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Inhibition of quorum sensing and biofilm formation in *Vibrio harveyi* by 4-fluoro-DPD; a novel potent inhibitor of AI-2 signalling†

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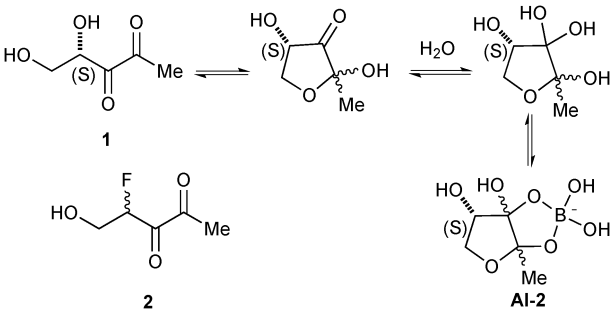
(*S*)-4,5-Dihydroxypentane-2,3-dione [(*S*)-DPD, (1)] is a precursor for AI-2, a quorum sensing signalling molecule for inter- and intra-species bacterial communication. The synthesis of its fluoro-analogue, 4-fluoro-5-hydroxypentane-2,3-dione (2) is reported. An intermediate in this route also enables a new, shorter synthesis of the native (*S*)-DPD. 4-Fluoro-DPD (2) completely inhibited bioluminescence and bacterial growth of *Vibrio harveyi* BB170 strain at 12.5 μM and 100 μM, respectively.

Biofilms are a common cause of persistent bacterial infections, are often recalcitrant to antimicrobial therapy and are rarely resolved *via* host immune defence mechanisms.[1–4] Biofilm formation is regulated through cell-to-cell communication between bacteria *via* the release of autoinducer signalling molecules, this process is referred to as quorum sensing (QS).[5,6] **AI-2** acts as a universal signalling molecule and is present in more than 70 species of bacteria.[7] Regulation of **AI-2** has been shown to play a significant role in biofilm formation in many bacterial species.[8–11] The ability to disrupt this signalling process and possibly prevent bacterial biofilm formation may therefore be advantageous in the treatment or prevention of infectious disease. Previous synthetic **AI-2** analogues have shown to have an inhibitory effect on biofilm formation in *Vibrio harveyi* and *E. coli*[11] as well as QS associated pyocyanin production in *Pseudomonas aeruginosa*, due to alterations in gene expression after exposure to extracellular **AI-2**.[12] The structures of (*S*)-DPD (**1**), and its boronate complex exist in equilibria of hydrated and cyclised forms in solution (Scheme 1).[13,14] *Vibrio harveyi*, an indicator bacterium which forms



Scheme 1 Autoinducer **AI-2**: acyclic and cyclic forms of (*S*)-DPD (**1**),[16] and its borate complex; F-DPD (**2**).

the 2,3-borate diester of the hydrated α-anomer of DPD, exhibits bioluminescence properties.[15–17]

Compounds that interfere with QS may provide a strategy for novel antibacterials.[18] Previously we reported a new synthesis and bioluminescence effect of the parent DPD.[19] Here, the synthesis of the novel 4-fluoro analogue of DPD (**2**, F-DPD) is reported, and shown to act as a powerful suppressor of bioluminescence and displays potent antibacterial activity. Fluorine is a common isosteric and isoelectronic substitution for a hydroxyl group, the differences being that F is only a H bond acceptor and the F–H bond is weaker than O–H. F-DPD (**2**) may be helpful in understanding the molecular mechanisms of **AI-2** based quorum sensing. We aimed to investigate whether (**2**) inhibits the bioluminescence, growth and biofilm formation of *Vibrio harveyi*.

The synthesis of the novel F-DPD, (**2**) is shown in Scheme 2. The key intermediates, (*R*)-1-(benzyloxy)-pent-3-yn-2-ol (**8**) and (*R*)-1-(4-methoxybenzyloxy)-pent-3-yn-2-ol (**9**), were prepared from (*R*)-glycidol (**3**) (Scheme 2a).[20–22] In addition, this intermediate (**9**) also enabled us to develop an improved, shorter synthesis of the native (*S*)-DPD, wherein conversion of (**9**) into isopropylidene (**10**), followed by oxidation provides dione (**11**) which we previously reported was trivially converted into (*S*)-DPD under mild-acid hydrolysis[19] (Scheme 2b).

Two approaches towards the synthesis of F-DPD (**2**) were pursued, the key step in each being the use of the deoxyfluorinating agent, Xtal-Fluor, to replace an hydroxyl group with a fluorine atom. In the

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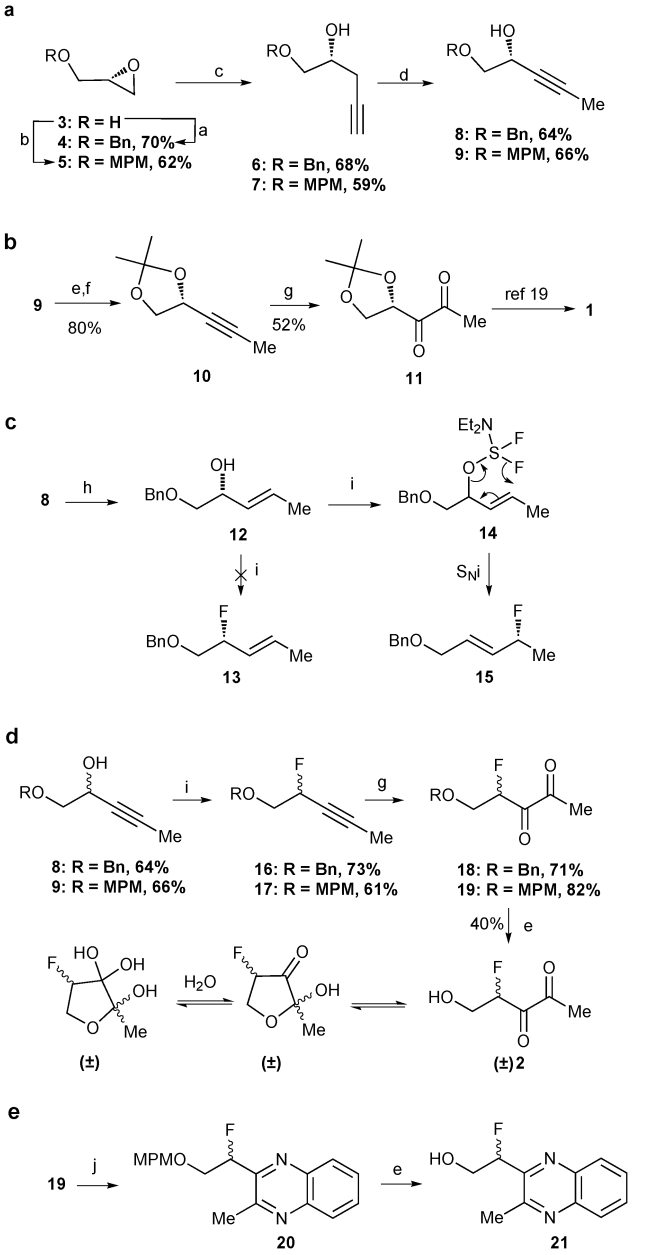
‡ Equal contribution to the research; MK & FF completed the synthesis and S Forbes the microbiology.



Scheme 2 Synthesis of F-DPD (**2**): (a) BnBr, NaH, dry DMF; (b) 4-MPMCl, NaH, TBAI, dry DMF; (c) lithium acetylide ethylenediamine complex (90%), dry DMSO; (d) potassium-*t*-butoxide, dry DMSO: (e) DDQ, DCM : H_2O [10 : 1]; (f) 2,2-dimethoxypropane, dry DMF, con. H_2SO_4 (cat); (g) RuO_2·H_2O, $NaIO_4$, CCl_4 : MeCN : H_2O [2 : 2 : 3]; (h) $LiAlH_4$, dry THF; (i) XtalFluor-E, $(Et)_3N$·3HF, TEA, −72 °C; (j) 1,2-phenylenediamine, MeOH.

first pathway (Scheme 2b), the benzyl analogue (**8**) was reduced to the alkene (**12**), but on reaction with Xtal-Fluor-E, the product was not the desired (*E*)-1-(benzyloxy)-2-fluoropent-3-ene (**13**) (Scheme 2c). The ^{19}F NMR spectrum confirmed the formation of a fluorinated compound, but the ^{1}H NMR data supported a double bond migration, confirmed by the presence of a dd for the methyl group (C-5) at 1.34 ppm ($^3J_{F–H5}$ 23.4 Hz, $^3J_{H4–H5}$ 6.6 Hz). This is consistent with the formation of (**15**), attributed to an S_N^i intramolecular substitution reaction *via* intermediate (**14**), although by ^{1}H NMR the compound was not pure (Scheme 2c).

To circumvent the vinylic migration, a second approach moved the fluorination step to the alkyne precursors (**8**) and (**9**). Fluorination of (±)-(**8**) or (±)-(**9**) with Xtal-Fluor-E, triethylamine trihydrofluoride and triethylamine at −72 °C gave the desired propargylic fluoro compounds (±)-(**16**) and (±)-(**17**) in 73% and 61% yield, respectively. Subsequent oxidative cleavage of (±)-(**17**) with $NaIO_4$ and RuO_2 gave α-diketone, 5-(4-methoxybenzyloxy)-4-fluoropentane-2,3-dione (±)-(**19**) in 82% (Scheme 2d). The ^{1}H NMR was readily assigned,[23] however further structural confirmation was provided by conversion into the quinoxaline derivative (±)-(**21**) by reaction of diketone (±)-(**19**) with 1,2-phenylenediamine, and subsequent deprotection of (±)-(**20**) with DDQ (Scheme 2e). Quinoxaline (±)-(**21**) was characterised by ^{1}H, ^{13}C and ^{19}F NMR spectroscopy.[24] On attempting to complete an enantiospecific synthesis of diketone (**18**), chiral GC of (**18**) showed racemisation of the product, attributed to facile enolisation, therefore (**2**) could only be made as a racemate. Thus, rationalising the selection of a route from racemic (**8**) or (**9**).

The oxidative deprotection of (±)-(**19**) with DDQ gave F-DPD (±)-(**2**) as an equilibrium mixture of non-hydrated (cyclic and acyclic) and hydrated (cyclic) compounds (Scheme 2c). GCMS showed two peaks for F-DPD (**2**): retention time (Rt) 6.77 min, *m*/*z* [134.0371]. and (Rt) 10.25 min, *m*/*z* 152.0 in a ratio of 1 : 3, consistent with the non-hydrated and hydrated forms of F-DPD (±)-(**2**). Despite being a low molecular weight compound, the ^{1}H NMR spectrum of (±)-(**2**) was very complex, attributed also to the cyclic forms existing as diastereoisomers. The ^{1}H NMR was similar to that for (*S*)-DPD (**1**),[16,17,19] however it was further complicated by H–F coupling. For acyclic F-DPD (±)-(**2**), the ^{1}H NMR spectrum included a ddd at 5.61 ppm for the CHF group with coupling constants of $^2J_{H–F}$ 55.6, $^3J_{H4–H5}$ 7.0 and $^3J_{H4–H5}$ 4.0 Hz. For the diastereoisomeric non-hydrated cyclic forms of F-DPD (±)-(**2**), the CHF groups appeared as a ddd at 5.09 ppm ($^2J_{F–H}$ 54.7, $^3J_{H4–H5}$ 5.3, $^3J_{H4–H5}$ 2.3 Hz) and a dt at 4.47 ppm ($^2J_{F–H}$ 46.5, $^3J_{F–H5}$ 5.1 Hz). For non-hydrated F-DPD (±)-(**2**), the ^{1}H-coupled ^{19}F NMR spectrum showed peaks at −202.7 ppm (acyclic), −192.5 ppm (cyclic) and −186.4 ppm (cyclic). In the ^{1}H-decoupled ^{19}F NMR spectrum, the diastereoisomeric hydrated cyclic forms were observed as ddd at −175.1 ppm ($^2J_{F–H}$ 58.5, $^3J_{F–H5}$ 31.1, $^3J_{F–H5}$ 27.1 Hz) and at −183.2 ppm ($^2J_{F–H}$ 57.2, $^3J_{F–H5}$ 35.4, $^3J_{F–H5}$ 21.8 Hz) in a ratio of 1 : 3. The spectroscopic data for each form of (±)-(**2**), is given in the ESI.†

Concentrations equal or greater than 12.5 μM F-DPD (**2**) resulted in greater than a 10 000-fold decrease in luminescence production in *Vibrio harveyi*, which corresponds to greater than 99.99% reduction (Fig. 1). Inhibition of luminescence production was also evident at lower F-DPD (**2**) concentrations albeit to a lesser extent. At the lowest test concentration (0.8 μM), luminescence was shown to be reduced by 37%. *Vibrio harveyi* planktonic growth was completely inhibited at 100 μM and 200 μM F-DPD (±)-(**2**) (Fig. 2). Slower growth of the bacterium, resulting in an increase in lag phase and delay in stationary phase, was evident at 12.5 μM, 25 μM and 50 μM F-DPD (±)-(**2**). When compared to the untreated culture (0 μM) changes in bacterial growth kinetics became more pronounced at higher F-DPD (±)-(**2**) concentrations. Inhibition of biofilm formation in *Vibrio harveyi* showed a clear dose response to increasing concentrations of F-DPD (±)-(**2**). At 200 μM, biofilm formation was reduced by over 90% in comparison to the untreated control (Fig. 3).

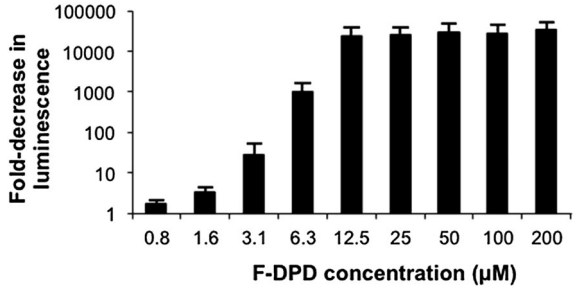


Fig. 1 Fold decrease in luminescence in *V. harveyi* BB170 grown in the presence of F-DPD (**2**) in comparison to an untreated control at peak luminescence. F-DPD (**2**) concentrations ranging from 0.2 μM to 6.25 μM did not affect specific bacterial growth rate or productivity in batch culture (see Fig. 2). Error bars show standard deviation of biological replicates, $n = 3$.

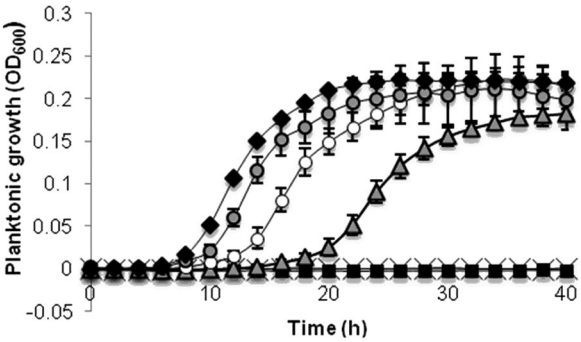


Fig. 2 Planktonic growth of *V. harveyi* BB170 grown in the presence of F-DPD (**2**). F-DPD (**2**) concentrations ranging between 0.2 μM and 200 μM were tested. For clarity the following selected concentrations have been included in this figure: 0 μM (black diamond), 12.5 μM (grey circle), 25 μM (white circle), 50 μM (grey triangle), 100 μM (black square) and 200 μM (black cross). F-DPD (**2**) concentrations ranging between 0.2 μM and 6.25 μM did not affect specific bacterial growth rate or productivity in batch culture (data not shown). Error bars show standard deviation of biological replicates, $n = 3$.

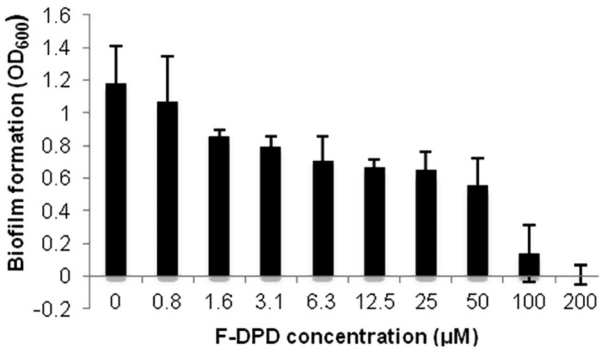


Fig. 3 Crystal violet biofilm assay of *V. harveyi* BB170 in the presence of F-DPD (**2**). F-DPD (**2**) concentrations ranging from 0.2 μM and 6.25 μM did not affect specific bacterial growth rate or productivity in batch culture (see Fig. 2). Error bars show standard deviation of biological replicates, $n = 3$.

A short synthesis of F-DPD (±)-(**2**) (alongside divergence to an improved synthesis of the native (*S*)-DPD) an isosteric analogue of quorum sensing DPD, is reported. Its quorum sensing properties to affect bioluminescence and bacterial growth of *Vibrio harveyi* BB170 strain were evaluated. F-DPD (±)-(**2**) displayed direct antibacterial activity at 100 μM and showed a pronounced ability to disrupt luminescence production in *Vibrio harveyi*, which is an **AI-2** signalling-mediated event. Furthermore F-DPD (±)-(**2**) has been shown to directly disrupt biofilm formation in the bacterium, possibly due to interference in the quorum sensing process. These data thus show F-DPD (±)-(**2**) to be an effective novel antibacterial and anti-biofilm agent in *Vibrio harveyi*.

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23 ^{1}H NMR (400 MHz, $CDCl_3$) of 5-(4′-methoxybenzyloxy)-4-fluoro-pentane-2,3-dione (**19**); δ 7.19 (2H, d, *J* 8.8 Hz, H–Ar), 6.88 (2H, dm, *J* 8.8 Hz, H–Ar), 5.72 (1H, ddd, $^{2}J_{4\text{-F}}$ 55.6, $^{3}J_{4-5a}$ 4.1, $^{3}J_{4-5b}$ 2.0 Hz, H-4), 4.52 (d, 1H, *J* 11.5 Hz, CH_2-Ar), 4.40 (d, 1H, *J* 11.5 Hz, CH_2-Ar), 4.11 (1H, ddd, $^{3}J_{5a-F}$ 34.9, $^{2}J_{5a-5b}$ 11.7, $^{3}J_{5a-4}$ 4.1 Hz, H-5a), 3.90 (1H, ddd, $^{3}J_{5b-F}$ 21.2, $^{2}J_{5b-5a}$ 11.7, $^{3}J_{5b-4}$ 2.0 Hz, H-5b), 3.81 (3H, s, OMe), 2.35 (3H, s, Me). ^{19}F NMR (375 MHz, $CDCl_3$) δ −201.4.
24 ^{1}H NMR (400 MHz, $CDCl_3$) of 2-fluoro-2-(3-methylquinoxalin-2-yl)-ethanol (**21**); δ 8.04 (2H, dd, $^{2}J_{H-H}$ 8.4, $^{4}J_{H-H}$ 1.2 Hz, H–Ar), 7.81–7.71 (2H, m, H–Ar), 5.84 (1H, dt, $^{2}J_{H-F}$ 46.5, $^{3}J_{H-H}$ 4.7 Hz, H-2), 4.48–4.32 (m, 2H, H-1), 2.87 (3H, d, *J* 2.3 Hz, Ar–Me). ^{19}F NMR (375 MHz, $CDCl_3$) δ −188.1. See ESI† for ^{13}C and ^{19}F NMR data.