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Synthesis and biological activity of a potent optically pure autoinducer-2 quorum sensing agonist

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ABSTRACT

Quorum sensing (QS) regulates population-dependent bacterial behaviours, such as toxin production, biofilm formation and virulence. Autoinducer-2 (AI-2) is to date the only signalling molecule known to foster inter-species bacterial communication across distantly related bacterial species. In this work, the synthesis of pure enantiomers of C4-propoxy-HPD and C4-ethoxy-HPD, known AI-2 analogues, has been developed. The optimised synthesis is efficient, reproducible and short. The (4*S*) enantiomer of C4-propoxy-HPD was the most active compound being approximately twice as efficient as (4*S*)-DPD and ten-times more potent than the (4*R*) enantiomer. Additionally, the specificity of this analogue to bacteria with LuxP receptors makes it a good candidate for clinical applications, because it is not susceptible to scavenging by LsrB-containing bacteria that degrade the natural AI-2. All in all, this study provides a new brief and effective synthesis of isomerically pure analogues for QS modulation that include the most active AI-2 agonist described so far.

1. Introduction

Cell-to-cell communication between bacteria, known as quorum sensing (QS) allows bacteria to sense the critical density of their population, and consequently synchronise their behaviour in order to adapt to changing environmental conditions [1]. QS is mediated by signalling molecules called autoinducers. Among autoinducers, autoinducer-2 (AI-2) is unique because it is recognised by many bacterial species, allowing bacteria from one species to sense and respond to the signal produced by other species [2,3]. AI-2 consists of (*S*)-4,5-dihydroxypentanedione ((4*S*)-DPD) **1**, the parent molecule and acyclic form, which in aqueous solution exists in a dynamic equilibrium with the two cyclic diastereomeric forms **2** and **3** that can be further hydrated to give tetrahydroxytetrahydrofuranes **4** and **5** (see Fig. 1). In the presence of boron, the *cis* borate diester **6** is formed. Even though these forms exist in equilibrium, each of the two known receptors for AI-2 is specific for only one of these different forms. LuxP receptors bind to (2*S*,4*S*)-THMF-borate **6** [4] and LsrB receptors bind to (2*R*,4*S*)-THMF **5** [5].

AI-2 regulates important bacterial processes, such as biofilm formation, toxin production and virulence, many of them implicated in human bacterial infections [2]. Thus, the discovery of new molecules for QS modulation has a huge potential for developing new strategies to treat bacterial infections without the risk of selecting for mechanisms of antibiotic resistance.

Several analogues of AI-2 have been described and some of them are highlighted in Fig. 2. None of the known DPD analogues are general agonists or antagonists, with different analogues having different activities in the assays with different receptors. Some of these analogues have been synthesised both in optically pure form and as racemic mixtures [6–12]. It has been shown that small structural differences in the analogues greatly influence their efficacy as QS modulators, with previous work demonstrating that the absolute configuration of these compounds is very important for QS activation. The configuration of DPD itself is very important for QS activity as its (4*R*) enantiomer is approximately 100 and 6 times less potent than the natural (4*S*)-DPD, for LuxP or LsrB responses, respectively [13,14].

In a previous work, we have synthesised DPD analogues with a new stereocenter at C-5 (4,5-dihydroxyhexanediones) [9]. These C5-

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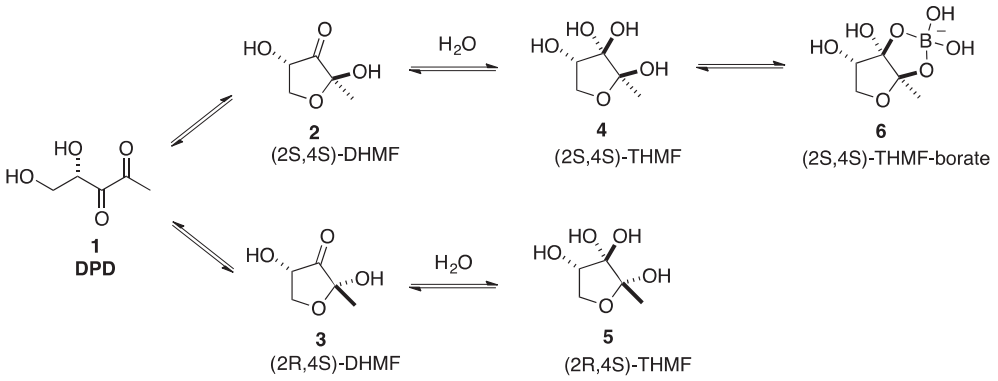


Fig. 1. Different forms of the AI-2 signal molecule in solution. The LuxP receptors bind the borated cyclic form – (2*S*,4*S*)-THMF-borate **6** – and the LsrB receptors bind the non-borated cyclic form – (2*R*,4*S*)-THMF **5**.

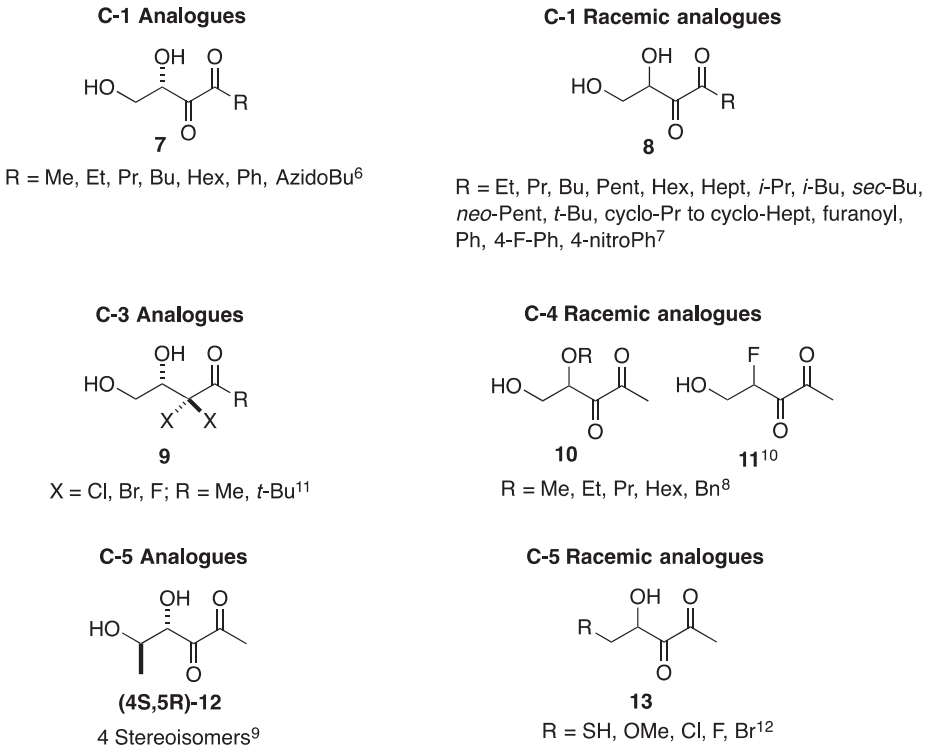
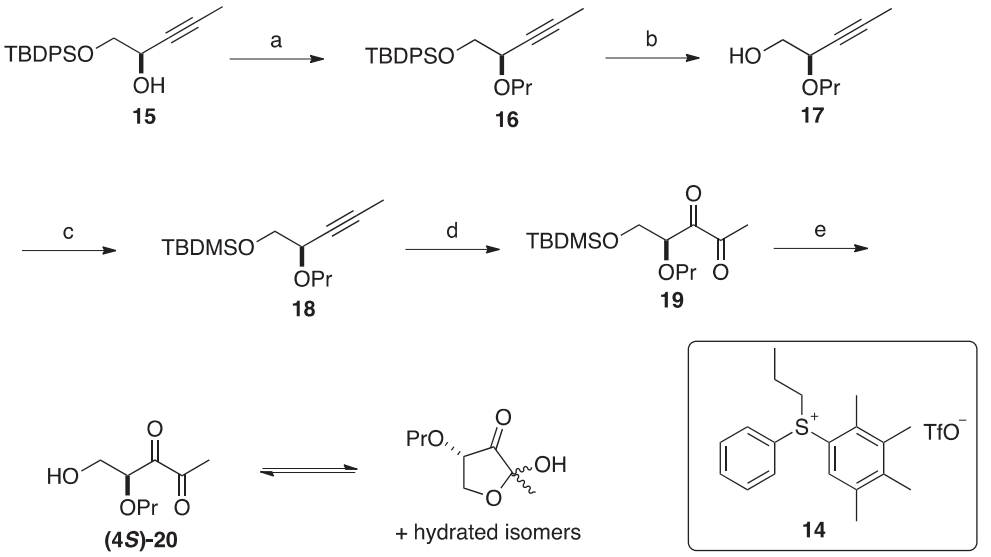


Fig. 2. Examples of AI-2 analogues previously studied.

analogues were synergistic agonists for LsrB and agonists for LuxP. The (4*S*,5*R*) **12** and (4*S*,5*S*)-isomers were clearly more active than the (4*R*)-isomers in the *Vibrio harveyi* assay (LuxP receptor), which reinforces the influence of the (4*S*) configuration in the QS activity of DPD analogues [9].

More recently, Tsuchikama et al reported a new family of DPD analogues, the C4-alkoxy-HPDs [8]. In this study, several racemic C-4 ethers **10** were synthesised, such as methyl, ethyl, propyl, hexyl, benzyl, using the corresponding alkyl halides and NaH. The QS modulator activity of these C4 ethers was tested using two reporter systems for the AI-2 receptors known: LsrB and LuxP. Interestingly, these analogues seemed to have specific agonist activity for LuxP receptors, as no activity in the LsrB reporter assay was detected. It was not possible to conclude if the analogues were able to bind LsrB because the *lsr*-dependent β-galactosidase reporter assay measures the expression of the *lsr* operon and not direct binding to LsrB. Intracellular phosphorylation of AI-2 by the kinase LsrK induces the start of the expression of the *lsr* operon by binding of AI-2-phosphate to the repressor protein [15,16]. However, the authors observed that the C4-hydroxy group is a critical factor for the LsrK-mediated phosphorylation, rendering the C4-hydroxy analogues unable to be phosphorylated [8]. Thus, the lack of phosphorylated DPD alone can be accountable for the lack of activity for the β-galactosidase LsrB-reporter assay.

From the analogues tested, C4-ethoxy-HPD and C4-propoxy-HPD were the most potent QS agonists and, significantly, were even more potent than DPD, with racemic C4-propoxy-HPD (rac-**20**) exhibiting the greatest LuxP-dependent QS activity. In pharmacology, it has been established that the use of the eutomer (more active isomer) rather than the racemic mixture is advantageous as only half the concentration is needed and there is no unbound distomer (less active isomer) free to act on other targets [17,18]. So, here we describe an optimised synthesis process for the pure *S*-enantiomer of C4-propoxy-HPD ((4*S*)-**20**) and C4-ethoxy-HPD analogues ((4*S*)-**25**). We took advantage of our previous work as we have previously reported the enantioselective syntheses of (4*R*)- and (4*S*)-DPD starting from methyl glycolate, where the optically enriched alcohol **15** was the key intermediate [13]. The optically pure enantiomer (4*S*)-**20** was the most efficient compound having approximately double the activity of DPD. Significantly, unlike DPD, this



a) **14**, NaH, DMF, 0°C/rt, 60%. b) TBAF, THF, rt, 71%. c) TBDMSCl, (*i*-Pr)$_2$NEt, DMAP, CH_2Cl_2,0°C/rt, 83%. d) $NaIO_4$, RuO_2, CCl_4/MeCN, H_2O, rt, 91%. e) D_2SO_4, DMSO-d_6/D_2O (1:4), rt.[8]

Scheme 1. Synthesis of **(4*S*)-20**.

agonist is not degraded by LsrB-harbouring bacteria, being a good clinical candidate for QS manipulation of bacteria with LuxP receptors.

2. Results and discussion

2.1. Chemical synthesis

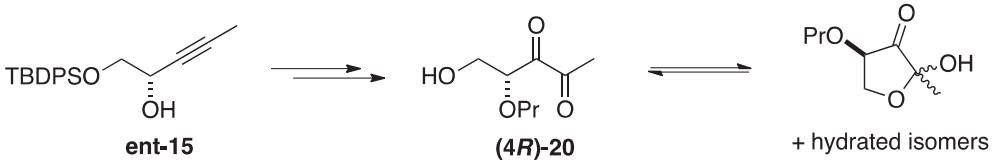
Having the ability to easily obtain both enantiomeric forms of **15** the optically enriched C4-propoxy DPD enantiomers were prepared (Schemes 1–3). The O-alkylation of racemic **15**, as reported by Tsuchikama et al, using sodium hydride and a propyl-1-halide afforded the propyl ether **16** in only 18% yield [8]. However, using *S*-1-propyl-*S*-phenyl-2,3,4,5-tetramethylphenylsulfonium triflate or tetrafluoroborate **14** as an alternative alkylating agent [19] the propyl ether **16** was obtained from **15** in a much better yield (60%, Scheme 1). Attempts to obtain the corresponding TBDMS protected alcohol **15** using our previous synthetic strategy failed [13], since selective hydrolysis of the TBDMS protected methyl glycolate to afford the TBDMS glycolic acid failed with lithium hydroxide and the TBDMS silyl ether was also removed. We therefore, proceeded with the TBPDPS protecting group for the rest of the synthesis, however, the cleavage of this bulkier silyl ether under acidic conditions at the end of the present synthesis was, as expected, more difficult than the cleavage of the less hindered TBDMS ether. Thus TBDPS protecting group was substituted for a TBDMS group before the final formation of the 1,2-dione moiety. Compound **16** was treated with TBAF in THF to afford alcohol **17** in 71% yield. Reprotection of **17** with TBDMSCl, diisopropylethylamine and DMAP afforded **18** in excellent yield (86%), with NMR data identical to the racemic product [8]. Oxidation of **18** with RuO_2/$NaIO_4$ as described earlier [8,13,14] afforded the diketone **19** (91% yield). Deprotection using deuterated sulfuric acid in deuterated DMSO and water [8] afforded (4*S*)-**20**. Similarly, alcohol ***ent*-15** was transformed into (4*R*)-**20** (Scheme 2)). The racemic C4-propoxy-HPD (rac-**20**) was also prepared for the biological assays, starting from racemic **15** obtained by reduction of the corresponding acetylenic ketone [13] with sodium borohydride.

In order to obtain the more active (4*S*)-**20** with higher enantiomeric excess, a different route was followed, as described in Scheme 3. The diol **21**, an intermediate of our DPD synthesis, was easily recrystallised to improve the e.e. to 99.84% [13]. The selective protection of the primary alcohol of **21** with TBDMSCl in pyridine afforded the monosylilated compound **22** in 72% yield. Alkylation using the sulfonium salt **14** as described above afforded the *O*-propyl alkylated product **18**, which was converted into (4*S*)-**20** following the steps described in Scheme 2. Alkylation of **22** with ethyl iodide following the procedure previously described in the literature [8], afforded **24** in 28% yield. In all alkylation reactions a minor product was observed in the NMR spectra, which we attributed to the migration of the silyl protecting group to the secondary hydroxyl group.

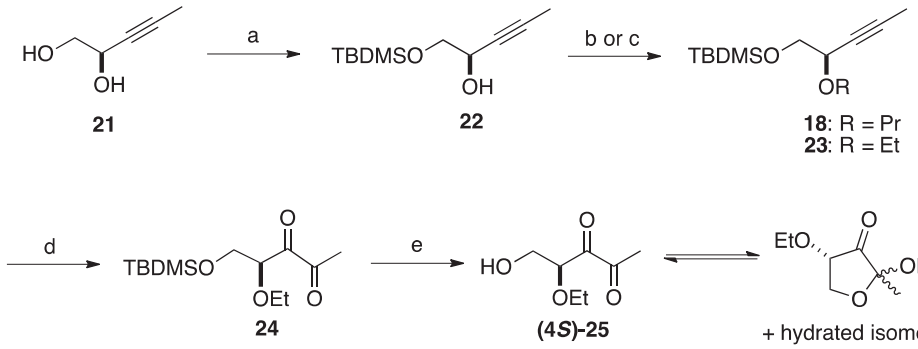
Oxidation with RuO_2/$NaIO_4$ followed by deprotection with deuterated sulfuric acid in deuterated DMSO and water [8] afforded enantiopure (4*S*)-**25** (Scheme 3).

2.2. Binding affinity of the DPD analogues to LuxP

The binding affinity of (4*S*)-**20**, (4*R*)-**20,** rac-**20** and (4*S*)-**25** compounds was assessed and compared to that of (4*S*)-DPD. To assess the affinity of these compounds to the LuxP receptor, a LuxP-FRET assay was performed. This assay uses a LuxP receptor that has a Cyan Fluorescent Protein (CFP) and a Yellow Fluorescent Protein (YFP) fused to each of its termini. A decrease in fluorescence resonance energy transfer is detected upon ligand binding to this modified LuxP, due to

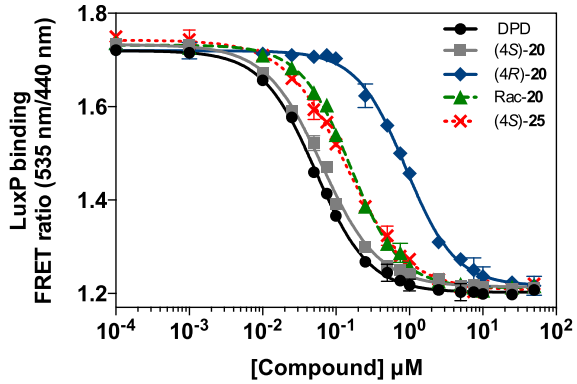


Scheme 2. Synthesis of **(4*R*)-20**.



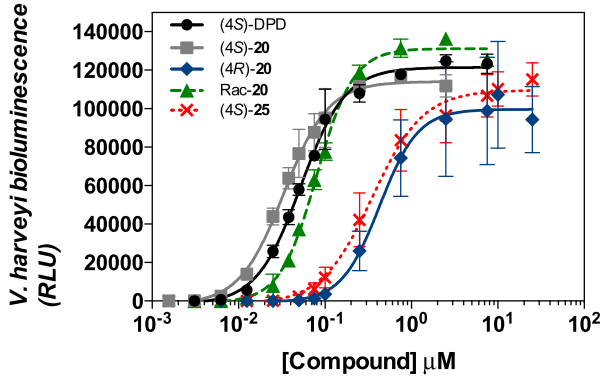
a) TBDMSCl, Pyr, DMAP, 0ºC/rt, 72%. b) **14**, NaH, DMF, 0ºC/rt, 60%. c) NaH, EtI, THF, 0ºC/rt, 28%.[8] d) $NaIO_4$, RuO_2, CCl_4/MeCN, H_2O, rt, 93%. e) D_2SO_4, DMSO-d_6/D_2O (1:4), rt.[8]

Scheme 3. Synthesis of **(4*S*)-25**.



Compound	Relative EC50 (µM)
(4*S*)-DPD	0.053 ± 0.001
(4*S*)-**20**	0.065 ± 0.002
(4*R*)-**20**	0.897 ± 0.034
Rac-**20**	0.157 ± 0.004
(4*S*)-**25**	0.137 ± 0.005

Fig. 3. Affinity of DPD and DPD analogues to LuxP. Values of half maximal effective concentration (EC50) were obtained from the fitted curves presented. A representative of two independent experiments is shown. Additional fitting parameters, and data from the repeated experiment, are shown in Table S1. Error bars represent the standard deviation of three technical replicates.



Compound	Relative EC50 (µM)
(4*S*)-DPD	0.053 ± 0.002
(4*S*)-**20**	0.034 ± 0.002
(4*R*)-**20**	0.429 ± 0.074
Rac-**20**	0.080 ± 0.002
(4*S*)-**25**	0.353 ± 0.038

Fig. 4. *In vivo* activity of the compounds tested. Bioluminescence produced by *V. harveyi* MM32 was measured in the presence of different concentrations of the tested compounds. A representative of 3 independent experiments is shown. The EC50 values were obtained from the fitted curves presented. Additional fitting parameters, and results from the repeats of this experiment, are shown in Table S2. Error bars represent the standard deviation of three technical replicates.

the increase in distance between the two termini [20,21]. The lowest the concentration able to induce this decrease, the better is the compound at binding LuxP.

Our results showed that (4*S*)-DPD and (4*S*)-**20** were the compounds with the highest affinity for LuxP (Fig. 3). The response curves and half maximal effective concentration (EC50) values for these compounds were very similar, showing that their affinity to bind LuxP was comparable. (4*R*)-**20** was the compound with lowest affinity having an EC50 more than ten-times higher than its enantiomer (4*S*)-**20**. This observation showed that the R-configured propoxy group on C4 was less favourable for binding to LuxP. Rac-**20** had an affinity in between the two enantiomers, as expected. (4*S*)-**25** also had high affinity for LuxP, with an EC50 value (0.137 ± 0.005) very similar to the one obtained for rac-**20** (0.157 ± 0.004) but higher than the one obtained for (4*S*)-DPD (0.053 ± 0.001). This was not expected since in the work of Tsuchikama et al. [8] the biological activity of the racemic mixture of this C4-ethoxy analogue was slightly higher than that of (4S)-DPD and lower than rac-**20**. Since no affinity measurement with the purified receptor was performed in that study, no direct comparisons can be made.

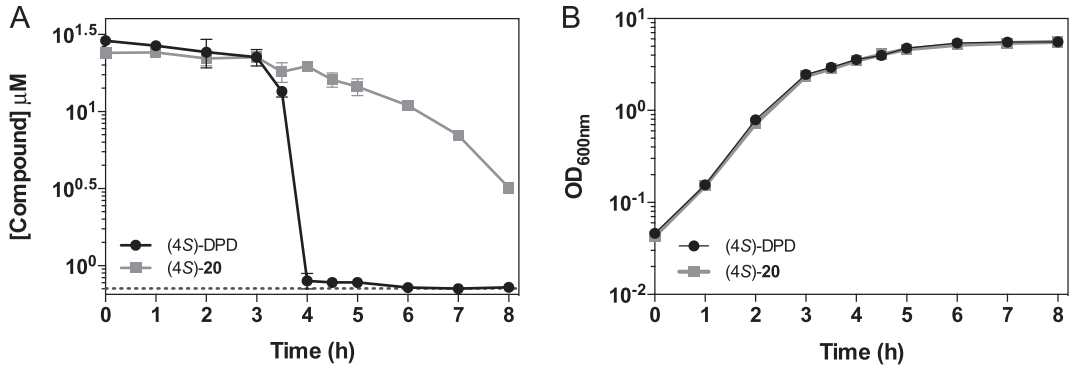


Fig. 5. Extracellular concentration of (4*S*)-DPD and (4*S*)-**20** in *E. coli* cultures. Cell-free culture fluids and bacterial suspension were collected at various time points to determine the extracellular concentration of the compounds (A) or the bacterial growth (B). A representative of three independent experiments is shown. Error bars represent the standard deviation of three technical replicates.

2.3. Biological activity of the DPD analogues

To assess the *in vivo* activity of the studied compounds, a *V. harveyi* bioassay was carried out. In *V. harveyi*, luminescence is produced in response to increasing concentrations of DPD. The MM32 *V. harveyi* strain, used in this assay, is a mutant that does not produce DPD, being, therefore, a good sensor to test the activity of DPD and its analogues. If an analogue is able to induce higher levels of light production at lower concentrations than DPD, then it is more active than the natural ligand.

Comparing the EC50 values obtained for all the compounds tested, the most active compound was (4*S*)-**20**, being approximately 2 times more active than (4*S*)-DPD (Fig. 4). (4*R*)-**20** was the least active compound. The racemic mixture of this analogue had an intermediate efficacy with an EC50 roughly two-times higher than (4*S*)-**20** (Fig. 4). The EC50 values obtained in the LuxP-FRET and *V. harveyi* assays were different. Nevertheless, the eudismic ratio, that reflects the difference in activity between the two enantiomers, is very similar in the two assays (13.8 ± 0.1 for LuxP-FRET and 12.6 ± 1.4 for *V. harveyi*) showing that in both assays the eutomer is approximately 10-fold more active than the distomer. (4*S*)-DPD and (4*S*)-**20** had a similar affinity towards the LuxP receptor (Fig. 3), but the latter seems to have a higher efficacy than (4*S*)-DPD *in vivo* (Fig. 4). Contrarily, (4*S*)-**25** that had a similar affinity as rac-**20**, was approximately four times less efficient *in vivo*. The difference in the EC50 of these analogues in the two assays are likely to be related to the different properties being assessed in these assays. In LuxP-FRET, only the affinity of the compounds to LuxP is determined, whereas in the *V. harveyi* bioassay we measure not only binding to LuxP, but also the *in vivo* effect of activating the QS network response to these compounds. Specifically, the EC50 values obtained with the *V. harveyi* assay could be related to different conformations that LuxP might assume upon binding to these different compounds. These potentially different conformations of LuxP could influence the affinity of this receptor to the membrane sensor histidine/kinase, leading to differences in the activation of the QS response and thus differences in the induction of light production. Additionally, possible differences in the stability of these analogues could also affect their *in vivo* efficacy.

The *V. harveyi* EC50 values for rac-**20**, (4*S*)-**20** and (4*S*)-**25** are in the expected order of magnitude given the EC50 values obtained for the racemic forms of C4-propoxy-HPD and C4-ethoxy-HPD obtained by Tsuchikama et al. [8]. However, the EC50 value obtained for (4*S*)-DPD in the present study is 20 times lower. Thus, although the analogues give somewhat similar values, our results suggest that these analogues are not so efficient when compared to (4*S*)-DPD. The differences observed could be due to the use of different quantification methods for the studied compounds. For this study NMR was used to try to overcome the errors in mass prediction or incomplete deprotection, since the final compounds are too unstable to be dried and thus are obtained and used in solution. Additionally, there are significant differences among the EC50 reported for DPD in different studies. Tsuchikama et al determined an EC50 of 1.07 µM for (4*S*)-DPD but EC50s of 0.87 µM and 0.25 µM have also been reported [8,12,22]. Moreover, in our previous work we obtained an EC50 of 0.076 µM for (4*S*)-DPD that is in the same order of magnitude as the ones obtained in the present work [9]. These differences are also likely to be potentiated by the intrinsic biological variability of the *V. harveyi* response. Therefore, we propose that, to compare the efficacy of different analogues, they should be tested on the same day with the same bacterial culture to determine relative biological responses between them. We have measured the activity of all the compounds studied here using the same culture of *V. harveyi* and repeated this assay on three different days. In all three experiments the relation between the EC50 obtained for the different compounds was the same (see results on Table S2 and Fig. S3). Importantly, our results support the major conclusion that the optically pure (4*S*)-**20** is the most potent agonist surpassing the efficacy of DPD. Moreover it has an eudismic ratio higher than 10.

2.4. Internalisation studies

In bacteria that possess LsrB receptors, DPD increases in the extracellular medium until a concentration threshold is reached. At this point, DPD is internalised and processed intracellularly [15,23]. To trap DPD inside the cell, a kinase – LsrK – phosphorylates DPD. Tsuchikama et al showed that C4-hydroxy analogues cannot be phosphorylated by LsrK, contrarily to DPD [8]. This led us to hypothesise that (4*S*)-**20** would not be degraded by LsrB-containing bacteria, as it cannot be trapped inside the cell by phosphorylation and should remain in the extracellular medium. To investigate this possibility, we determined the extracellular levels of (4*S*)-**20** and (4*S*)-DPD through time after adding 40 µM of these two compounds to actively growing cultures of *Escherichia coli*, a bacterium able to degrade AI-2 via a LsrB-dependent mechanism. To measure the quantities of the compounds added without the interference of DPD produced by the bacteria we used an *E. coli* strain unable to produce DPD (mutant for DPD synthase, LuxS).

Indeed, as previously observed, (4*S*)-DPD is readily depleted from the extracellular medium as *E. coli* reaches stationary phase [15]. Contrarily, (4*S*)-**20** remains in the extracellular medium (Fig. 5). These results show that, unlike DPD, the levels of (4*S*)-**20** are not significantly altered by bacteria with LsrB receptors. This could be an advantage for clinical applications, as it means that bacteria with LsrB receptors are not able to degrade this analogue. Thus, lower concentrations of analogue, in comparison with DPD, should be needed to induce a stable and durable QS activation. This might prove to be of importance to manipulate QS in pathogens such as *Vibrio cholerae*, for example. *V. cholerae* is the causative agent of cholera in humans and it is known that at high cell densities and hence, at high AI-2 and CAI-1 (cholerae

autoinducer-1) concentrations, virulence and biofilm formation are repressed [24]. So, addition of synthetic CAI-1 and AI-2 can decrease virulence in *V. cholerae*. Actually, it has been shown that addition of synthetic CAI-1 decreases the expression of toxic co-regulated pilus, a canonical virulence factor [25]. So, we propose that the addition of (4*S*)-**20** together with CAI-1 would be more advantageous than the addition of AI-2, both because (4*S*)-**20** is not labile to degradation by intestinal LsrB-containing bacteria like *E. coli,* and because of its higher efficacy inducing LuxP-mediated QS responses.

3. Conclusions

Both enantiomers of the C4-propoxy-HPD and the *S*-enantiomer of C4-ethoxy-HPD analogues were synthesised. The key O-alkylation reaction has been improved and affords yields of 60%, by using an electrophilic sulfonium salt instead of the alkyl iodide (in the case of C4-propoxy-HPD). These syntheses were short, efficient and reproducible. The *S*-enantiomer of C4-propoxy-HPD was the most efficient compound being almost twice as active as DPD and ten-times better than the *R*-enantiomer. Moreover, bacteria with LsrB receptors do not degrade this compound, making it a good candidate for clinical treatment of LuxP-harbouring bacteria, such as *V. cholerae* that expresses less virulence factors in the presence of high concentrations of DPD. The efficient synthesis of optically pure DPD analogues, here described, will allow the use of lower concentrations of compounds for modulation of QS, without contamination by the less active enantiomer.

4. Materials and methods

4.1. Chemical synthesis

4.1.1. Materials

^{1}H NMR spectra were obtained at 400 MHz in $CDCl_3$ or D_2O with chemical shift values (δ) in ppm downfield from tetramethylsilane in the case of $CDCl_3$, and ^{13}C NMR spectra were obtained at 100.61 MHz in $CDCl_3$. Assignments are supported by 2D correlation NMR studies. Medium pressure preparative column chromatography: silica gel Merck 60H. Analytical TLC: Aluminium-backed silica gel Merck 60 F254. Specific rotations ($[\alpha]_D^{20}$) were measured using an automatic polarimeter. Reagents and solvents were purified and dried according to the literature [26]. All reactions were carried out under an inert atmosphere (argon), except when the solvents were undried. The enantiomeric excesses were determined by HPLC on a Waters 600E/U6K instrument using a Daicel Chiralpack AD-H column.

4.1.2. Preparation of (2R)-1-(tert-Butyldiphenylsilyloxy)-2-propoxy-3-pentyne **16**

The protocol described in Ref. [17] was followed.

4.1.3. Preparation of (2R)-2-propoxypent-3-yn-1-ol **17**

To a solution of **16** (0.4 g, 1.05 mmol) in THF (4 mL), at rt, was added TBAF 1 M (1.16 mL, 1.15 mmol). After 1 h, water (5 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 4 mL), dried ($MgSO_4$), concentrated and the residue was purified by flash column chromatography (30/70 AcOEt/Hex). Alcohol **17** was obtained as a colourless oil (0.106 g, 71%). $[\alpha]_D^{20} = -81.3$ (c 0.98, CH_2Cl_2, 86% e.e.). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.11–4.07 (m, 1H), 3.73 (ddd, *J* = 8.1, 8.1, 15.4 Hz, 1H), 3.67 (d, *J* = 5.7 Hz, 2H), 3.36 (ddd, *J* = 7.2, 7.2, 14.4 Hz, 1H), 1.86 (s, 3H), 1.63 (m, 2H), 0.94 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 82.9, 75.4, 70.8, 70.6, 65.4, 22.7, 10.5, 3.5.

The same procedure afforded ***ent*-17**, starting from ***ent*-16** [13].

4.1.4. Preparation of (2R)-1-(tert-Butyldimethylsilyloxy)-2-propoxy-3-pentyne **18**

To a solution of alcohol **17** (0.161 g, 1.1 mmol) in CH_2Cl_2 (2 mL) was added (*i*-Pr)$_2$NEt (0.394 mL, 2.2 mmol), TBDMSCl (0.256 g, 1.8 mmol) and a catalytic amount of DMAP at 0 °C. The reaction mixture was stirred at rt overnight. Water was added (5 mL) and the mixture was extracted with CH_2Cl_2 (3 × 5 mL), dried ($MgSO_4$), concentrated and the residue was purified by flash column chromatography (10/90 AcOEt/Hex) to afford **18** [8] as a colourless oil (0.241 g, 83%). $[\alpha]_D^{20} = -39.7$ (c 1.5, CH_2Cl_2, 86% e.e.), $[\alpha]_D^{20} = -48.3$ (c 1.09, CH_2Cl_2, 99.84% e.e.). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.06–4.02 (m, 1H), 3.76–3.64 (m, 3H), 3.40–3.34 (m, 1H), 1.83 (d, *J* = 2.0 Hz, 3H), 1.63–1.58 (m, 3H), 0.94–0.90 (m, 3H), 0.89 (s, 9H), 0.079 (s, 3H), 0.073 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 82.0, 76.6, 71.3, 71.1, 66.6, 25.9, 22.8, 18.4, 10.5, 3.6, −5.1, −5.2.

The same procedure afforded ***ent*-18**, $[\alpha]_D^{20} = +40.9$ (c 1.7, CH_2Cl_2, 86% e.e.).

4.1.5. Preparation of (4S)-5-(tert-Butyldimethylsilyloxy)-4-propoxy-2,3-pentadione **19**

To compound **18** (0.096 g, 0.37 mmol) dissolved in CCl_4 (2.8 mL) and MeCN (2.8 mL) was added a solution of $NaIO_4$ (0.180 mg, 0.84 mmol) in H_2O (3 mL) and $RuO_2{\cdot}H_2O$ (1.2 mg, 0.009 mmol) and the reaction mixture was stirred vigorously until all starting material had been consumed (TLC). The mixture was extracted with AcOEt (3 × 15 mL), filtered by a very short silica pad and concentrated under vacuum to give the bright yellow oil **19** [8] (0.098 g, 91%). $[\alpha]_D^{20} = +26.2$ (c 1.0, CH_2Cl_2, 86% e.e.), $[\alpha]_D^{20} = +36.8$ (c 1.03, CH_2Cl_2, 99.84% e.e.), ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.68 (dd, *J* = 5.32, 5.32 Hz, 1H), 4.02 (dd, *J* = 5.9, 10.4 Hz, 1H), 3.84 (dd, *J* = 5.08, 10.4 Hz, 1H), 3.52–3.47 (m, 1H), 3.44–3.38 (m, 1H), 2.33 (s, 3H), 1.64–1.55 (m, 2H), 0.93–0.89 (m, 3H), 0.83 (s, 9H), 0.04 (s, 3H), 0.03 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.24, 199.20, 80.3, 72.9, 63.9, 25.7, 24.2, 22.9, 18.2, 10.3, −5.5, −5.6. HRMS calcd. for $C_{14}H_{30}O_5SiNa$ 329.1755, found 329.1753 (M + H_2O + Na).

The same procedure afforded ***ent*-19**, $[\alpha]_D^{20} = -28.3$ (c 1.2, CH_2Cl_2, 86% e.e.).

4.1.6. Preparation of (4S)-4-Propoxy-5-hydroxy-2,3-pentadione **20** and (R)-4-Propoxy-5-hydroxy-2,3-pentadione ent-**20**

The procedure described in Ref. [8] was followed. The characterisation data was the same as previously described in the same reference for the racemic compounds.

4.1.7. (2R)-1-(tert-Butyldimethylsilyloxy)-3-pentyn-2-ol **22**

To a solution of diol **21** [13] (0.265 g, 2.6 mmol) in pyridine (2 mL) was added TBDMSCl (0.358 g, 2.4 mmol) and a catalytic amount of DMAP at 0 °C. The reaction mixture was stirred at rt for 4 h. Water was added (5 mL) and the mixture was extracted with AcOEt (3 × 5 mL), dried ($MgSO_4$), concentrated and the residue was purified by flash column chromatography (10/90 to 30/70 AcOEt/Hex) to afford **22** as a colourless oil (0.406 g, 72%). $[\alpha]_D^{20} = -6.2$ (c 2.0, CH_2Cl_2, 99.84% e.e.). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.35–4.32 (m, 1H), 3.72 (dd, *J* = 10.0, 3.6 Hz, 1H), 3.58 (dd, *J* = 10.0, 7.6 Hz, 1H), 2.57 (d, *J* = 4.1 Hz, 1H), 1.82 (d, *J* = 2.1 Hz, 3H), 0.89 (s, 9H), 0.081 (s, 3H), 0.077 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 81.8, 67.3, 63.3, 25.8, 18.3, 3.5, −5.3.

4.1.8. (2R)-1-(tert-Butyldimethylsilyloxy)-2-ethoxy-3-pentyne **23**

The procedure described in Ref. [8] was followed. $[\alpha]_D^{20} = -37.2$ (c 1.14, CH_2Cl_2, 99.84% e.e.). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.07–4.03 (m, 1H), 3.81–3.67 (m, 3H), 3.52–3.44 (m, 1H), 1.83 (d, *J* = 2.1 Hz, 3H), 1.21 (t, *J* = 7.0 Hz, 3H), 0.89 (s, 9H), 0.078 (s, 3H), 0.074 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 82.0, 76.5, 71.2, 66.6, 64.7, 25.8, 18.4, 15.1, 3.6, −5.1, −5.2.

4.1.9. (4S)-5-(tert-Butyldimethylsilyloxy)-4-ethoxy-2,3-pentadione **24**

To compound **23** (0.015 g, 0.06 mmol) dissolved in CCl_4 (0.5 mL) and MeCN (0.5 mL) was added a solution of $NaIO_4$ (0.030 mg, 0.14 mmol) in H_2O (0.5 mL) and $RuO_2{\cdot}H_2O$ (0.18 mg, 0.0014 mmol)

and the reaction mixture was stirred vigorously until all starting material had been consumed (TLC). The mixture was extracted with AcOEt (3 × 10 mL), filtered by a very short silica pad and concentrated under vacuum to give the bright yellow oil **24** [8] (0.015 g, 88%). $[\alpha]_D^{20}$ = +32.8 (c 0.43, CH_2Cl_2, 99.84% e.e.). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.71 (dd, J = 5.4, 5.4 Hz, 1H), 4.01 (dd, J = 5.6, 10.4 Hz, 1H), 3.83 (dd, J = 5.2, 10.4 Hz, 1H), 3.61–3.57 (m, 1H), 3.54–3.39 (m, 1H), 2.33 (s, 3H), 1.21 (t, J = 7.0 Hz, 3H), 0.85 (s, 9H), 0.04 (s, 3H), 0.03 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.16, 199.15, 80.0, 66.6, 64.0, 25.7, 24.2, 18.2, 15.2, −5.5, −5.6.

4.1.10. (4S)-4-Ethoxy-5-hydroxy-2,3-pentadione **25**

The procedure described in Ref. [8] was followed. The characterisation data was the same as previously described for the racemic compound in the same reference.

4.2. Biological assays

All biological assays were performed with the enantiomer (4S)-**20** that was obtained with 99.84% of enantiomeric excess as previous tests showed no differences between this enantiomer and the one obtained with 86% enantiomeric excess.

4.2.1. LuxP-FRET assay

In vitro response of LuxP-FRET protein was measured as previously described [9,13], optimised for 96 well plate reading using a multilabel counter (1420 Victor 3, Perkin Elmer). Serial dilutions of test compounds were performed in MiliQ water and added to 12.5 μg ml^{-1} of CFP-LuxP-YFP chimeric protein in 25 mM of sodium phosphate buffer (pH 8.0), 35 mM NaCl, and 1 mM boric acid. Samples (2.5 μl) were added to 280 μl of reaction volume and FRET ratio was calculated (535/440 nm). Relative EC50 values were determined by fitting a four parameter logistic model in a custom equation created in GraphPad Prism version 6 (GraphPad Software, La Jolla, California, USA) to the dose-response curves obtained. Fitting parameters for all the curves obtained using LuxP-FRET assays are presented on Table S1.

4.2.2. Bioluminescence assay in Vibrio harveyi

V. harveyi in vivo response was measured using MM32 reporter strain grown in AB (autoinducer bioassay medium; detailed composition in supplementary information) as previously reported [9,13]. Serial dilutions of the tested compounds were performed in MiliQ water. Light emission was measured in a GloMax Explorer microplate luminometer (Promega, USA) after 5 h of incubation at 30 °C. Relative EC50 values were determined by fitting a four parameter logistic model in a custom equation created in GraphPad Prism version 6 (GraphPad Software, La Jolla, California, USA) to the dose-response curves obtained. The concentrations of each compound used were adjusted to have at least two concentrations before and after the linear slope [27]. To test the same concentration range for all the compounds, so that we could compare their EC50 values, all the compounds shared at least 9 out of the 12 concentrations. Fitting parameters for all the curves obtained using this bioassay are presented on Table S2.

4.2.3. Internalisation studies

E. coli KX1290 (Δ*luxS*) [15] was grown overnight in LB supplemented with 100 mM MOPS buffer, pH 7.0 at 37 °C, 240 rpm. Overnight cultures were diluted in fresh medium until an OD_{600nm} of approximately 0.05 and grown at 37 °C, 240 rpm. At the specified time points, bacterial suspensions were collected for growth assessment (at OD_{600nm}) and for compound detection. For compound detection, bacterial suspensions were filtered through multiscreen filter plates (Millipore) and stored at −20 °C, overnight. A LuxP-FRET assay was employed to detect DPD and DPD analogues following the protocol described above. To determine the concentration of compound in the cell-free supernatants, results were compared against a calibration curve obtained with different DPD concentrations.

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Appendix A. Supplementary material

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