J|A|C|S COMMUNICATIONS

Published on Web 10/13/2009

Revisiting AI-2 Quorum Sensing Inhibitors: Direct Comparison of Alkyl-DPD Analogues and a Natural Product Fimbrolide

Colin A. Lowery,[†] Takumi Abe,[†] Junguk Park,[†] Lisa M. Eubanks,[†,‡] Daisuke Sawada,[†] Gunnar F. Kaufmann,[†] and Kim D. Janda*[,†,‡]

The Skaggs Institute for Chemical Biology and Departments of Chemistry and Immunology & Microbial Science, The Scripps Research Institute, Worm Institute for Research and Medicine (WIRM), The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, California 92037

Received August 6, 2009; E-mail: kdjanda@scripps.edu

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

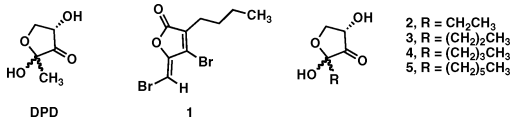
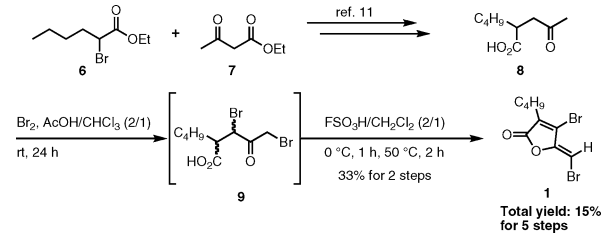


Figure 1. Structures of the DPD signal and QS modulators.

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 Δ*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*, has hindered the study of putative AI-2 QS systems. We have reported a panel of DPD analogues 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 (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-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**).

Scheme 1. Synthesis of Furanone **1**



Of the several syntheses of **1** reported, based on the short sequence we pursued 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 this route, **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, 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 $BF_3 \cdot Et_2O$ (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 **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 ∼1 and then diluted 1:1 into fresh medium containing the test compounds and incubated for 30 min.

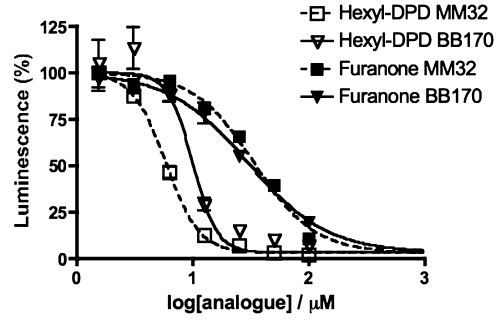


Figure 2. Inhibition of bioluminescence in *V. harveyi* by **1** (closed symbols) and **5** (open symbols). The same trend was observed in the two *V. harveyi* strains examined: MM32 (- - -) and BB170 (—).

BB170 cells (ATCC BAA-1121, Δ*luxN*), a strain capable of producing luminescence through the AI-2 pathway but not through the acyl

[†] The Skaggs Institute for Chemical Biology and Departments of Chemistry and Immunology & Microbial Science.
[‡] WIRM.

10.1021/ja9066783 CCC: $40.75

homoserine lactone (AHL) pathway, were 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 analogues in these assays, and as a general trend, 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 ensure the inhibitory effects were exerted

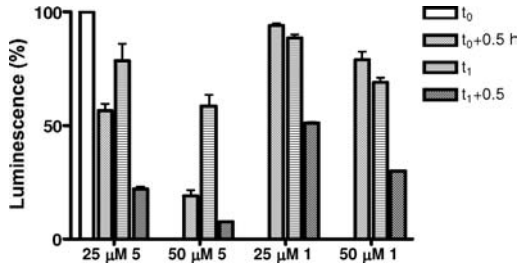


Figure 3. Time course of luminescence inhibition by **5** and **1**. Compounds were added at t_0 and t_1 = 2 h (i.e., after 2 h reading). Luminescence was measured at t_0+0.5 h (0.5 h), t_1 (2 h), and t_1+0.5 h (2.5 h) after initial addition of inhibitor.

on the AI-2 response system, rather than DPD production. Thus, using MM32 cells in the presence of 1 μM synthetic DPD, **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 ~4-fold more active than **1**. Note that in most reported assays involving **1**, including those reported herein, the bacterial culture was not grown in the presence of furanone **1** but rather only incubated for a brief time,[7,9] as this effectively avoids detrimental effects on bacterial growth 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 a 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, **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), which suggests a difference in mechanism of action between **1** and hexyl-DPD. Furanone **1** has been shown to interact with the QS master regulator protein LuxR to prevent induction of the target genes and covalently modify the DPD synthase, LuxS.[12] Thus, it is evident that there is a covalent interaction between the furanone and its target proteins, which is in accord with the observed activity reported herein. In contrast, our data are suggestive of a noncovalent mechanism for hexyl-DPD, 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 **1** on the QS of *S. typhimurium* in parallel with our panel of DPD analogues using the reporter strain Met844, a strain possessing AI-2 regulated β-galactosidase activity. However, **1**, at 10 μM, neither exerted effects on the AI-2-dependent β-galactosidase activity nor inhibited bacterial growth. 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 analogues represent, to the best of our knowledge, the only reported compounds effective against both defined AI-2-QS systems.

We have previously demonstrated the absence of toxicity of DPD and the corresponding C1-substituted DPD analogues 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 **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 analogues, 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 its cytotoxic effects against mammalian cells. We also present a direct comparison between the naturally occurring fimbrolide and our recently developed panel of DPD analogues. Our DPD analogues not only are more potent than the fimbrolide against the QS of *V. harveyi* but also are active against the AI-2 QS of *S. typhimurium*. Thus, we present a viable alternative to the widely accepted use of fimbrolide-derived compounds as "gold standard" antagonists of AI-2 based QS and also show that our panel of analogues represents the only known compounds active against the two well-defined AI-2 based QS systems.

Acknowledgment. We thank the NIH (AI079503), the Skaggs Institute for Chemical Biology, and a Sanofi-Aventis Graduate Fellowship (C.A.L.) for support and Prof. Bonnie Bassler (Princeton University) for providing *S. typhimurium* Met844.

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

References

(1) Fuqua, W. C.; Winans, S. C.; Greenberg, E. P. *J. Bacteriol.* **1994**, *176*, 269–75.
(2) Rasmussen, T. B.; Givskov, M. *Int. J. Med. Microbiol.* **2006**, *296*, 149–61. Suga, H.; Smith, K. M. *Curr Opin Chem Biol* **2003**, *7*, 586–91.
(3) Waters, C. M.; Bassler, B. L. *Annu. Rev. Cell Dev. Biol.* **2005**, *21*, 319–46.
(4) Lowery, C. A.; Park, J.; Kaufmann, G. F.; Janda, K. D. *J. Am. Chem. Soc.* **2008**, *130*, 9200–1.
(5) Ganin, H.; Tang, X.; Meijler, M. M. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3941–4.
(6) Ren, D.; Bedzyk, L. A.; Ye, R. W.; Thomas, S. M.; Wood, T. K. *Appl. Environ. Microbiol.* **2004**, *70*, 2038–43. Ren, D.; Zuo, R.; Wood, T. K. *Appl. Microbiol. Biotechnol.* **2005**, *66*, 689–95.
(7) Defoirdt, T.; Crab, R.; Wood, T. K.; Sorgeloos, P.; Verstraete, W.; Bossier, P. *Appl. Environ. Microbiol.* **2006**, *72*, 6419–23.
(8) Manefield, M.; Harris, L.; Rice, S. A.; de Nys, R.; Kjelleberg, S. *Appl. Environ. Microbiol.* **2000**, *66*, 2079–84.
(9) Ren, D.; Sims, J. J.; Wood, T. K. *Environ. Microbiol.* **2001**, *3*, 731–736.
(10) Beechan, C. M.; Sims, J. J. *Tetrahedron Lett.* **1979**, *20*, 1649–1652. Caine, D.; Ukachukwu, V. C. *J. Org. Chem.* **1985**, *50*, 2195–2198. de March, P.; Font, J.; Gracia, A.; Qingying, Z. *J. Org. Chem.* **1995**, *60*, 1814–1822. Haval, K. P.; Argade, N. P. *Synthesis* **2007**, 2198–2202.
(11) Manny, A. J.; Kjelleberg, S.; Kumar, N.; de Nys, R.; Read, R. W.; Steinberg, P. *Tetrahedron* **1997**, *53*, 15813–15826.
(12) Defoirdt, T.; Miyamoto, C. M.; Wood, T. K.; Meighen, E. A.; Sorgeloos, P.; Verstraete, W.; Bossier, P. *Environ Microbiol* **2007**, *9*, 2486–95. Zang, T.; Lee, B. W.; Cannon, L. M.; Ritter, K. A.; Dai, S.; Ren, D.; Wood, T. K.; Zhou, Z. S. *Bioorg. Med. Chem. Lett.* **2009**, in press.
(13) Janssens, J. C.; Steenackers, H.; Robijns, S.; Gellens, E.; Levin, J.; Zhao, H.; Hermans, K.; De Coster, D.; Verhoeven, T. L.; Marchal, K.; Vanderleyden, J.; De Vos, D. E.; De Keersmaecker, S. C. *Appl. Environ. Microbiol.* **2008**, *74*, 6639–48.
(14) Kuehl, R.; Al-Bataineh, S.; Gordon, O.; Luginbuehl, R.; Otto, M.; Textor, M.; Landmann, R. *Antimicrob. Agents Chemother.* **2009**, 4159–4166.

JA9066783