Bacterial Communication

Synthesis and Biological Validation of a Ubiquitous Quorum-Sensing Molecule**

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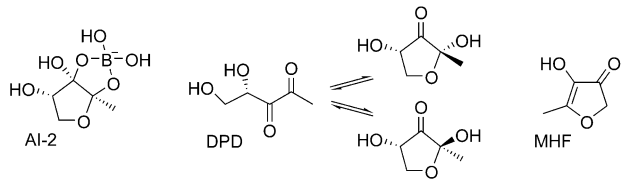
Cell-to-cell communication is used by single-cell organisms to coordinate their behavior and function in such a way that they can adapt to changing environments and possibly compete with multicellular organisms. Chemical communication amongst bacteria has been termed "quorum sensing".[1] Examples of quorum-sensing-controlled behaviors are biofilm formation, virulence-factor expression, antibiotic production, and bioluminescence. These processes are useful only when a group of bacteria carries them out simultaneously. For example, bioluminescence produced by the marine bacteria *Vibrio fischeri* is beneficial to a number of marine animals that host this species, but only when a sufficient number of bacteria synchronize their production of light. The host can use the light to seek out food sources and mates as well as for defense against predators, and in turn, protection and nutrients are provided for the symbiotic bacterial species.[2]

Quorum sensing is regulated by autoinducers that can be categorized into three classes:[3,4] 1) acyl homoserine lactones (AHLs) are produced by over 70 species of Gram-negative bacteria, and differences within this class of autoinducers occur in the acyl side chain; 2) autoinducing peptides (AIPs) are generally employed by Gram-positive bacteria, and they typically consist of 5–10 amino acid residues, with a subgroup of AIPs containing a cyclic thiolactone moiety; 3) a third type of autoinducer, termed AI-2, is used by both Gram-positive and -negative species.

A wide variety of bacteria has been shown to produce and respond to AI-2,[3,4] and this molecule is regarded by some as a species-nonspecific autoinducer.[5,6] The structure of this purported universal signaling molecule has recently been solved by X-ray crystallography in a complex with the *Vibrio harveyi* sensor protein LuxP (Scheme 1). In this structure, AI-2 is found as a furanosyl borate diester formed from reaction of the ring-closed form of (*R*)-4,5-dihydroxy-2,3-pentanedione (DPD) and boric acid.[7] This is a highly unusual structure as boron has not commonly been assigned a functional role in biological systems.[8] The generation of AI-2 has been proposed to proceed through conversion of the ribose moiety of *S*-ribosylhomocysteine (RH) into DPD by the enzyme LuxS, but the method of formation of AI-2 is still uncertain as the generation of DPD can only be inferred from the indirect detection of the homocysteine by-product with Ellman's reagent and through the reaction of generated DPD with 1,2-phenylenediamine.[9–12]



Scheme 1. Structures of AI-2, (*R*)-4,5-dihydroxy-2,3-pentanedione (DPD), and 4-hydroxy-5-methyl-3-(2*H*)-furanone (MHF).

Consequently, we have become interested in the chemical synthesis of DPD, as its total synthesis would allow critical questions to be addressed that cannot be answered with biosynthesis, including: 1) is DPD the penultimate "precursor" of AI-2 and thus important in the quorum-sensing cascade, 2) must DPD be chaperoned into its active cyclic confirmation by the LuxS protein or does this process simply occur on a chemical timescale, and 3) is boron complexation to DPD an essential requirement for the activity seen with AI-2?

Herein, we describe the first chemical synthesis of DPD, in enantiopure form, and prove that this structure is the ligand precursor of AI-2; when complexed to boron this previously hypothesized precursor induces bioluminescence in *V. harveyi* with an activity almost identical to that reported for biosynthetic preparations of the complex.

AI-2 activity has been observed in a number of species, including *V. harveyi*, *Escherichia coli*, *Helicobacter pylori*, *Salmonella typhimurium*, *Shigella flexneri*, *Borrelia burgdorferi*, and *Neisseria meninginitidis*;[6,13] however, information is sparse concerning the regulation and turnover of this autoinducer. An assay that has been used extensively to investigate AI-2 production in bacteria involves a *V. harveyi* mutant (BB170) that lacks the LuxN receptor for type 1 autoinducers (AHLs) but does contain the sensor protein (LuxP) for AI-2.[14] Addition of biosynthetically produced AI-2 has been shown to induce bioluminescence in this mutant.

The relatively compact five-carbon framework of DPD suggests at first glance that the synthesis should be straightforward. However, this simple analysis is misleading in that its skeletal backbone is densely functionalized in a contiguous array containing two hydroxy and two keto moieties. This arrangement suggests that DPD is a highly reactive molecule that could exist at physiological pH values in an equilibrium

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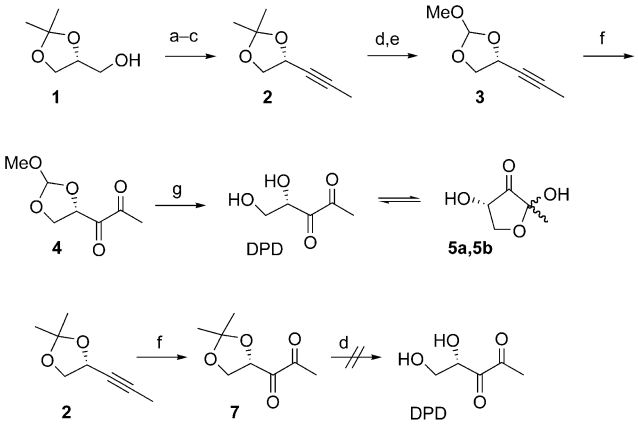
between the linear and two anomeric cyclized forms (Scheme 1).[15] Indeed, many attempts to synthesize DPD have been thwarted by such instability and reactivity.[9,10]

Our approach to synthesize DPD commenced from (*R*)-(−)-2,2-dimethyl-1,3-dioxolane-4-methanol (**1**; Scheme 2). A



Scheme 2. Synthesis of DPD. Reagents and conditions: a) oxalyl chloride, DMSO, CH_2Cl_2; then Et_3N; b) CBr_4, Ph_3P, CH_2Cl_2; c) *t*BuLi, MeI, THF; d) 60% AcOH; e) $CH(OMe)_3$ (neat), H_2SO_4 (cat.); f) $KMnO_4$, acetone, buffer (aq); g) H_2O, pH 6.5 (K_2HPO_4/KH_2PO_4 (0.1 M), NaCl (0.15 M)), 24 h. DMSO = dimethylsulfoxide, THF = tetrahydrofuran.

high-yielding Swern oxidation with oxalyl chloride resulted in the aldehyde, which was converted into alkyne **2** by applying a Corey–Fuchs homologation following work reported by Witulski et al.[16]

Oxidation assisted by potassium permanganate in a buffered water/acetone mixture yielded diketone **7**, but regrettably deprotection of **7** mediated by acetic acid did not lead to pure DPD. Instead, a mixture of compounds was obtained; purification of the more polar fragments resulted in fractions that showed significant AI-2-like activity in *V. harveyi* strain BB170. Extensive purification attempts failed to yield a pure compound. Interestingly, a less-polar fraction of this mixture did provide a crystalline compound (**8**), which was examined by X-ray crystallography and revealed to be a dimeric triacetal combination of DPD and one of its two anomeric furanone isomers (Figure 1). We entertained hopes

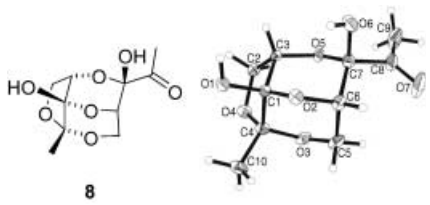


Figure 1. Compound **8** and its solid-state structure determined by X-ray crystallography.[17]

that **8** might be a pro-AI-2 molecule, but unfortunately it only displayed a low light-inducing activity in *V. harveyi* strain BB170; this activity could be the result of slow hydrolysis of the acetal bond with concomitant formation of DPD or simply of binding to LuxP, albeit with lower affinity than AI-2.

Further efforts to synthesize DPD focused on altering the diol-protecting group. The use of benzyl or silyl ether protection did not cede satisfactory results, whereas conversion of the acetonide moiety into a more acid-labile methoxymethylene acetal protecting group (yielding **3**) proved to be successful. Oxidation of **3** led to diketone **4**. Compound **4** was dissolved in phosphate-buffered D_2O (pH 6.5) and the slow formation of DPD, along with its ring-closed equilibrium anomers, could be clearly monitored by ^{1}H NMR spectroscopy. When the concentration of **4** was kept moderate (less than 3 mM, or 0.5 mg mL^{-1}), only a minimal amount (< 1%) of polymerized by-products was observed and DPD was stable for at least one month at room temperature. At higher concentrations the stability of DPD decreased significantly. Additional evidence for the clean formation of DPD and its two cyclic anomers **5a** and **5b** was obtained by addition of 1,2-phenylenediamine (1.5 equiv), which led to a Maillard reaction and the formation of a single quinoxaline derivative. We noted that, in the presence of 1,2-phenylenediamine, the disappearance of the three compounds in equilibrium was nearly complete within 30 minutes, as monitored by ^{1}H NMR spectroscopy (see the Supporting Information).

To examine the biological activity of synthetic DPD, freshly prepared DPD was diluted in autoinducer-bioassay medium and tested for AI-2 activity (Figure 2).[14] As a reference substance we used 4-hydroxy-5-methyl-3-(2*H*)-furanone (MHF, Scheme 1), a compound similar in structure

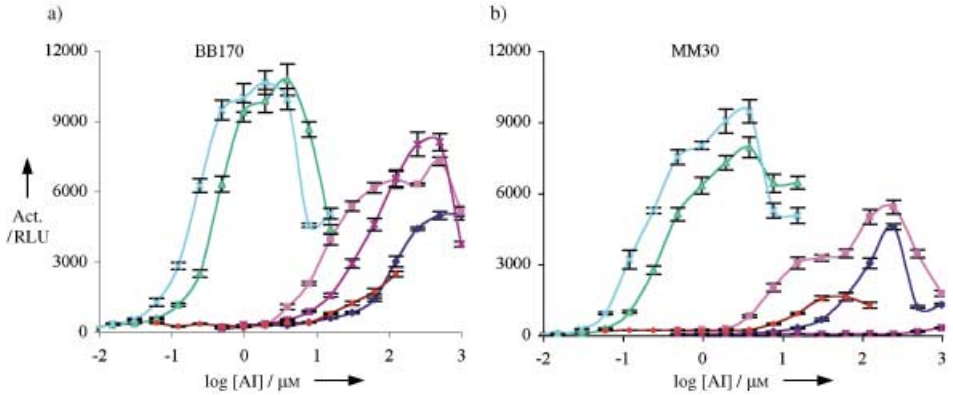


Figure 2. Induction of bioluminescence in a) *V. harveyi* strain BB170 and b) *V. harveyi* strain MM30 with autoinducers (AI): MHF (◆), MHF with boric acid (■), DPD (△), DPD with boric acid (×), only boric acid (*), compound **8** (●).

to cyclized DPD that has been observed as a by-product of the in vitro degradation of RH.[9,10] MHF has been shown to be roughly 1000-times less active than biosynthetic AI-2. Schauder et al.[9] determined EC_{50} values of approximately 80 nM for biosynthetic AI-2 versus approximately 100 μM for MHF, while Winzer et al.[10] reported higher values (ca. 1 μM versus ca. 1 mM) but a comparable difference in activities. Figure 2 shows light-inducing activity in *V. harveyi* mutants BB170[14] and MM30[18] with different concentrations of synthetic DPD or MHF. In our assays we observed a roughly 500-fold higher activity for synthetic DPD than for MHF (EC_{50} = ca. 250 nM for DPD versus ca. 125 μM for MHF), a result indicative of the strong relationship between DPD and AI-2.

Addition of boric acid to synthetic DPD did slightly increase the activity of DPD in both strains BB170 and MM30. Figure 2, however, clearly shows that addition of boric acid by itself induces bioluminescence in BB170 and *not* in MM30, an expected result since MM30 lacks the synthase LuxS. Addition of boric acid to MHF did significantly increase the activity of MHF in both bacterial strains, but its EC_{50} value remained roughly 50-times lower than that of DPD with boric acid. One explanation could be that a small amount of the MHF is converted into AI-2 upon reaction with borate. Additional evidence for the necessity of boron for induction of bioluminescence was demonstrated by using BB170 grown in a boron-free autoinducer-bioassay medium. These bacteria showed no bioluminescence until boric acid was added to the medium. Finally, preliminary studies that we have carried out with ^{1}H and ^{11}B NMR spectroscopy as well as mass spectrometry confirm that synthetic DPD is capable of chelating borate at high (> 8) pH values.

The described synthesis of enantiopure DPD and our biochemical examination of its properties substantiates the argument that it is the true precursor to AI-2 and adds a unique analytical tool to the quorum-sensing field. From our studies it can be deduced that DPD does not require LuxS to direct it into a chemical state that can be readily utilized by LuxP and will result in induction of bioluminescence. Furthermore, chelation of boron by cyclic DPD appears to be a requirement for full induction of bioluminescence. Additional studies will be required to determine whether alternative metals can coordinate to cyclic DPD and generate bioluminescence. Synthetic DPD is stable at moderate concentrations in buffer solution. This observation is in contrast with previous reports from research groups that were unable to synthesize DPD or claimed that the compound was too unstable to isolate. Our straightforward synthetic route could be used to produce DPD as a reliable standard to readily investigate bacterial coordination of gene expression, biofilm formation, and other AI-2 quorum-sensing-regulated processes. Our findings also provide a foundation for a structure- and mechanism-based approach to develop an innovative antimicrobial therapy targeting the disruption of quorum-sensing networks.

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[17] CCDC-221569 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB21EZ, UK; fax: (+44)1223-336-033; or deposit@ccdc.cam.ac.uk). See the Supporting Information for experimental details.
[18] *V. harveyi* strain MM30[13a] is a a $LuxS^{-}$ mutant that is not able to generate AI-2.

