Chemical Synthesis of (S)-4,5-Dihydroxy-2,3-pentanedione, a Bacterial Signal Molecule Precursor, and Validation of Its Activity in Salmonella typhimurium*"[S]

We describe an original, short, and convenient chemical synthesis of enantiopure (S)-4,5-dihydroxy-2,3-pentanedione (DPD), starting from commercial methyl (S)-(-)-2,2-dimethyl-1,3-dioxolane-4-carboxylate. DPD is the precursor of autoinducer (AI)-2, the proposed signal for bacterial interspecies communication. AI-2 is synthesized by many bacterial species in three enzymatic steps. The last step, a LuxS-catalyzed reaction, leads to the formation of DPD, which spontaneously cyclizes into AI-2. AI-2-like activity of the synthesized molecule was ascertained by the Vibrio harveyi bioassay. To further validate the biological activity of synthetic DPD and to explore its potential in studying DPD (AI-2)-mediated signaling, a Salmonella typhimurium luxS mutant was constructed. Expression of the AI-2 regulated lsr operon can be rescued in this luxS mutant by addition of synthetic DPD or genetic complementation. Biofilm formation by S. typhimurium has been reported to be defective in a luxS mutant, and this was confirmed in this study to test DPD for chemical complementation. However, biofilm formation of the luxS mutant cannot be restored by addition of DPD. In contrast, introduction of luxS under control of its own promoter complemented biofilm formation. Further results demonstrated that biofilm formation of the luxS mutant cannot be restored with luxS under control of the strong nptII promoter. This indicates that altering the intrinsic promoter activity of luxS affects Salmonella biofilm formation. Conclusively, we synthesized biologically active DPD. Using this chemical compound in combination with genetic approaches opens new avenues in studying AI-2-mediated signaling.

Bacteria possess an arsenal of chemical signal molecules that enable them to communicate within and between species. As such, these unicellular organisms are able to behave in a multicellular way. When a few bacteria release such signal molecules into the environment, their concentration remains below detection limits. However, when the signal molecules accumulate and the concentration reaches a threshold level, they induce the population to cooperate in diverse behaviors such as bioluminescence, virulence, and biofilm formation, by activating or repressing target genes (1--5). This phenomenon is referred to as quorum sensing.

A broad variety of quorum-sensing signal molecules has been identified in the past 20 years that can roughly be divided into (i) acyl-homoserine lactones in Gram-negatives (6), (ii) process oligopeptides in Gram-positives (7), and (iii) AI-2 in both Gram-negatives and Gram-positives (8). In contrast to the first two types of signal molecules, which are usually involved in intraspecies communication, the AI-2 molecule has been proposed to serve as a “universal” signal for interspecies communication (9, 10). The synthase for AI-2 production, LuxS, is widely conserved among Gram-negative and Gram-positive bacteria (9). Many genera of bacteria, including Salmonella typhimurium, produce AI-2 (11).

The biosynthetic pathway leading to AI-2 production is part of the activated methyl cycle (12). AI-2 is produced from S-adenosylmethionine in three enzymatic steps, the last step being catalyzed by LuxS, using S-ribosylhomocysteine as a substrate and yielding (S)-4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine (12). The unstable DPD spontaneously cyclizes into AI-2. The structural analysis of the Vibrio harveyi AI-2-binding protein LuxP in complex with the active AI-2 ligand (13) revealed the chemical identity of the V. harveyi AI-2 as a furanosyl borate diester. Recently, it was shown that the S. typhimurium AI-2-binding protein recognizes another DPD derivative, i.e. 2-methyl-2,3,3,4-tetrahydroxy-tetrahydrofuran (14).

In S. typhimurium, AI-2 is produced and released during exponential growth. It subsequently is reinternalized by the bacteria via the Lsr (LuxS-regulated) ABC transporter (15, 16). AI-2 induces transcription of the lsrACDBFGE operon, of which the first four genes encode the Lsr transport apparatus. Additionally, a Salmonella luxS mutant has been reported to be defective in biofilm formation on gallstones (17). However, whether this is the result of a signaling role of AI-2 is a current topic of debate (8, 18). Winzer et al. (19) advocated that AI-2 is not a signal molecule in organisms other than V. harveyi but rather is a discarded by-product of S-ribosylhomocysteine recycling. Changes in gene expression due to inactivation of luxS could be due to defects in the methionine metabolism, rather than to the role of AI-2 as a signal. If, however, wild-type phenotypes in luxS mutants can be restored by the addition of exogenous AI-2, a role of luxS as signal molecule synthase...
could be justified. Therefore, there is a need for chemically well-defined AI-2 signal molecules.

In this study we present an original, short, and convenient chemical synthesis of enantiopure DPD, starting from commercial methyl (S)-(-)-2,2-dimethyl-1,3-dioxolane-4-carboxylate. In addition to demonstrating the biological activity of the chemically synthesized DPD by means of the *Vibrio* bioassay, we succeeded in rescuing the expression of an AI-2 regulated target gene in a *Salmonella* luxS mutant by adding chemically synthesized DPD. The availability of synthetic DPD in combination with a genetic approach, allowed us to reveal the complexity of *Salmonella* biofilm formation.

**EXPERIMENTAL PROCEDURES**

**Synthesis of DPD**

The synthesis route to DPD, starting from methyl (S)-(-)-2,2-dimethyl-1,3-dioxolane-4-carboxylate (Fluka), is depicted in Fig. 1A (2). The key step is the ozonolysis (iv) of (S)-1,2-dihydroxy-4-methyl-4-penten-3-one (see Fig. 1A, 5). 0.78 mg (6 μmol) of the enone 5 was dissolved in 2 ml of methanol and cooled to −78 °C. A stream of 0.75 g/h (15.7 mmol/h) O₃ in O₂ was bubbled through the solution for 0.5 min with a total flow of 10.8 dm³/h. Next, the excess of ozone was removed from the solution with a stream of nitrogen gas and dimethyl sulfide (see Fig. 1A, 6). The volatiles (methanol, dimethyl sulfide) were removed by evaporation. NMR showed the signals of DPD and its anomers, accompanied by minor traces of remaining formaldehyde hydrate, which is split off during ozonolysis, methanol, dimethyl sulfide, and Me₃SO or dimethyl sulfoxide. Details can be found in the supplemental data.

**Detection of DPD**

*Equilibrium Solution of DPD in Water—DPD (see Fig. 1A, 1) and its cyclic anomic products (1b and 1c) were studied by one- and two-dimensional (COSY, 1H NMR spectroscopy, 1H NMR (400 MHz, D₂O; with a total flow of 10.8 dm³/h. Next, the excess of ozone was removed from the solution with a stream of nitrogen gas and dimethyl sulfide was added (5–10 eq). The reaction mixture was allowed to warm up and kept at room temperature for 24 h. Before purification of 3 ml of water, the volatiles (methanol, dimethyl sulfide) were removed by evaporation.

NMR showed the signals of DPD and its anomers, accompanied by minor traces of remaining formaldehyde hydrate, which is split off during ozonolysis, methanol, dimethyl sulfide, and Me₃SO or dimethyl sulfoxide. Details can be found in the supplemental data.

**Biofilm Formation**

Biofilms were grown essentially by the method of Stepesovic et al. (23) with modifications specified in the supplemental data. One of these modifications is the device used for biofilm formation, i.e. a platform carrying 96 polystyrene pegs (Nunc number 445497) that fits as a microtitter plate lid with a peg hanging into each microtitter plate well (Nunc number 269787), as described previously (24, 25). Synthetic DPD was added to the growth medium at the time of inoculation and when changing the medium. Alternatively, a batch experiment was set up in which a mixture of 3 ml of water, the volatiles (methanol, dimethyl sulfide) were removed by evaporation. NMR showed the signals of DPD and its anomers, accompanied by minor traces of remaining formaldehyde hydrate, which is split off during ozonolysis, methanol, dimethyl sulfide, and Me₃SO or dimethyl sulfoxide. Details can be found in the supplemental data.

**Chemical Synthesis and Validation of DPD**

*Chemical Synthesis of DPD* 

Starting from commercial methyl (S)-(-)-2,2-dimethyl-1,3-dioxolane-4-carboxylate (Fluka, 1A, 2), DPD was obtained in four steps (Fig. 1A). First, the methyl ester, 2, was transformed into the amide, 3, which was subsequently transformed to olefin, 4, with isopropenylmagnesium bromide in a Grignard reaction. Hydrolysis of the dioxolane ring in 4 was performed on an acid Dowex resin to yield the enone 5. Ozone treatment of the double bond in 5 and in situ reductive cleavage of the ozonide with dimethyl sulfide yielded DPD. A simple workup, with addition of water and evaporation of volatiles resulted in an aqueous solution of DPD. In principle, it would be possible to reverse the order of the deprotection and ozonolysis (Fig. 1A, iii and iv), but the deprotection turned out to be much less clean when performed as the last step.

**Characterization of DPD**

Besides its biological activity (see below), evidence for the formation of DPD was collected in different ways. Based on 1H NMR spectroscopic measurements (see supplemental data), the aqueous product solution contains DPD, 1, along with its two cyclic equilibrium anomers (Fig. 1A, 1b and 1c), which result...
from the ring closure of the open form of DPD. The 400-MHz spectrum fully matches the previously reported spectrum of Meijler et al. (26) (see Fig. 1 of the supplemental data). It is possible that in the aqueous solution the ketone group in the anomers 1b and 1c is further hydrated to the corresponding geminal diols (Fig. 1A, 1d and 1e); such a hydration has been reported for the structurally related cyclized 3-deoxyglycosones (27). Note that a hydrated form of DPD has been prepared by Semmelhack et al. (28); it has been proposed to be of biological significance in *S. typhimurium* (14).

Further evidence for the formation of DPD and the presence of its equilibrium products was obtained by using 1,2-phenylenediamine (Fig. 1B) (29, 30). Addition of this diamine to the aqueous solution resulted in the disappearance of DPD and its equilibrium products, and led to the formation a single, stable quinoxaline derivative 6 through a Maillard reaction, with only a few other signals remaining in the spectrum. This proves that at least 80% of the products of the aqueous equilibrium mixture are derived from DPD 1. NMR data on this derivatization can be found in the supplemental data.

**Biological Activity of Synthetic DPD in the Vibrio Bioassay**

The biological activity of different concentrations of synthetic DPD was determined in the *V. harveyi* bioassay for AI-2 (31) (Fig. 2). As a reference, MHF, which is structurally related to DPD, was included in the test (Fig. 2). The bioluminescence-inducing capacity of synthetic DPD clearly exceeds that of MHF (Fig. 2). As a reference, MHF, which is structurally related to synthetic DPD was observed at a concentration of 300 μM, whereas the active uptake and modification system for AI-2, encoded by the *lsr* operon, is regulated by AI-2 30 μM added DPD. In the absence of DPD, expression of the *lsrA::lux* fusion in the *Salmonella* luxS mutant occurred at a concentration of ~300 μM added DPD. In the absence of DPD, expression of the *lsrA::lux* fusion in the *luxS* mutant (luxS null), was much lower than in the *luxS* wild-type background (wild-type luxS). Addition of 72 μM DPD restores *lsrA* expression in the *luxS* mutant.

For comparison with genetic complementation, we transferred the *luxS* gene to the *luxS* mutant fusion strain. pCMPG5664 carries the *luxS* gene driven by its own, weak promoter (33). Introduction of the parent vector without *luxS* did not affect *lsrA* expression in a *luxS* null background (data not shown). Introduction of pCMPG5664 (*luxS* null + *luxS*) induced the expression of *lsrA::lux* fusions to a level comparable with that observed when wild-type *luxS* is present on the chromosome (Fig. 3). Supplying synthetic DPD to the *S. typhimurium* *lsrA::lux* strains that were wild-type for *luxS* on the chromosome or that were *luxS* null with *luxS* complemented in *trans*, showed an increased level of *lsrA::lux* expression, as compared with endogenous levels of AI-2. These results corroborate those previously obtained by Tago et al. (15) with enzymatically synthesized AI-2. They showed that the *lsr* operon, under the conditions applied, was not fully induced by the endogenously produced AI-2.

**The Effect of Synthetic DPD on *S. typhimurium* Biofilm Formation**

To investigate the role of *luxS* and DPD in *Salmonella* biofilm formation, we tested *S. typhimurium* wild type and the *luxS* mutant (CMPG5602) for their ability to form biofilms on polystyrene pegs. The *Salmonella* luxS mutant is impaired in forming mature biofilms. Introduction of a functional *luxS* gene driven by its own promoter (pCMPG5664) in the *luxS* mutant restores biofilm formation to the wild-type level (Fig. 4A). However, synthetic DPD cannot rescue the biofilm formation defect of the *Salmonella* luxS mutant at 72 μM concentration, which is sufficient to restore *lsrA* expression in a *luxS* mutant (Figs. 3 and 4A).
Alternative ways of adding DPD to the biofilm assay were applied in an attempt to rescue biofilm formation in the luxS mutant. DPD concentrations were increased, the incubation time of biofilm formation was extended to 7 days, and the incubation conditions of biofilm formation were adapted to enhance contact with DPD (slightly rocking). Alternatively, complementation with synthetic DPD was performed in a fed batch experiment, in which the concentration of DPD was increased stepwise during 48 h in the assay for biofilm formation. Eventually, DPD supplementation was restricted to the time of inoculation. In none of these cases was restoration of biofilm formation in the luxS mutant observed (not shown).

To exclude an indirect effect of the activated methyl cycle, in which LuxS plays a role (12, 19), as a possible cause of the lack of restoration of biofilm formation by DPD, the biofilm medium was supplemented with methionine, cysteine, or S-adenosylmethionine. However, no rescue of biofilm formation in the Salmonella luxS mutant was observed. Addition of S-adenosylmethionine even completely abrogated biofilm formation in Salmonella wild type (not shown).

To explore the hypothesis that controlled synthesis of AI-2 at a given time point, and therefore tight control of LuxS activity, is required for S. typhimurium biofilm formation as suggested from the previous experiments, we tested whether introducing luxS under the control of the strong constitutive nptII promoter (34) (pCMPG5643), resulted in restoration of Salmonella biofilm formation in the luxS mutant. Interestingly, introduction of luxS driven by this nptII promoter (pCMPG5643) cannot rescue biofilm formation in the luxS mutant (Fig. 4B). On the other hand, wild-type Salmonella containing the same construct, i.e., pCMPG5643, is able to form a biofilm in the same amount as wild type not containing this plasmid (Fig. 4B). As such, it can be further stated that the chromosomal wild-type luxS allele, i.e., driven by its own promoter, is dominant in the luxS merodiploid S. typhimurium strain containing two luxS copies, one driven by its own promoter and one by the nptII promoter. This experiment also allows excluding possible interference of a particular antibiotic added in the biofilm assay, because introduction of pCMPG5643 in wild-type Salmonella and consequently adding of the corresponding antibiotic, does not affect biofilm formation.

**DISCUSSION**

A most exciting development in recent years is the discovery that bacteria communicate thereby mimicking a multicellular organization. The AI-2 signal molecule is an exciting chemical signal, because it is produced and interpreted by a variety of bacteria (31). In many cases, luxS orthologs have been shown to be essential for AI-2 synthesis (9). The precursor of AI-2, DPD, is ubiquitous (9, 12, 14, 19). However, because of the additional metabolic role of LuxS (19), it is not clear whether all the bacteria that produce AI-2 actually use it as a signal molecule. To elucidate this, a chemically defined AI-2 is required. We designed a chemical synthesis of DPD, because the lack of...
chemically defined AI-2 (or its precursor DPD) hampered further studies on AI-2 signaling. Indeed, at the time we initiated our DPD synthesis, only partial purification of AI-2 by methanol-dichloromethane extraction from conditioned medium had been described (35). Recently, an affinity column with a borate resin showed effective in providing a method for concentrating and purifying V. harveyi AI-2 from the biosynthetic product (32). In vitro enzymatic production of AI-2 starting from S-adenosylhomocysteine is limited in concentration of end product by the low solubility of S-adenosylhomocysteine and results in a mixture of AI-2, MHF, homocysteine, and adenine (12, 18). During the preparation and handling of this manuscript, two synthetic procedures for DPD synthesis have been published (26, 28).

We here have described a unique synthesis of enantiopure DPD, starting from the commercially available methyl (S)-(−)-2,2-dimethyl-1,3-dioxolane-4-carboxylate in four straightforward steps. The end product of the synthesis is a solution containing DPD as an equilibrium mixture with its cyclization products, among which are 2 anomic furanones and possibly hydration products (Fig. 1A), as shown by NMR and 1,2-phenylenediamine derivatization. A convincing proof for DPD synthesis is that our NMR spectrum fully matches that of the synthetic DPD.

The biological activity of our synthetic DPD was shown using the V. harveyi bioreporter. Biosynthetic AI-2 has been reported to possess EC_{50} values ranging from 80 nM (12) to 1 μM (19). Our synthetic DPD showed a half-maximal activation in the V. harveyi bioassay at a concentration of 125 nM. This is in the range of the EC_{50} of DPD reported by Meijler et al. (250 nM) (26).

The report on Salmonella luxS mutants being impaired in biofilm formation on gallstones (17) offered an interesting, more complex phenotype to evaluate biological activity of our DPD. The suggested role of LuxS (AI-2) in bacterial biofilm development is ambiguous. For instance, one study found a role for the LuxS system in Streptococcus mutans, but two other studies indicated that LuxS is not required for biofilm formation in S. mutans (40–42). Our results showed that Salmonella
biofilm formation on polystyrene is dependent on an intrinsically regulated luxS, because a Salmonella luxS mutant, which is impaired in biofilm formation, could be rescued by the introduction of a plasmid containing wild-type luxS but not by luxS driven by the strong nptII promoter. In line with this, addition of synthetic DPD cannot rescue the biofilm formation defect of the Salmonella luxS mutant. Although in planktonic state, S. typhimurium luxS expression was reported to be weak and constitutive (33), during biofilm formation this might not be the case. The sessile lifestyle associated with persistence within a biofilm is distinct from the lifestyles of planktonic culture, resulting in different gene expression (43, 44). Our results indicate that not AI-2 as such, but altering the intrinsic promoter activity of luxS, changes expression in time and/or intensity of Salmonella genes, among which are genes crucial for biofilm formation. The role of LuxS in the activated methyl cycle seems not to be a major player in biofilm formation, because supplementation with methionine, cysteine, or S-adenosylmethionine could not restore biofilm formation in a Salmonella luxS mutant. The observation that wild-type luxS is dominant in a merodiploid strain containing a wild-type luxS allele and a luxS allele engineered for strong constitutive expression further suggests a complex mechanism of LuxS-mediated biofilm formation. We will follow a genome-wide approach to disentangle the role of luxS regulation and its influence on the different levels of the biofilm formation cascade. Synthetic DPD offers a convenient, powerful tool to assist in this endeavor.

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