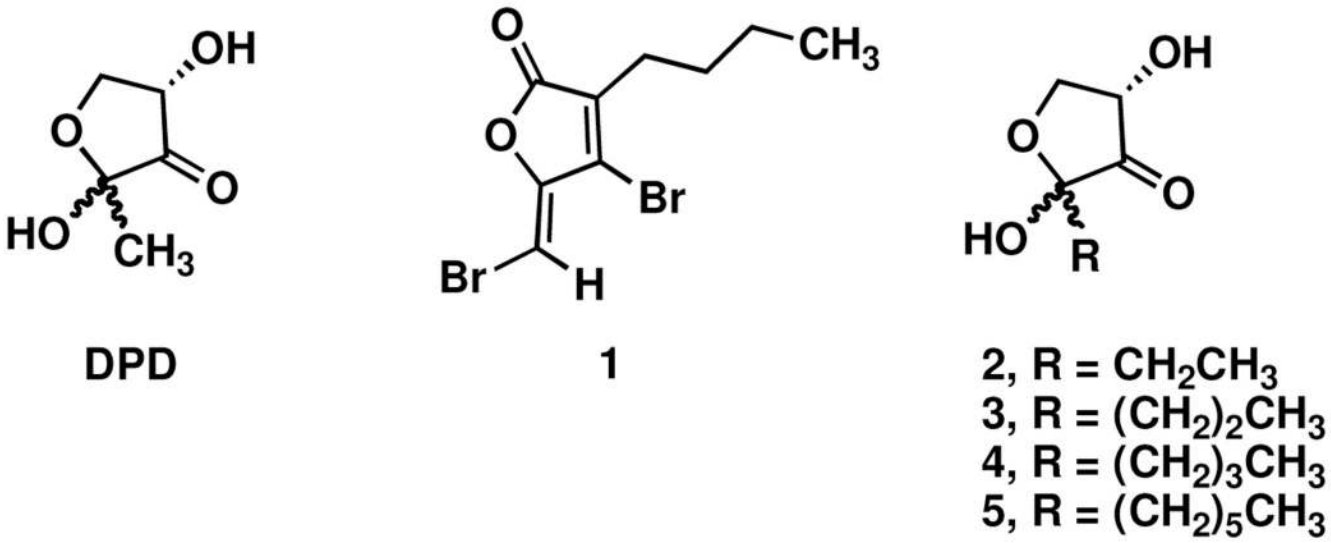



**Figure 1.**
Structures of the DPD signal and QS modulators.

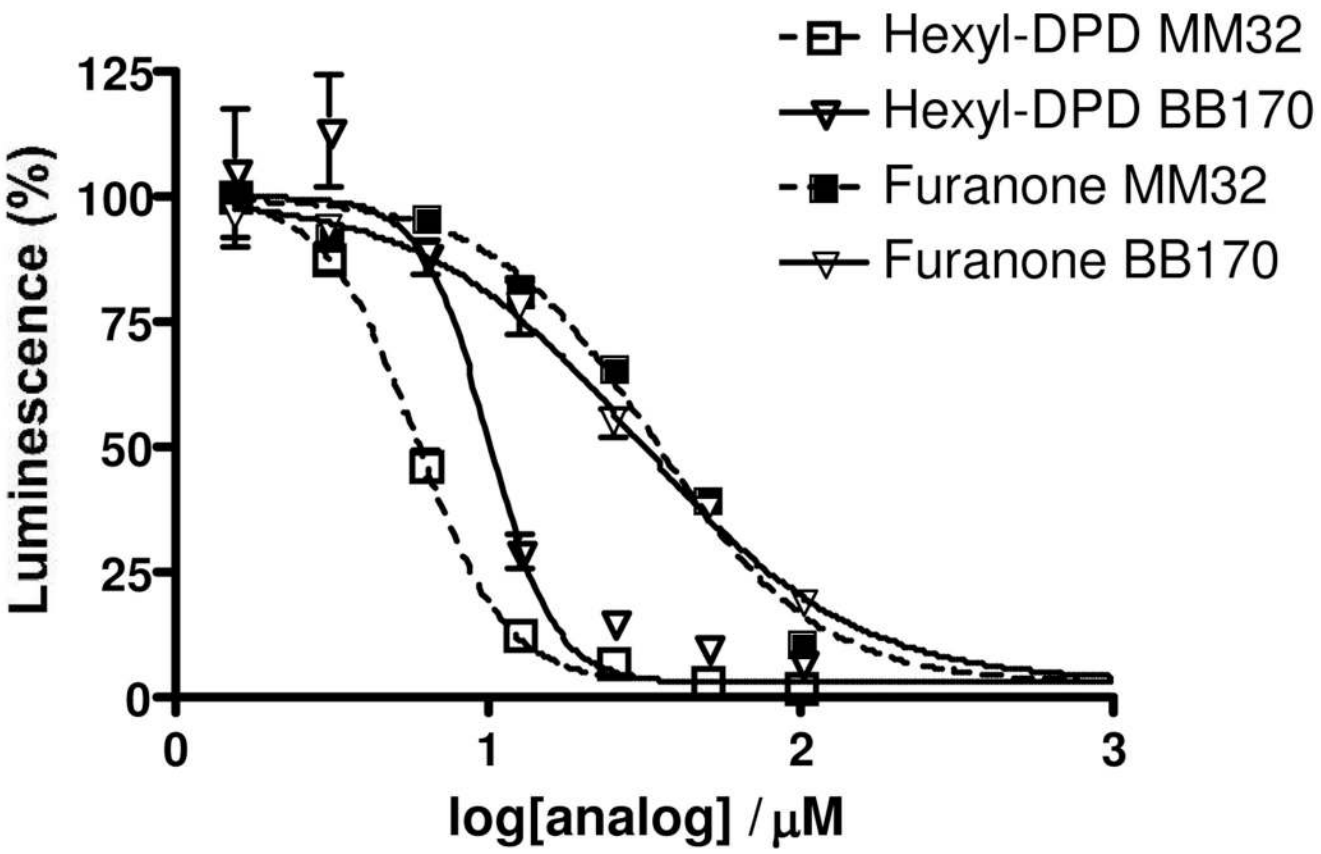



**Figure 2.**
Inhibition of bioluminescence in *V. harveyi* by furanone **1** (closed symbols) and hexyl-DPD **5** (open symbols). The same trend was observed in the two *V. harveyi* strains examined: MM32 (dashed lines) and BB170 (solid lines).

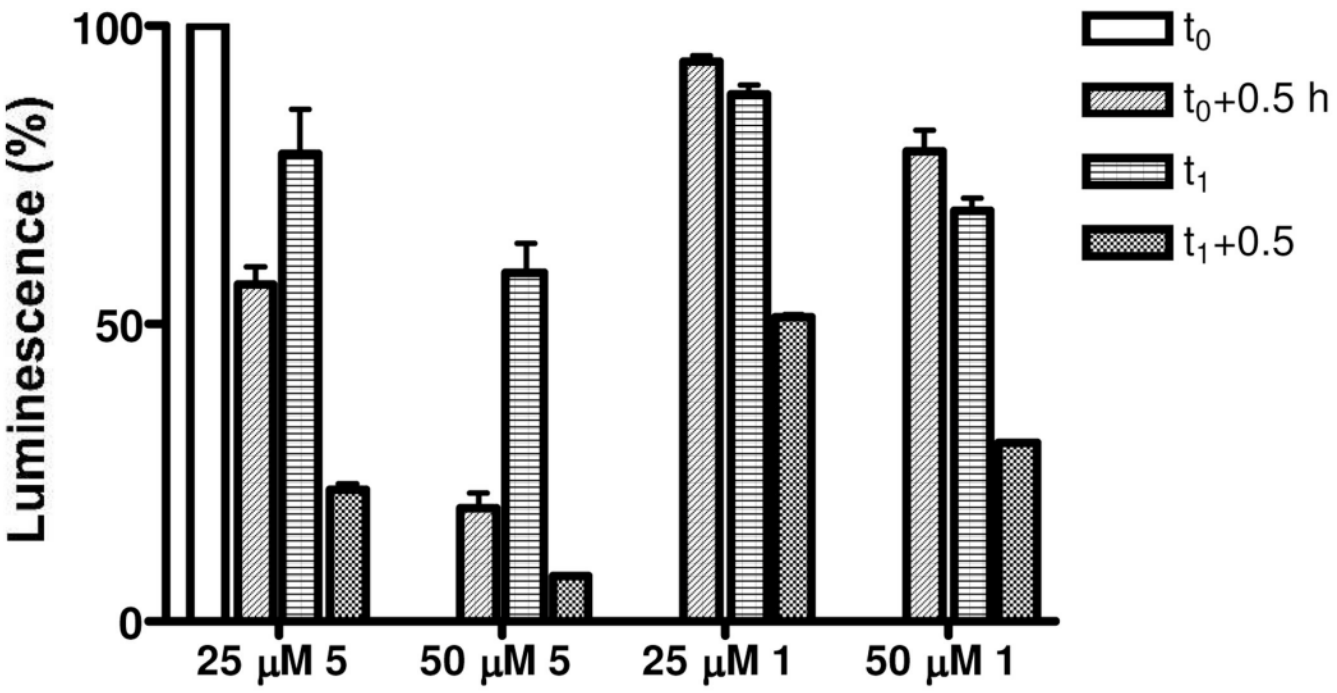
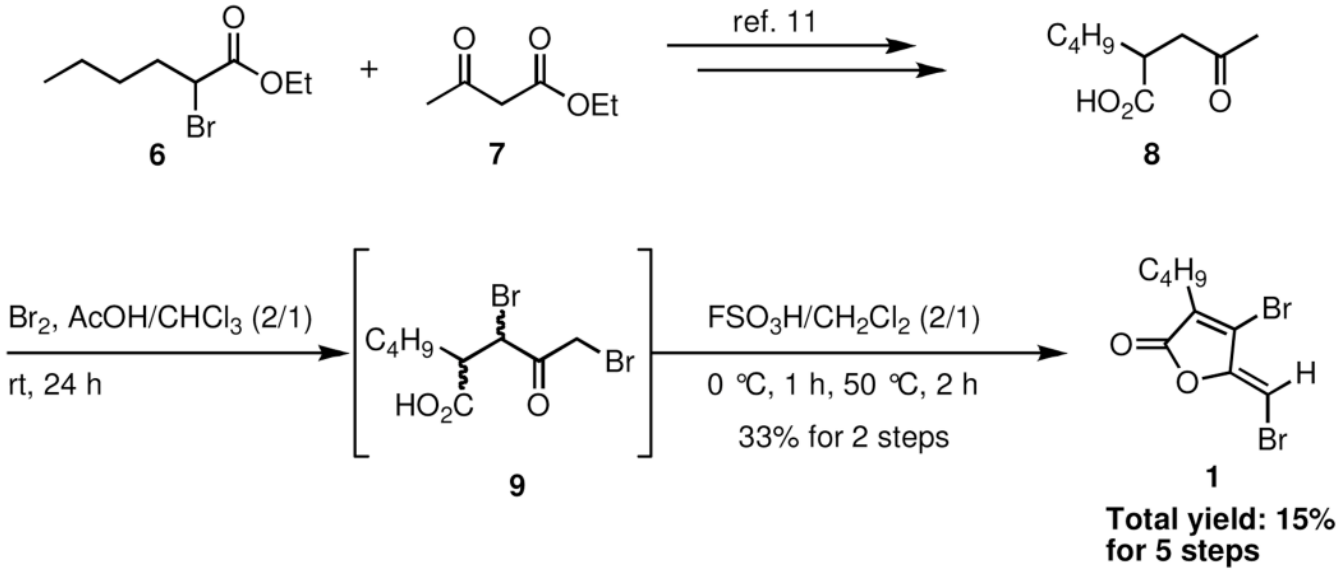



**Figure 3.**
Time course of luminescence inhibition by hexyl-DPD **5** and furanone **1**. Compounds were added at $t_0$ and $t_1$= 2 h (i.e. immediately after the 2 h reading). Luminescence was measured at $t_0$+0.5 h (0.5 h), $t_1$ (2h), and $t_1$+0.5h (2.5 h) after initial addition of inhibitor.




**Scheme 1.**
Synthesis of furanone **1**.