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 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.

*Keywords:* AI-2; DPD; DPD analogues; DPD agonists; Quorum Sensing

1. Introduction

34

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.

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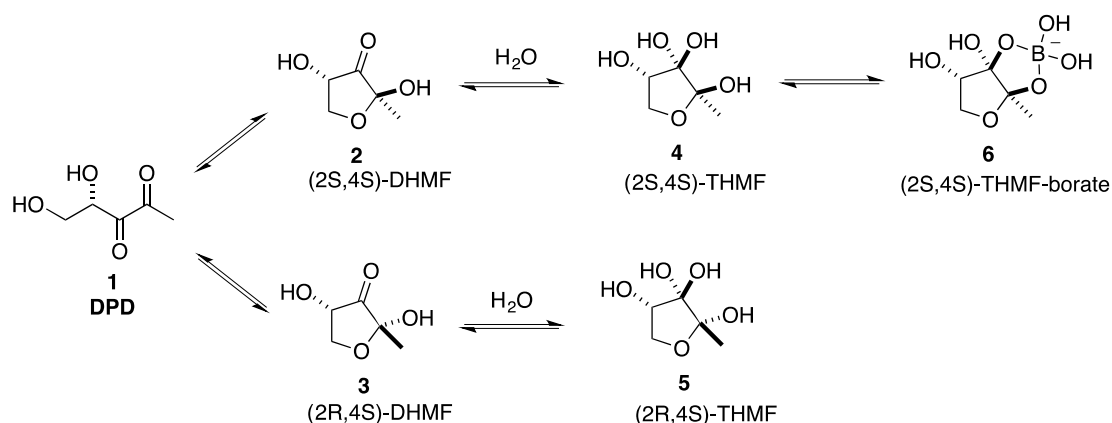
Fig. 1. Different forms of the AI-2 signal molecule in solution. The LuxP receptors bind the borated cyclic form – (2S,4S)-THMF-borate **6** – and the LsrB receptors bind the non-borated cyclic form – (2R,4S)-THMF **5**.

59

60   Several analogues of AI-2 have been described and some of them are highlighted in
61   Fig. 2. None of the known DPD analogues are general agonists or antagonists, with
62   different analogues having different activities in the assays with different receptors.
63   Some of these analogues have been synthesized both in optically pure form and as
64   racemic mixtures.[6-12] It has been shown that small structural differences in the
65   analogues greatly influence their efficacy as QS modulators, with previous work
66   demonstrating that the absolute configuration of these compounds is very important for
67   QS activation. The configuration of DPD itself is very important for QS activity as its
68   (4*R*) enantiomer is approximately 100 and 6 times less potent than the natural (4*S*)-
69   DPD, for LuxP or LsrB responses, respectively.[13,14]
70   In a previous work, we have synthesised DPD analogues with a new stereocenter at C-5
71   (4,5-dihydroxyhexanediones).[9] These C5-analogs were synergistic agonists for LsrB
72   and agonists for LuxP. The (4*S*,5*R*) **12** and (4*S*,5*S*)-isomers were clearly more active
73   than the (4*R*)-isomers in the *Vibrio harveyi* assay (LuxP receptor), which reinforces the
74   influence of the (4*S*) configuration in the QS activity of DPD analogs.[9]
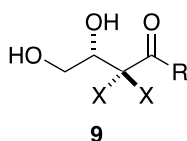75

C-1 Analogues

7
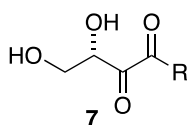
R = Me, Et, Pr, Bu, Hex, Ph, AzidoBu[6]

C-1 Racemic analogues

8
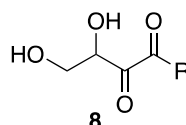
R = Et, Pr, Bu, Pent, Hex, Hept, *i*-Pr, *i*-Bu, *sec*-Bu,
neo-Pent, *t*-Bu, cyclo-Pr to cyclo-Hept, furanoyl,
Ph, 4-F-Ph, 4-nitroPh[7]

C-3 Analogues

9

X = Cl, Br, F; R = Me, *t*-Bu[11]

C-4 Racemic analogues

10   **11**[10]
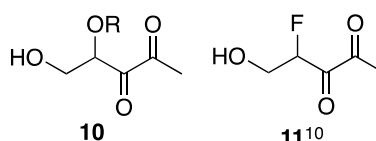
R = Me, Et, Pr, Hex, Bn[8]

C-5 Analogues

(4S,5R)-12
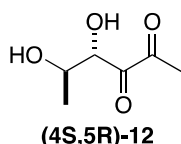
4 Stereoisomers[9]

C-5 Racemic analogues

13

R = SH, OMe, Cl, F, Br[12]

77   **Fig. 2.** Examples of AI-2 analogues previously studied.
78

79 More recently, Tsuchikama et al reported a new family of DPD analogues, the C4-
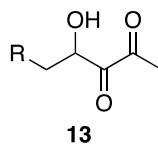80 alkoxy-HPDs.[8] In this study, several racemic C-4 ethers **10** were synthesised, such as
81 methyl, ethyl, propyl, hexyl, benzyl, using the corresponding alkyl halides and NaH.
82 The QS modulator activity of these C4 ethers was tested using two reporter systems for
83 the AI-2 receptors known: LsrB and LuxP. Interestingly, these analogues seemed to
84 have specific agonist activity for LuxP receptors, as no activity in the LsrB reporter
85 assay was detected. It was not possible to conclude if the analogues were able to bind
86 LsrB because the *lsr*-dependent β-galactosidase reporter assay measures the expression
87 of the *lsr* operon and not direct binding to LsrB. Intracellular phosphorylation of AI-2
88 by the kinase LsrK induces the start of the expression of the *lsr* operon by binding of
89 AI-2-phosphate to the repressor protein.[15,16] However, the authors observed that the C4-
90 hydroxy group is a critical factor for the LsrK-mediated phosphorylation, rendering the
91 C4-hydroxy analogues unable to be phosphorylated.[8] Thus, the lack of phosphorylated
92 DPD alone can be accountable for the lack of activity for the β-galactosidase LsrB-
93 reporter assay.
94 From the analogues tested, C4-ethoxy-HPD and C4-propoxy-HPD were the most
95 potent QS agonists and, significantly, were even more potent than DPD*,* with racemic
96 C4-propoxy-HPD (rac-**20**) exhibiting the greatest LuxP-dependent QS activity. In
97 pharmacology, it has been established that the use of the eutomer (more active isomer)
98 rather than the racemic mixture is advantageous as only half the concentration is needed
99 and there is no unbound distomer (less active isomer) free to act on other targets.[17,18]
100 So, here we describe an optimized synthesis process for the pure *S*-enantiomer of C4-
101 propoxy-HPD ((4*S*)-**20**) and C4-ethoxy-HPD analogues ((4*S*)-**25**). We took advantage
102 of our previous work as we have previously reported the enantioselective syntheses of
103 (4*R*)- and (4*S*)-DPD starting from methyl glycolate, where the optically enriched
104 alcohol **15** was the key intermediate.[13] The optically pure enantiomer (4*S*)-**20** was the
105 most efficient compound having approximately double the activity of DPD.
106 Significantly, unlike DPD, this agonist is not degraded by LsrB-harbouring bacteria,
107 being a good clinical candidate for QS manipulation of bacteria with LuxP receptors.
108
109 **2. Results and discussion**
110
111 *2.1. Chemical synthesis*
112

113   Having the ability to easily obtain both enantiomeric forms of **15** the optically enriched

114   C4-propoxy DPD enantiomers were prepared (schemes 1 to 3). The O-alkylation of

115   racemic **15**, as reported by Tsuchikama et al, using sodium hydride and a propyl-1-

116   halide afforded the propyl ether **16** in only 18% yield.[8] However, using *S*-1-propyl-*S*-

117   phenyl-2,3,4,5-tetramethylphenylsulfonium triflate or tetrafluoroborate **14** as an

118   alternative alkylating agent[19] the propyl ether **16** was obtained from **15** in a much better

119   yield (60%, scheme 1). Attempts to obtain the corresponding TBDMS protected alcohol

120   **15** using our previous synthetic strategy failed,[13] since selective hydrolysis of the

121   TBDMS protected methyl glycolate to afford the TBDMS glycolic acid failed with

122   lithium hydroxide and the TBDMS silyl ether was also removed. We therefore,

123   proceeded with the TBPDPS protecting group for the rest of the synthesis, however,

124   the cleavage of this bulkier silyl ether under acidic conditions at the end of the present

125   synthesis was, as expected, more difficult than the cleavage of the less hindered

126   TBDMS ether. Thus TBDPS protecting group was substituted for a TBDMS group

127   before the final formation of the 1,2-dione moiety. Compound **16** was treated with

128   TBAF in THF to afford alcohol **17** in 71% yield. Reprotection of **17** with TBDMSCl,

129   diisopropylethylamine and DMAP afforded **18** in excellent yield (86%), with NMR

130   data identical to the racemic product.[8] Oxidation of **18** with $RuO_2/NaIO_4$ as described

131   earlier[8,13,14] afforded the diketone **19** (91% yield). Deprotection using deuterated

132   sulfuric acid in deuterated DMSO and water[8] afforded (4*S*)-**20**. Similarly, alcohol **ent-**

133   **15** was transformed into (4*R*)-**20** (scheme 2)). The racemic C4-propoxy-HPD (rac-**20**)

134   was also prepared for the biological assays, starting from racemic **15** obtained by

135   reduction of the corresponding acetylenic ketone[13] with sodium borohydride.

136   In order to obtain the more active (4*S*)-**20** with higher enantiomeric excess, a different

137   route was followed, as described in scheme 3. The diol **21**, an intermediate of our DPD

138   synthesis, was easily recrystallized to improve the e.e. to 99.84%.[13] The selective

139   protection of the primary alcohol of **21** with TBDMSCl in pyridine afforded the

140   monosylilated compound **22** in 72% yield. Alkylation using the sulfonium salt **14** as

141   described above afforded the *O*-propyl alkylated product **18**, which was converted into

142   (4*S*)-**20** following the steps described in scheme 2. Alkylation of **22** with ethyl iodide

143   following the procedure previously described in the literature,[8] afforded **24** in 28%

144   yield. In all alkylation reactions a minor product was observed in the NMR spectra,

145   which we attributed to the migration of the silyl protecting group to the secondary

146   hydroxyl group.

147   Oxidation with $RuO_2/NaIO_4$ followed by deprotection with deuterated sulfuric acid in

148   deuterated DMSO and water[8] afforded enantiopure (4*S*)-**25** (scheme 3).
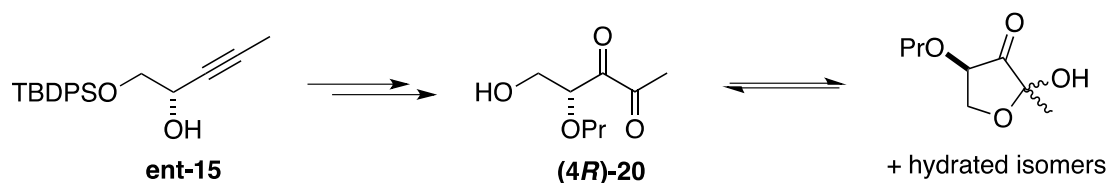
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150   **Scheme 1.**
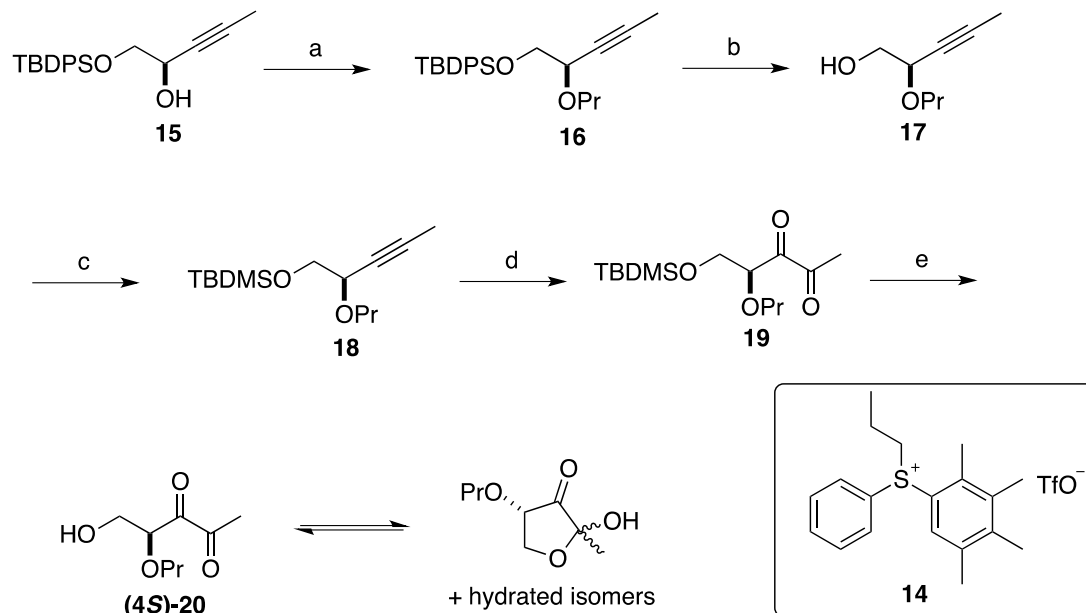
a) **14**, NaH, DMF, 0°C/rt, 60%. b) TBAF, THF, rt, 71%. c) TBDMSCl, (*i*-Pr)$_2$NEt, DMAP, CH$_2$Cl$_2$,0°C/rt,
151   83%. d) NaIO$_4$, RuO$_2$, CCl$_4$/MeCN, H$_2$O, rt, 91%. e) D$_2$SO$_4$, DMSO-d$_6$/D$_2$O (1:4), rt.[8]
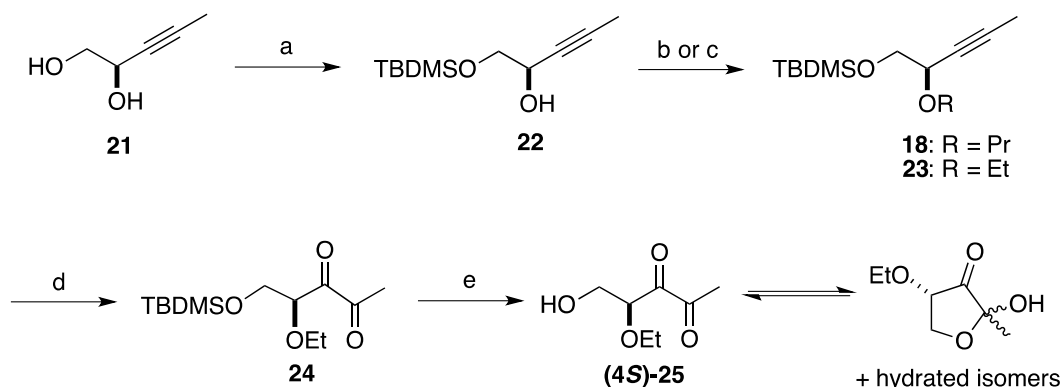
152

153   **Scheme 2.**

155

156   **Scheme 3.**

a) TBDMSCl, Pyr, DMAP, 0°C/rt, 72%. b) **14**, NaH, DMF, 0°C/rt, 60%. c) NaH, EtI, THF, 0°C/rt, 28%.[8] d) NaIO$_4$, RuO$_2$, CCl$_4$/MeCN, H$_2$O, rt, 93%. e) D$_2$SO$_4$, DMSO-d$_6$/D$_2$O (1:4), rt.[8]
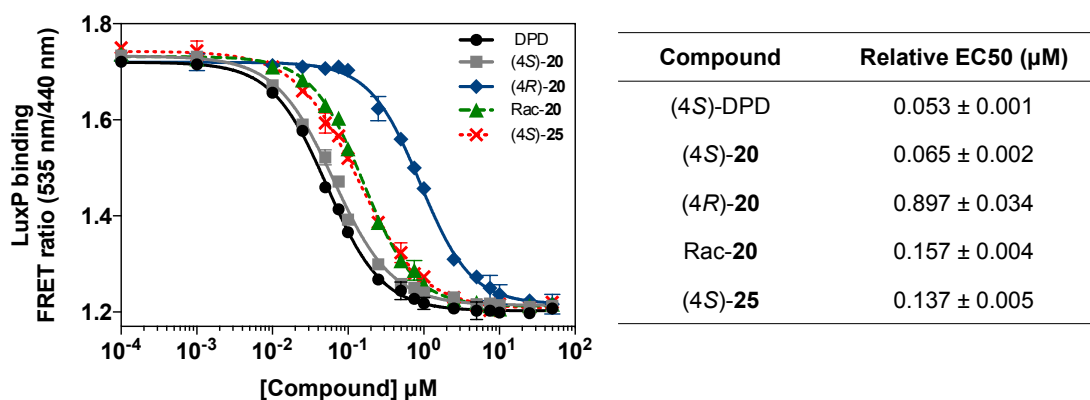
2.2. Binding affinity of the DPD analogues to LuxP

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 the increase in distance between the two termini.[20,22] The lowest the concentration able to induce this decrease, the better is the compound at binding LuxP.

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

174 the repeated experiment, are shown in table S1. Error bars represent the standard
175 deviation of three technical replicates.

176

177 Our results showed that (4*S*)-DPD and (4*S*)-**20** were the compounds with the highest
178 affinity for LuxP (Fig. 3). The response curves and half maximal effective
179 concentration (EC50) values for these compounds were very similar, showing that their
180 affinity to bind LuxP was comparable. (4*R*)-**20** was the compound with lowest affinity
181 having an EC50 more than ten-times higher than its enantiomer (4*S*)-**20**. This
182 observation showed that the R-configured propoxy group on C4 was less favourable for
183 binding to LuxP. Rac-**20** had an affinity in between the two enantiomers, as expected.
184 (4*S*)-**25** also had high affinity for LuxP, with an EC50 value (0.137 ± 0.005) very similar
185 to the one obtained for rac-**20** (0.157 ± 0.004) but higher than the one obtained for (4*S*)-
186 DPD (0.053 ± 0.001). This was not expected since in the work of Tsuchikama et al[8] the
187 biological activity of the racemic mixture of this C4-ethoxy analogue was slightly
188 higher than that of (4S)-DPD and lower than rac-**20**. Since no affinity measurement
189 with the purified receptor was performed in that study, no direct comparisons can be
190 made.

191

192 *2.3. Biological activity of the DPD analogues*

193

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

200

Compound	Relative EC50 (µM)
(4S)-DPD	0.053 ± 0.002
(4S)-20	0.034 ± 0.002
(4R)-20	0.429 ± 0.074
Rac-20	0.080 ± 0.002
(4S)-25	0.353 ± 0.038
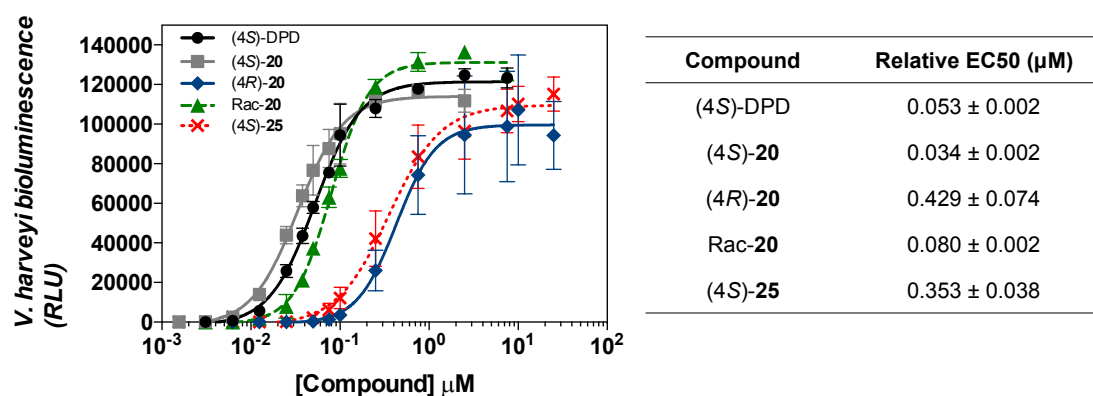
Fig. 4. *In vivo* activity of the compounds tested. Bioluminescence produced by *V. harveyi* MM32 was measured in the presence of different concentrations of the tested compounds. A representative of 3 independent experiments is shown. The EC50 values were obtained from the fitted curves presented. Additional 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.

Comparing the EC50 values obtained for all the compounds tested, the most active compound was (4S)-**20**, being approximately 2 times more active than (4S)-DPD (Fig. 4). (4R)-**20** was the least active compound. The racemic mixture of this analogue had an intermediate efficacy with an EC50 roughly two-times higher than (4S)-**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. (4S)-DPD and (4S)-**20** had a similar affinity towards the LuxP receptor (Fig. 3), but the latter seems to have a higher efficacy than (4S)-DPD in vivo (Fig. 4). Contrarily, (4S)-**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

228   affinity of this receptor to the membrane sensor histidine/kinase, leading to differences

229   in the activation of the QS response and thus differences in the induction of light

230   production. Additionally, possible differences in the stability of these analogues could

231   also affect their *in vivo* efficacy.

232   The *V. harveyi* EC50 values for rac-**20**, (4*S*)-**20** and (4*S*)-**25** are in the expected order

233   of magnitude given the EC50 values obtained for the racemic forms of C4-propoxy-

234   HPD and C4-ethoxy-HPD obtained by Tsuchikama et al.[8] However, the EC50 value

235   obtained for (4*S*)-DPD in the present study is 20 times lower. Thus, although the

236   analogues give somewhat similar values, our results suggest that these analogues are

237   not so efficient when compared to (4*S*)-DPD. The differences observed could be due to

238   the use of different quantification methods for the studied compounds. For this study

239   NMR was used to try to overcome the errors in mass prediction or incomplete

240   deprotection, since the final compounds are too unstable to be dried and thus are

241   obtained and used in solution. Additionally, there are significant differences among the

242   EC50 reported for DPD in different studies. Tsuchikama et al determined an EC50 of

243   1.07 μM for (4*S*)-DPD but EC50s of 0.87 μM and 0.25 μM have also been

244   reported.[8,12,23] Moreover, in our previous work we obtained an EC50 of 0.076 μM for

245   (4*S*)-DPD that is in the same order of magnitude as the ones obtained in the present

246   work.[9] These differences are also likely to be potentiated by the intrinsic biological

247   variability of the *V. harveyi* response. Therefore, we propose that, to compare the

248   efficacy of different analogues, they should be tested on the same day with the same

249   bacterial culture to determine relative biological responses between them. We have

250   measured the activity of all the compounds studied here using the same culture of *V.*

251   *harveyi* and repeated this assay on three different days. In all three experiments the

252   relation between the EC50 obtained for the different compounds was the same (see

253   results on table S2 and Fig. S3). Importantly, our results support the major conclusion

254   that the optically pure (4*S*)-**20** is the most potent agonist surpassing the efficacy of DPD.

255   Moreover it has an eudismic ratio higher than 10.

256

257   *2.4. Internalisation studies*

258

259   In bacteria that possess LsrB receptors, DPD increases in the extracellular medium until

260   a concentration threshold is reached. At this point, DPD is internalised and processed

261   intracellularly.[15,24] To trap DPD inside the cell, a kinase – LsrK- phosphorylates DPD.

262   Tsuchikama et al showed that C4-hydroxy analogues cannot be phosphorylated by

263   LsrK, contrarily to DPD.[8] This led us to hypothesise that (4*S*)-**20** would not be degraded

264   by LsrB-containing bacteria, as it cannot be trapped inside the cell by phosphorylation

265   and should remain in the extracellular medium. To investigate this possibility, we

266   determined the extracellular levels of (4*S*)-**20** and (4*S*)-DPD through time after adding

267   40 μM of these two compounds to actively growing cultures of *Escherichia coli*, a

268   bacterium able to degrade AI-2 via a LsrB-dependent mechanism. To measure the

269   quantities of the compounds added without the interference of DPD produced by the

270   bacteria we used an *E. coli* strain unable to produce DPD (mutant for DPD synthase,

271   LuxS).

272
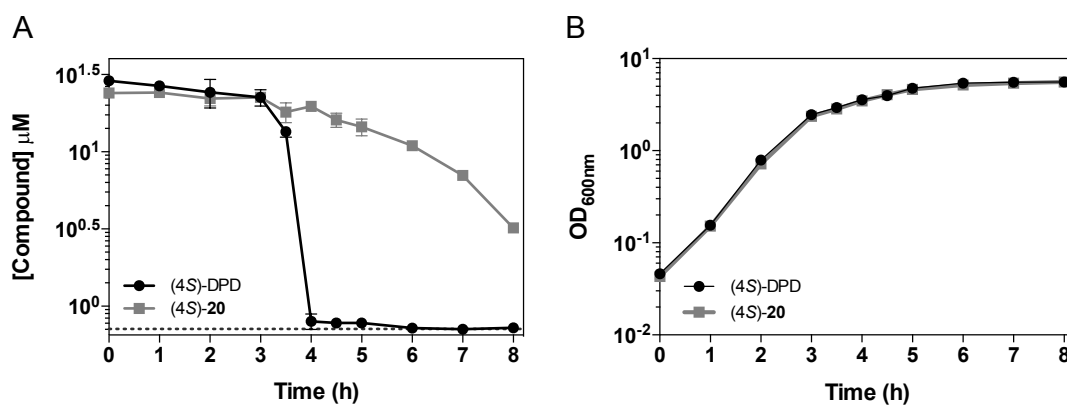
273

274   **Fig. 5.** Extracellular concentration of (4*S*)-DPD and (4*S*)-**20** in *E. coli* cultures. Cell-

275   free culture fluids and bacterial suspension were collected at various time points to

276   determine the extracellular concentration of the compounds (A) or the bacterial growth

277   (B). A representative of three independent experiments is shown. Error bars represent

278   the standard deviation of three technical replicates.

279

280   Indeed, as previously observed, (4*S*)-DPD is readily depleted from the extracellular

281   medium as *E. coli* reaches stationary phase.[15] Contrarily, (4*S*)-**20** remains in the

282   extracellular medium (Fig. 5). These results show that, unlike DPP, the levels of (4*S*)-

283   **20** are not significantly altered by bacteria with LsrB receptors. This could be an

284   advantage for clinical applications, as it means that bacteria with LsrB receptors are not

285   able to degrade this analogue. Thus, lower concentrations of analogue, in comparison

286   with DPD, should be needed to induce a stable and durable QS activation. This might

287   prove to be of importance to manipulate QS in pathogens such as *Vibrio cholerae*, for

288   example. *V. cholerae* is the causative agent of cholera in humans and it is known that

289   at high cell densities and hence, at high AI-2 and CAI-1 (cholerae autoinducer-1)

290   concentrations, virulence and biofilm formation are repressed.[25] So, addition of

291   synthetic CAI-1 and AI-2 can decrease virulence in *V. cholerae*. Actually, it has been

292   shown that addition of synthetic CAI-1 decreases the expression of toxic co-regulated

293   pilus, a canonical virulence factor.[26] So, we propose that the addition of (4*S*)-**20**

294   together with CAI-1 would be more advantageous than the addition of AI-2, both

295   because (4*S*)-**20** is not labile to degradation by intestinal LsrB-containing bacteria like

296   *E. coli,* and because of its higher efficacy inducing LuxP-mediated QS responses.

297

298   **3. Conclusions**

299   Both enantiomers of the C4-propoxy-HPD and the *S*-enantiomer of C4-ethoxy-HPD

300   analogues were synthesised. The key O-alkylation reaction has been improved and

301   affords yields of 60%, by using an electrophilic sulfonium salt instead of the alkyl

302   iodide (in the case of C4-propoxy-HPD). These syntheses were short, efficient and

303   reproducible. The *S*-enantiomer of C4-propoxy-HPD was the most efficient compound

304   being almost twice as active as DPD and ten-times better than the *R*-enantiomer.

305   Moreover, bacteria with LsrB receptors do not degrade this compound, making it a

306   good candidate for clinical treatment of LuxP-harbouring bacteria, such as *V. cholerae*

307   that expresses less virulence factors in the presence of high concentrations of DPD. The

308   efficient synthesis of optically pure DPD analogues, here described, will allow the use

309   of lower concentrations of compounds for modulation of QS, without contamination by

310   the less active enantiomer.

311

312   **4. Materials and methods**

313

314   *4.1. Chemical synthesis*

315

316   *4.1.1. Materials*

317   ^{1}H NMR spectra were obtained at 400 MHz in $CDCl_3$ or D_2O with chemical shift values

318   (δ) in ppm downfield from tetramethylsilane in the case of $CDCl_3$, and ^{13}C NMR spectra

319   were obtained at 100.61 MHz in $CDCl_3$. Assignments are supported by 2D correlation

320   NMR studies. Medium pressure preparative column chromatography: silica gel Merck

321   60H. Analytical TLC: Aluminium-backed silica gel Merck 60 F254. Specific rotations

322 ($[\alpha]^{20}_D$) were measured using an automatic polarimeter. Reagents and solvents were

323 purified and dried according to the literature.[27] All reactions were carried out under an

324 inert atmosphere (argon), except when the solvents were undried. The enantiomeric

325 excesses were determined by HPLC on a Waters 600E/U6K instrument using a Daicel

326 Chiralpack AD-H column.

327

328 *4.1.2. Preparation of (2R)-1-(tert-Butyldiphenylsilyloxy)-2-propoxy-3-pentyne 16*

329 The protocol described in ref. 17 was followed.

330

331 *4.1.3. Preparation of (2R)-2-propoxypent-3-yn-1-ol 17.*

332 To a solution of **16** (0.4 g, 1.05 mmol) in THF (4 mL), at rt, was added TBAF 1M (1.16

333 mL, 1.15 mmol). After 1 h, water (5 mL) was added and the mixture was extracted with

334 CH_2Cl_2 (3 x 4 mL), dried ($MgSO_4$), concentrated and the residue was purified by flash

335 column chromatography (30/70 AcOEt/Hex). Alcohol **17** was obtained as a colourless

336 oil (0.106 g, 71%). $[\alpha]_D^{20}$ = -81.3 (c 0.98, CH_2Cl_2, 86% e.e.). ^{1}H NMR (400 MHz,

337 $CDCl_3$): 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,

338 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

339 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.

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

341

342 *4.1.4. Preparation of (2R)-1-(tert-Butyldimethylsilyloxy)-2-propoxy-3-pentyne 18.*

343 To a solution of alcohol **17** (0.161 g, 1.1 mmol) in CH_2Cl_2 (2 mL) was added (i-Pr)$_2$NEt

344 (0.394 mL, 2.2 mmol), TBDMSCl (0.256 g, 1.8 mmol) and a catalytic amount of

345 DMAP at 0 ºC. The reaction mixture was stirred at rt overnight. Water was added (5

346 mL) and the mixture was extracted with CH_2Cl_2 (3 x 5 mL), dried ($MgSO_4$),

347 concentrated and the residue was purified by flash column chromatography (10/90

348 AcOEt/Hex) to afford **18**[8] as a colourless oil (0.241 g, 83%). $[\alpha]_D^{20}$ = -39.7 (c 1.5,

349 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,

350 $CDCl_3$): 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

351 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,

352 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,

353 3.6, -5.1, -5.2.

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

355

356 *4.1.5. Preparation of (4S)-5-(tert-Butyldimethylsilyloxy)-4-propoxy-2,3-pentadione* **19**.

357 To compound **18** (0.096 g, 0.37 mmol) dissolved in CCl_4 (2.8 mL) and MeCN (2.8 mL)

358 was added a solution of $NaIO_4$ (0.180 mg, 0.84 mmol) in H_2O (3 mL) and $RuO_2.H_2O$

359 (1.2 mg, 0.009 mmol) and the reaction mixture was stirred vigorously until all starting

360 material had been consumed (TLC). The mixture was extracted with AcOEt (3 x 15

361 mL), filtered by a very short silica pad and concentrated under vacuum to give the bright

362 yellow oil **19**[8] (0.098 g, 91%). $[\alpha]_D^{20} = +26.2$ (c 1.0, CH_2Cl_2, 86% e.e.), $[\alpha]_D^{20} = +36.8$

363 (c 1.03, CH_2Cl_2, 99.84% e.e.),. ^1H NMR (400 MHz, $CDCl_3$): δ 4.68 (dd, $J = 5.32$, 5.32

364 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

365 (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

366 (s, 9H), 0.04 (s, 3H), 0.03 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.24, 199.20, 80.3,

367 72.9, 63.9, 25.7, 24.2, 22.9, 18.2, 10.3, -5.5, -5.6. HRMS calcd. for $C_{14}H_{30}O_5SiNa$

368 329.1755, found 329.1753 ($M + H_2O + Na$).

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

370

371 *4.1.6. Preparation of (4S)-4-Propoxy-5-hydroxy-2,3-pentadione* **20** *and (R)-4-*

372 *Propoxy-5-hydroxy-2,3-pentadione ent-***20**.

373 The procedure described in ref. 8 was followed. The characterisation data was the same

374 as previously described in the same reference for the racemic compounds.

375

376 *4.1.7. (2R)-1-(tert-Butyldimethylsilyloxy)-3-pentyn-2-ol* **22**.

377 To a solution of diol **21**[13] (0.265 g, 2.6 mmol) in pyridine (2 mL) was added TBDMSCl

378 (0.358 g, 2.4 mmol) and a catalytic amount of DMAP at 0 ºC. The reaction mixture was

379 stirred at rt for 4h. Water was added (5 mL) and the mixture was extracted with AcOEt

380 (3 x 5 mL), dried ($MgSO_4$), concentrated and the residue was purified by flash column

381 chromatography (10/90 to 30/70 AcOEt/Hex) to afford **22** as a colourless oil (0.406 g,

382 72%). $[\alpha]_D^{20} = -6.2$ (c 2.0, CH_2Cl_2, 99.84% e.e.). ^1H NMR (400 MHz, $CDCl_3$): δ 4.35-

383 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,

384 $J = 4.1$ Hz, 1H), 1.82 (d, $J = 2.1$ Hz, 3H), 0.89 (s, 9H), 0.081 (s, 3H), 0.077 (s, 3H). ^{13}C

385 NMR (100 MHz, $CDCl_3$): δ 81.8, 67.3, 63.3, 25.8, 18.3, 3.5, -5.3.

386

387 *4.1.8. (2R)-1-(tert-Butyldimethylsilyloxy)-2-ethoxy-3-pentyne* **23**.

388 The procedure described in ref. 8 was followed. $[\alpha]_D^{20} = -37.2$ (c 1.14, CH_2Cl_2, 99.84%

389 e.e.). ^1H NMR (400 MHz, $CDCl_3$): δ 4.07-4.03 (m, 1H), 3.81-3.67 (m, 3H), 3.52-3.44

390 (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),

391 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,

392 15.1, 3.6, -5.1, -5.2.

393

394 *4.1.9. (4S)-5-(tert-Butyldimethylsilyloxy)-4-ethoxy-2,3-pentadione **24***.

395 To compound **23** (0.015 g, 0.06 mmol) dissolved in CCl$_4$ (0.5 mL) and MeCN (0.5 mL)

396 was added a solution of NaIO$_4$ (0.030 mg, 0.14 mmol) in H$_2$O (0.5 mL) and RuO$_2$.H$_2$O

397 (0.18 mg, 0.0014 mmol) and the reaction mixture was stirred vigorously until all

398 starting material had been consumed (TLC). The mixture was extracted with AcOEt (3

399 x 10 mL), filtered by a very short silica pad and concentrated under vacuum to give the

400 bright yellow oil **24**[8] (0.015 g, 88%). $[\alpha]_D^{20}$ = +32.8 (c 0.43, CH$_2$Cl$_2$, 99.84% e.e.). ^{1}H

401 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,

402 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,

403 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

404 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.

405

406 *4.1.10. (4S)-4-Ethoxy-5-hydroxy-2,3-pentadione **25***.

407 The procedure described in ref. 8 was followed. The characterisation data was the same

408 as previously described for the racemic compound in the same reference.

409

410 *4.2. Biological assays*

411

412 All biological assays were performed with the enantiomer (4S)-**20** that was obtained

413 with 99.84% of enantiomeric excess as previous tests showed no differences between

414 this enantiomer and the one obtained with 86% enantiomeric excess.

415

416 *4.2.1. LuxP-FRET assay*

417 *In vitro* response of LuxP-FRET protein was measured as previously described,[9,13]

418 optimized for 96 well plate reading using a multilabel counter (1420 Victor 3, Perkin

419 Elmer). Serial dilutions of test compounds were performed in MiliQ water and added

420 to 12.5 µg ml^{-1} of CFP-LuxP-YFP chimeric protein in 25mM of sodium phosphate

421 buffer (pH 8.0), 35mM NaCl, and 1 mM boric acid. Samples (2.5 µl) were added to 280

422 µl of reaction volume and FRET ratio was calculated (535/440 nm). Relative EC50

423 values were determined by fitting a four parameter logistic model in a custom equation

424   created in GraphPad Prism version 6 (GraphPad Software, La Jolla, California,USA)

425   to the dose-response curves obtained. Fitting parameters for all the curves obtained

426   using LuxP-FRET assays are presented on table S1.

427

428   *4.2.2. Bioluminescence assay in Vibrio harveyi*

429   *V. harveyi in vivo* response was measured using MM32 reporter strain grown in AB

430   (autoinducer bioassay medium; detailed composition in supplementary information) as

431   previously reported.[9,13] Serial dilutions of the tested compounds were performed in

432   MiliQ water. Light emission was measured in a GloMax Explorer microplate

433   luminometer (Promega, USA) after 5h of incubation at 30°C. Relative EC50 values

434   were determined by fitting a four parameter logistic model in a custom equation created

435   in GraphPad Prism version 6 (GraphPad Software, La Jolla, California,USA) to the

436   dose-response curves obtained. The concentrations of each compound used were

437   adjusted to have at least two concentrations before and after the linear slope.[28] To test

438   the same concentration range for all the compounds, so that we could compare their

439   EC50 values, all the compounds shared at least 9 out of the 12 concentrations. Fitting

440   parameters for all the curves obtained using this bioassay are presented on table S2.

441

442   *4.2.3. Internalisation studies*

443   *E. coli* KX1290 (Δ*luxS*)[15] was grown overnight in LB supplemented with 100 mM

444   MOPS buffer, pH 7.0 at 37ºC, 240 rpm. Overnight cultures were diluted in fresh

445   medium until an OD_{600nm} of approximately 0.05 and grown at 37ºC, 240 rpm. At the

446   specified time points, bacterial suspensions were collected for growth assessment (at

447   OD_{600nm}) and for compound detection. For compound detection, bacterial suspensions

448   were filtered through multiscreen filter plates (Millipore) and stored at -20ºC, overnight.

449   A LuxP-FRET assay was employed to detect DPD and DPD analogues following the

450   protocol described above. To determine the concentration of compound in the cell-free

451   supernatants, results were compared against a calibration curve obtained with different

452   DPD concentrations.

453

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513

514

Supplementary material for:

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

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This file includes:

Autoinducer bioassay medium composition;

Methods for the determination of enantiomeric excess;

Figures S1 to S3;

Tables S1 and S2;

References.

Autoinducer bioassay (AB) medium composition

The medium was prepared as previously[1]. AB medium consists of 0.3 M NaCl, 0.05 M $MgSO_4$, and 0.2% (w/v) vitamin-free casamino acids adjusted to pH 7.5 with KOH. After sterilization, the medium was allowed to cool and 10 ml of sterile 1 M potassium phosphate (pH 7.0), 10 ml of 0.1 M L-arginine and 20 ml of glycerol 50% (v/v) per liter were added.

Determination of enantiomeric excess

The determination of the enantiomeric excess was performed by HPLC, using the method described in reference 2, by converting the racemic and optically active diols **21** into the corresponding dibenzoates. The conditions employed and the chromatograms obtained are detailed below.

Conditions:

HPLC: Waters Alliance e2695/Waters 2998

Column: Chiralpak AD-H 0.46X25 cm DAIC 19325;

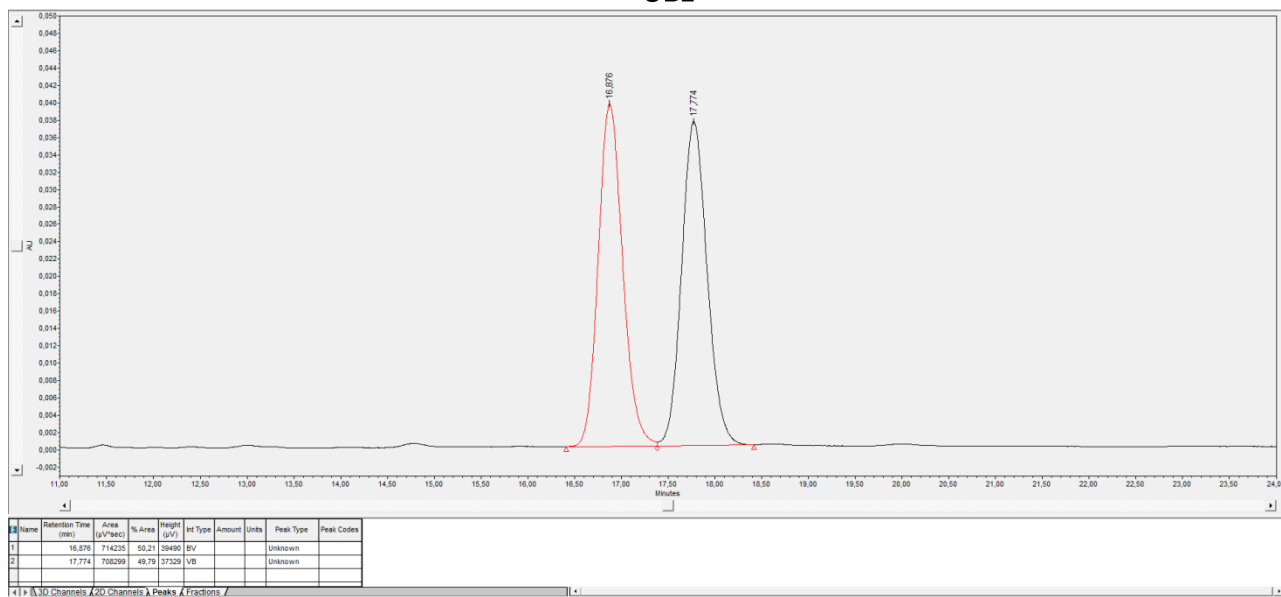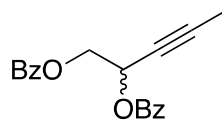Flow: 0.5 ml/min; Eluent: Isopropanol/n-Hexane 5/95. UV detection at 230 nm. 25ºC

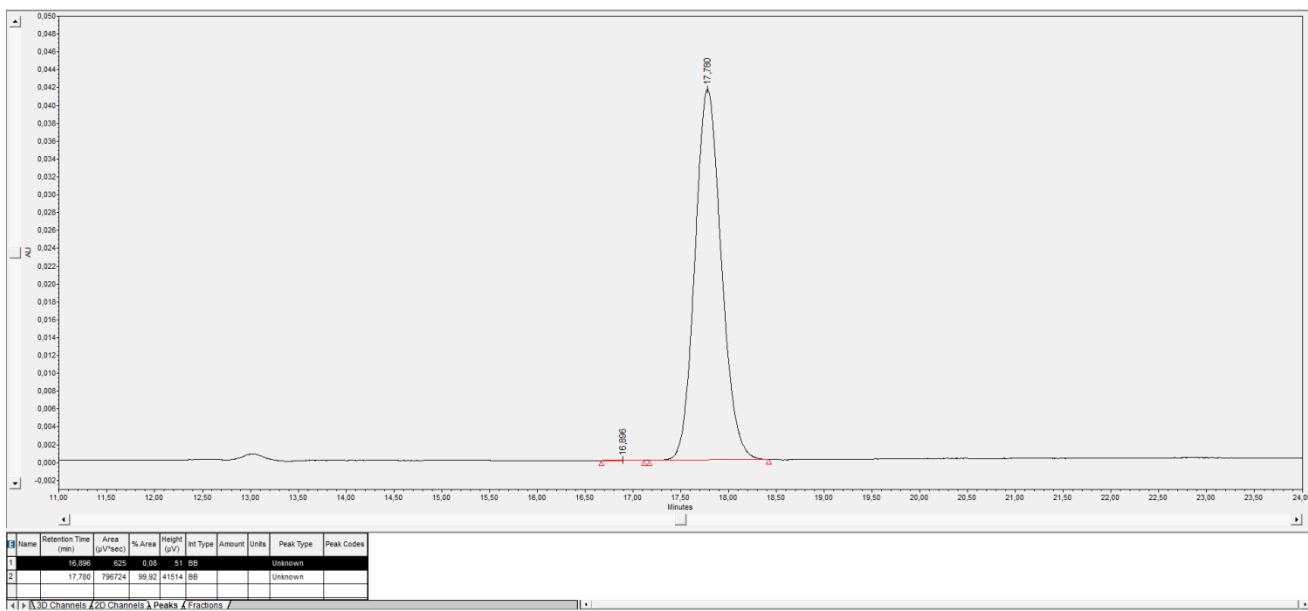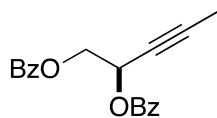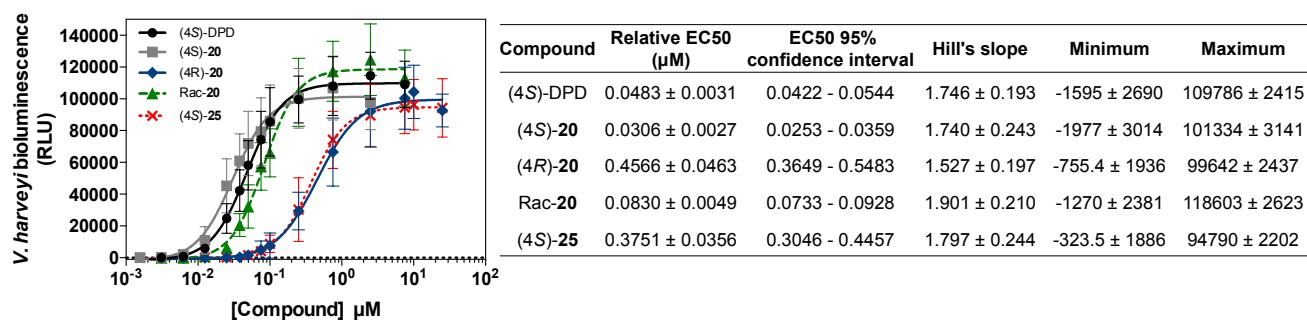**Fig. S1.** Chromatogram of the racemic dibenzoate of **21**.

Fig. S2. Chromatogram of the optically active dibenzoate of **21**.

Table S1. Fitting parameters for the determination of the relative EC50 of the studied compounds using data from the LuxP-FRET assay.

Compound	Relative EC50 (μM)	EC50 95% confidence interval	Hill's slope	Minimum	Maximum
(4*S*)-DPD	0.052 ± 0.002	0.048 - 0.056	1.241 ± 0.061	1.211 ± 0.004	1.736 ± 0.006
	0.053 ± 0.001	0.050 - 0.055	1.191 ± 0.033	1.202 ± 0.002	1.720 ± 0.003
(4*S*)-**20**	0.071 ± 0.006	0.060 - 0.082	1.417 ± 0.160	1.226 ± 0.008	1.717 ± 0.011
	0.065 ± 0.002	0.061 - 0.069	1.125 ± 0.037	1.214 ± 0.003	1.732 ± 0.004
(4*R*)-**20**	0.857 ± 0.053	0.751 - 0.963	1.067 ± 0.066	1.229 ± 0.009	1.769 ± 0.005
	0.897 ± 0.034	0.829 - 0.965	1.293 ± 0.062	1.216 ± 0.006	1.720 ± 0.003
Rac-**20**	0.154 ± 0.004	0.146 - 0.161	1.228 ± 0.031	1.220 ± 0.003	1.760 ± 0.003
	0.157 ± 0.004	0.149 - 0.165	1.277 ± 0.032	1.212 ± 0.003	1.732 ± 0.003
(4*S*)-**25**	0.147 ± 0.004	0.140 - 0.154	1.149 ± 0.026	1.212 ± 0.002	1.744 ± 0.003
	0.137 ± 0.005	0.127 - 0.146	1.037 ± 0.032	1.207 ± 0.004	1.743 ± 0.004

Table S2. Fitting parameters for the determination of the relative EC50 of the studied compounds using data from the *Vibrio harveyi* MM32 assay.

Compound	Relative EC50 (μM)	EC50 95% confidence interval	Hill's slope	Minimum	Maximum
(4*S*)-DPD	0.038 ± 0.001	0.036 - 0.041	2.052 ± 0.125	-835.1 ± 1550	117120 ± 1319
	0.053 ± 0.002	0.048 - 0.057	1.692 ± 0.119	-1737 ± 1862	121426 ± 1715
	0.062 ± 0.004	0.053 - 0.070	1.597 ± 0.177	-1727 ± 2179	91483 ± 2113
(4*S*)-**20**	0.024 ± 0.002	0.021 - 0.028	2.072 ± 0.227	-1824 ± 2540	112679 ± 2421
	0.034 ± 0.002	0.030 - 0.037	1.687 ± 0.148	-1762 ± 2105	114026 ± 2276
	0.039 ± 0.002	0.035 - 0.043	1.556 ± 0.127	-1895 ± 1340	77879 ± 1539
(4*R*)-**20**	0.444 ± 0.056	0.330 - 0.559	2.230 ± 0.463	-205.7 ± 2756	104781 ± 3478
	0.429 ± 0.074	0.278 - 0.580	1.956 ± 0.516	-689.7 ± 3565	99665 ± 4388
	0.545 ± 0.096	0.349 - 0.741	2.152 ± 0.695	-252.3 ± 2601	70788 ± 3406
Rac-**20**	0.076 ± 0.004	0.068 - 0.084	1.815 ± 0.171	-2028 ± 2365	131047 ± 2508
	0.080 ± 0.002	0.076 - 0.084	2.055 ± 0.107	-850.4 ± 1202	131249 ± 1312
	0.099 ± 0.005	0.090 - 0.109	1.871 ± 0.148	-990.4 ± 1299	93572 ± 1535
(4*S*)-**25**	0.296 ± 0.039	0.217 - 0.374	1.955 ± 0.418	-566.8 ± 2925	95788 ± 3184
	0.353 ± 0.038	0.275 - 0.430	1.474 ± 0.192	-1704 ± 2473	109617 ± 2763
	0.528 ± 0.046	0.434 - 0.622	3.022 ± 0.578	762.6 ± 1424	78542 ± 1865

Compound	Relative EC50 (µM)	EC50 95% confidence interval	Hill's slope	Minimum	Maximum
(4S)-DPD	0.0483 ± 0.0031	0.0422 - 0.0544	1.746 ± 0.193	-1595 ± 2690	109786 ± 2415
(4S)-20	0.0306 ± 0.0027	0.0253 - 0.0359	1.740 ± 0.243	-1977 ± 3014	101334 ± 3141
(4R)-20	0.4566 ± 0.0463	0.3649 - 0.5483	1.527 ± 0.197	-755.4 ± 1936	99642 ± 2437
Rac-20	0.0830 ± 0.0049	0.0733 - 0.0928	1.901 ± 0.210	-1270 ± 2381	118603 ± 2623
(4S)-25	0.3751 ± 0.0356	0.3046 - 0.4457	1.797 ± 0.244	-323.5 ± 1886	94790 ± 2202

Fig. S3. Determination of the EC50 values using the data from the three independent experiments. By joining the data from the three biological replicates (each with technical triplicates) we observe that the EC50 value of DPD is 1.6 times superior to the one of (4S)-**20**, confirming what we observe in each of the experiments. This reflects the robustness of our results.

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