



## Furanone quorum-sensing inhibitors with potential as novel therapeutics against *Pseudomonas aeruginosa*

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1 Furanones; the Quorum Sensing  
2 Inhibitors as Potential Therapeutics  
3 against *Pseudomonas aeruginosa*

4  
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22 [Abstract](#)

23 Microorganisms use quorum sensing (QS), a cell density dependent process, to communicate.  
24 This QS mode of interchange leads to the production of a variety of virulence factors, co-  
25 ordination of complex bacterial behaviours, such as swarming motility, degradation of host  
26 tissue and biofilm formation. QS is implicated in numerous human infections and,  
27 consequently, researchers have sought ways of effectively inhibiting the process in  
28 pathogenic bacteria. Two decades ago, furanones were the first class of chemical compounds  
29 identified as *Pseudomonas aeruginosa* QS inhibitors (QSIs). *P. aeruginosa* is a ubiquitous  
30 organism, capable of causing a wide range of infections in humans, including eye and ear  
31 infections, wound infections and potentially fatal bacteraemia and thus novel treatments  
32 against this organism are greatly needed. This review provides a brief background on QS and  
33 the use of furanones as QSIs. Based on the effectiveness of action, both *in vivo* and *in vitro*,  
34 we will explore the use of furanones as potential antimicrobial therapeutics and conclude  
35 with open questions.

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## 43 Introduction

44 Quorum sensing (QS) is a cell density-dependent method of bacterial communication and co-  
45 ordination, which is seen in many species, including *P. aeruginosa*, *V. fischeri* and *E.*  
46 *carotovora* [1]. QS systems in these organisms are often associated with altered phenotypes  
47 [2] and the regulation of complex processes such as biofilm formation [3–7], swarming  
48 motility [8] and virulence gene expression including those coding for degradative enzymes  
49 [9]. In brief, quorum sensing bacteria constitutively express low molecular weight signalling  
50 molecules known as autoinducers. In gram-negative organisms, the primary autoinducers are  
51 a group of molecules known as the N-acyl homoserine lactones (AHL)[10]. These AHLs are  
52 produced and released by bacteria and accumulate in their extracellular environment. Once a  
53 critical environmental AHL concentration is reached, the signalling molecules will interact  
54 with their cognate receptors and induce the expression of their target genes. The regulation  
55 of these target genes can result in the production of molecules, such as endotoxins and  
56 degradative enzymes [11–13] or down regulation of surface antigens, such as OprH [14] to  
57 avoid detection by a host's immune system. Bacteria often have multiple QS systems, which  
58 work together in order to govern the organism's virulence and behaviour. A good example of  
59 this is the model gram-negative organism *P. aeruginosa*, which utilises 3 distinct quorum  
60 sensing systems known as the Las, Rhl, PQS systems and one recently proposed system, the  
61 IQS system. [15] . The Las QS system is responsible for the production of many virulence  
62 factors including the LasA, LasB, and Apr protease enzymes [16, 17]. While the Las QS system  
63 is only one QS system used by *P. aeruginosa*, it is typically thought of as being at the top of  
64 the QS hierarchy, primarily due to the fact that activation of the Las system causes a  
65 subsequent activation of both the Rhl and PQS systems[18, 19]. Activation of the Rhl QS  
66 system governs the transcription of a distinct set of virulence associated genes, such as those

67 involved in rhamnolipid, pyocyanin, and hydrogen cyanide production [20, 21]. As is the case  
68 with the Las quorum sensing system, the Rhl QS system is not wholly independent and it can  
69 be activated by the Las system, but can also suppress the activation of the PQS system. This  
70 interrelation of the various QS systems allows *P. aeruginosa* to compensate for the loss or  
71 inhibition of one QS system and still remain virulent. The complexity of bacterial QS and the  
72 ability of many species to compensate for the loss of one or more QS systems makes  
73 exploiting the inhibition of quorum sensing a particularly challenging task.

74 An overview of the QS process in *P. aeruginosa* is presented in Figure 1 For a comprehensive  
75 overview of bacterial quorum sensing, the reader is referred to two recent reviews [22, 23]

76

77 **Figure 1. Interrelation of the 3 primary quorum sensing systems (Las, Rhl and PQS) in the**  
78 **model gram-negative microorganism *Pseudomonas aeruginosa*. 1. Octadecanoyl**  
79 **homoserine lactone (OdDHL) accumulates in the extracellular environment and once a**  
80 **critical threshold is reached it diffuses back into the cell. 2. Here, the LasR interacts with,**  
81 **and causes dimerization of, the transcriptional activator, LasR . 3-4. The LasR/OdDHL**  
82 **complex then initiates transcription of numerous genes including *lasI* (thus producing more**  
83 **OdDHL), *lasA* (LasA protease) and *lasB* (LasB elastase). These products then either diffuse**  
84 **out or are actively effluxed from the cell. 5. The LasR/OdDHL complex also activates**  
85 **transcription of the *rhIR* and *rhII* genes. 6. Transcription of *rhIR* results in the production of**  
86 **the transcriptional regulator protein RhIR and transcription of *rhII* results in production of**  
87 **Butyryl homoserine lactone (BHL). 7. BHL diffuses from the cell and accumulates in the**  
88 **extracellular environment resulting in a second positive feedback loop. 8. As with OdDHL,**  
89 **BHL enters the cell and binds with RhIR to cause a conformational change. The RhIR/BHL**  
90 **complex then promotes the transcription of *rhII* leading to an increased production of BHL**  
91 **as well as the transcription of genes causing production of rhamnolipids and pyocyanin. 9.**  
92 **The RhIR/BHL complex also plays a role in negatively regulating the PQS system. 10-12.**  
93 **Finally, the PQS quorum sensing system, responsible for the production of pyocyanin and**  
94 **hydrogen cyanide is activated by the LasR/OdDHL complex causing the transcription of *pqsH***  
95 **gene that results in the production of the *Pseudomonas* quinolone signal (PQS) [24]. 13-14.**  
96 **PQS, in conjunction with the transcriptional regulator, PqsR, activates transcription of the**  
97 ***pqsABCDE* operon, positively regulating further transcription of *rhIR* and, via the *pqsABCDE***  
98 **gene products, increasing expression of genes associated with production of hydrogen**  
99 **cyanide, rhamnolipids, biofilm and pyocyanin.**

100

101 QS is important not only for bacterial survival but also in allowing bacteria to colonise and  
102 infect a host by, for example, promoting bacterial adhesion to host cells [25] Additionally, QS–  
103 controlled behaviours, such as biofilm formation and virulence factor production, have been  
104 implicated in numerous health conditions including cystic fibrosis, development of chronic  
105 wounds and colonisation/infection of medical implants [26–28]. Biofilms, in particular are  
106 highly problematic in these conditions.

107 Biofilm formation is a process by which planktonic cells form organised communities. They  
108 are encased in a self–produced polymeric substance, often attached to a biotic or abiotic  
109 surface [29]. Briefly, biofilms are formed when planktonic bacteria attach, first reversibly then  
110 irreversibly, to a surface. This attachment results in changes in the expression of multiple  
111 genes involved in the biofilm formation process, such as the genes involved in the production  
112 of the polymeric matrix that provides the biofilm structure and some virulence factors [30,  
113 31]. Bacterial cells begin to aggregate and form microcolonies, encased in the extracellular  
114 polymeric substance. These polymers are continually produced and gradually accumulate,  
115 while bacterial cells divide providing much of the three–dimensional structure of the biofilm.  
116 Finally, once the biofilm has matured, large quantities of surfactants, such as rhamnolipid, are  
117 produced. These surfactants allow the biofilm to disperse, thus returning some of its cells to  
118 a planktonic state [32]. Cells that have become planktonic again are then free to relocate and  
119 seed new biofilms. This dispersal may be brought about through the mature biofilm  
120 experiencing a damaging mechanical force or it may be triggered by environmental cues, such  
121 as an abundance of nutrients [33].

122 The role of QS in biofilm formation has been debated for some time with many published  
123 results providing contradictory findings. Despite this, QS has been implicated in many stages

124 of biofilm development in *P. aeruginosa*. For example, QS-controlled twitching motility is  
125 involved in the formation of the microcolonies in the early stages of biofilm formation[34]. As  
126 well as having a role in microcolony formation through the regulation of twitching motility,  
127 QS has also been shown to be involved in the process of biofilm maturation and the formation  
128 and maintenance of its three-dimensional structure [32], thus making QS an attractive target  
129 for new therapies.

130 One method of ameliorating the negative effect of processes, such as biofilm formation, is to  
131 inhibit the QS processes that control them, using compounds known as quorum sensing  
132 inhibitors (QSI). QSI include a range of natural and synthetic compounds that prevent  
133 autoinducers from binding to their cognate receptors, suppress the production of new  
134 autoinducers, or enzymatically degrade autoinducers before they are detected (also known  
135 as quorum quenching). In this review, we will focus on the potential utility of furanones as  
136 QSI. For a more general review of quorum sensing inhibitors, particularly their use as  
137 antibiofilm agents, the reader is referred to the recent work of Brackman and Coenye [35].

138

### 139 [Furanones](#)

140 Furanones are a family of structurally related molecules characterised by the presence of a 5  
141 membered heterocyclic furan ring. Furanones are common in nature and are found in a wide  
142 variety of marine and terrestrial plants, including the red marine macroalga *Delisia pulchra*,  
143 strawberries and coffee [36, 37]. Some furanones are produced as a by-product of cooking  
144 food, for example in the Maillard reaction when baking bread, where they also act as flavour  
145 compounds [38, 39]. Furanones are also found in a number of fermented food products,  
146 including wine and Japanese soy sauce [40]. One of the final natural sources of furanones are

147 fungi and species, such as *Aspergillus spp.* They are capable of producing furanone derivative  
148 compounds [41], which represent a potential new avenue of enquiry into QS inhibition.

149 As well as being present in a number of natural sources, furanones can be chemically  
150 synthesised. Furanone derivatives have been chemically synthesised since the 1980s and  
151 synthesis often began with relatively simple compounds, such as ketones and dimethyl  
152 acetals or other simple organic precursors [42, 43]. Alternatively, modifications can be made  
153 to existing furanone compounds, as well as additions to existing furanone structures as will  
154 be discussed later in this review. Both natural and synthetic furanones have been shown to  
155 effectively inhibit QS but synthetic furanones offer the possibility of precise control over  
156 compound structure and, therefore, control of any potential off-target effects.

#### 157 [Efficacy of furanones against non-human pathogens](#)

158 One of the earliest natural furanones to be discovered and characterised was 2,5-dimethyl-4-  
159 hydroxy-3(2H)-furanone. It is found in pineapple and was reported in 1965 by Rodin *et al.*  
160 [44]. Using gas chromatography and nuclear magnetic resonance spectroscopy, they  
161 determined that this aromatic compound must contain a cyclic five-membered ring structure  
162 in addition to an arrangement of side groups that the authors were unable to definitively  
163 characterise, despite similarities to a previously synthesised compound tetrahydro-3,4,-  
164 furandione [44, 45]. In spite of the characterisation of this and other furanone compounds,  
165 the structural similarities between furanones and gram-negative QS molecules was not  
166 realised until after the identification of autoinduction by Nealson *et al.* in 1970 and the  
167 structural characterisation of the chemical autoinducers (now known as N-acyl homoserine  
168 lactones or AHL) in *Photobacterium fischeri* by Eberhard *et al.* in 1981 [46, 47]. The *P. fischeri*  
169 autoinducer used to control luciferase production consisted of a five-membered ring with a



170 six-carbon chain and was identified as N-(3-oxo-hexanoyl)-3-aminodihydro-2(3H)-furanone.  
171 Although the structure of furanones and AHL had both been known for more than a decade  
172 at this point, investigations into their anti-quorum sensing properties began only in 1993  
173 following the discovery of halogenated furanones in the marine alga *Delisea pulchra* [36]. As  
174 with the naturally occurring fruit furanones, these natural halogenated furanones were  
175 structurally similar to AHL. The similarity of the five membered ring structure found in both  
176 furanones and AHL, along with the ability of *D. pulchra* to resist epiphytic colonisation,  
177 prompted studies into the effects of these seaweed-derived furanones on AHL-mediated  
178 bacterial behaviours. Some of the first experiments showed furanone treatment greatly  
179 reduced *Serratia liquifaciens* swarming motility, as well as *Vibrio* spp. bioluminescence (up to  
180 100-fold) [48], providing evidence that furanones could modulate QS-mediated behaviours.  
181 Further investigation of QSI revealed that seaweed-derived furanones interfered with AHL  
182 mediated processes via displacement of the AHL signalling molecule from its cognate  
183 receptor. Using tritium-labelled AHL, Manefield *et al.* [49] showed that the addition of  
184 furanone to cultures of *Escherichia coli* pHK724 (overexpressing a recombinant LuxR AHL  
185 receptor protein) resulted in a dose-dependent decrease in the proportion of labelled AHL in  
186 the cells when compared to untreated cells. They also showed that addition of two  
187 different, although unnamed, furanones to a culture of *E. coli* pHK724 that was already  
188 saturated with 0.5  $\mu$ M exogenous autoinducer reduced the proportion of autoinducer bound  
189 to the LuxR receptor by over 75%. Two-dimensional gel electrophoresis then revealed that  
190 protein synthesis in furanone-treated cells was largely unaffected, save for reductions in the  
191 abundance of several Lux QS system proteins tentatively identified as LuxA, LuxB and LuxD,  
192 as well as OmpF, DnaK and Glutamate-ammonia ligase.

193 It has been shown by Welch *et al.* (2000) and Manfield *et al.* (2001) that, in *Erwinia*  
194 *carotovora*, treatment with an unnamed, seaweed derived, halogenated furanone (30  $\mu$ M)  
195 reduced the secretion of virulence factors, including protease and cellulase. Furthermore, a  
196 dose-dependent response was observed between 10  $\mu$ M and 40  $\mu$ M, leading to a reduction  
197 in carbapenem production that was suggested to result from furanone-mediated  
198 interference in the quorum sensing-regulated production of the CarA and CarC proteins  
199 required for carbapenem-3-carboxylic acid production[50–52].

200 These reports showed that not only can natural furanones effectively inhibit quorum sensing,  
201 but that they could, in theory, be used to attenuate the virulence of some microorganisms.  
202 This leads logically to consideration of the potential of furanones in the battle against human  
203 pathogens.

#### 204 [Efficacy of furanones against human pathogens](#)

205 In recent years, the majority of research into furanone-mediated QSI has focused primarily  
206 on the effects of these compounds on human pathogens and, in particular, the model  
207 organisms *E. coli* and *Pseudomonas aeruginosa*.

#### 208 **Efficacy of furanones against *Escherichia coli***

209 A natural furanone, known as (5Z)-4-bromo-5-(bromoethylene)-3-butyl-2(5H)-furanone  
210 (Figure 3), was demonstrated to inhibit bioluminescence in the marine organism *Vibrio*  
211 *harveyi* at concentrations between 16.13  $\mu$ M and 32.26  $\mu$ M. The same furanone also  
212 attenuated biofilm formation in *E. coli*, reducing average biofilm thickness by 55% and the  
213 number of viable cells by 87 % at a concentration of 164  $\mu$ M. Additionally, lower furanone  
214 concentrations (64.5  $\mu$ M) significantly reduced *E. coli* swarming motility [53]. This important  
215 work clearly demonstrated that furanones could interfere with QS processes and that the

216 phenomenon could be used to combat virulence in human pathogens. Investigations into the  
217 effects of synthetic brominated furanones showed that these compounds could also reduce  
218 *E. coli* biofilm thickness and surface coverage by up to 50% [54] and that, when applied at 50  
219  $\mu\text{M}$ , could inhibit swarming motility and reduce biofilm production by up to 40 % in the food–  
220 borne pathogen *E.coli* O103: H2 [55, 56].

### 221 **Efficacy of furanones against *Pseudomonas aeruginosa***

222 Natural furanones greatly reduce the production of *P. aeruginosa* virulence factors including  
223 protease (up to 43 %), chitinase and pyoverdine (by almost 100%) [57–59]. However, it  
224 becomes clear that the efficacy, and effect, of furanones in decreasing bacterial virulence may  
225 not be consistent. For example, the near total prevention of pyoverdine production by C-30,  
226 a synthetic derivative (Figure 3) of the natural seaweed–derived furanone, was reported by  
227 Hentzer *et al.* in 2003 [58]. The later work of Ren *et al.* (2005), however, demonstrated that  
228 both *P. aeruginosa* PAO1 and JB2 exhibited an increase in siderophore production when  
229 exposed to the natural furanone (5Z)-4-bromo-5-(bromoethylene)-3-butyl-2(5H)-furanone at  
230 concentrations between 20  $\mu\text{g mL}^{-1}$  and 100  $\mu\text{g mL}^{-1}$  (64.5–322.5  $\mu\text{M}$ ) [60]. This discrepancy  
231 is most likely due to the differences in structures of the furanone used. The natural furanone  
232 used by Ren *et al.* has a hydrocarbon chain bound to the furanone ring [61], whereas this is  
233 substituted for a single hydrogen in the synthetic furanone, C-30 [58] (Figure 3). Nonetheless,  
234 the observed differences in effects mediated by these two molecules suggest that alterations  
235 in the chemical structure of furanones or their derivatives result in significant differences in  
236 their QSI activity.

237 **Figure 3. Comparison of natural and synthetic furanone structures: (A) naturally occurring**  
238 **(seaweed–derived) furanone (5Z)-4-bromo-5-(bromoethylene)-3-butyl-2(5H)-furanone, (B)**  
239 **the synthetic furanone known as C-30. Both compounds exhibit similarities in structure,**  
240 **consisting primarily of a five–membered furan ring, however, C-30 lacks a hydrocarbon**

241 **chain. The compounds have different biological activities, leading to the conclusion that**  
242 **changes in side group structure play a key role in determining furanone compound efficacy**  
243 **as QSI.**

244

245 In addition to their ability to reduce virulence factor production in a range of microorganisms,  
246 one of the most important effects of furanone compounds on *P. aeruginosa* is their ability to  
247 interfere with biofilm formation. Investigations in the early 2000s showed that two synthetic  
248 furanone derivatives in particular, C-56 and C-30, were highly effective in inhibiting *P.*  
249 *aeruginosa* biofilm formation [57, 58]. These furanones interfered with quorum sensing in  
250 both planktonic cultures and, more importantly, in established biofilms. While furanone C-56  
251 did not appear to affect the initial bacterial attachment stage of biofilm formation, cells  
252 treated with 5  $\mu\text{g mL}^{-1}$  (28.5  $\mu\text{M}$ ) C-56 developed significantly thinner biofilms, exhibiting an  
253 approximate 37% reduction in overall thickness. The synthetic furanone-treated biofilms also  
254 appeared to be more sparsely populated than untreated biofilms and overall the data  
255 suggested that furanones might have a significant long-term effect on both biofilm formation  
256 and maintenance. This idea was further supported by the recent work of Choi *et al.* in 2014  
257 [59] who showed that, in PAO1, treatment with a natural furanone 4-hydroxy-2,5-dimethyl-  
258 3(2H)-furanone (HDMF) reduced rhamnolipid production. Rhamnolipids are essential for  
259 maintenance of biofilm structure and integrity [32, 59] and PAO1 cells grown in the presence  
260 of either 0.1  $\mu\text{M}$  or 1  $\mu\text{M}$  HDMF formed biofilms, but with reduced biofilm biomass (27.8%  
261 and 42.6%, respectively). Treatment of 48 hour PAO1 biofilms with either 0.1  $\mu\text{M}$  or 1  $\mu\text{M}$   
262 HDMF also led to subsequent 66.3% and 84.8% losses, respectively, of biofilm biomass over  
263 the following 48 hours, whereas untreated biofilms lost only ~50% of their biomass in the  
264 same time period. This data suggests that HDMF treatment accelerates the natural, post 48  
265 hour, loss of biomass observed in mature *P. aeruginosa* biofilms. Biofilm morphology was also

266 changed, as furanone–treated late–stage biofilms presented as poorly populated monolayers  
267 in comparison to the more natural 3D architecture and dense cell population of the untreated  
268 controls.

269 Kim *et al.* (2008) showed that a synthetic furanone compound, named 5a, could efficiently  
270 reduce the initial attachment of planktonic *P. aeruginosa* cells to glass slides [62]. While no  
271 mechanism for the phenomenon was reported by the authors, the differences observed  
272 suggest that, although different furanones may inhibit QS and biofilm production, they may  
273 do so in quite different ways, possibly by acting upon different elements of the QS–mediated  
274 processes that lead to biofilm formation.

275 Numerous other furanone compounds can also effectively reduce biofilm formation in PAO1.  
276 Reductions of up to 43 % were shown by Shetye *et al.* [54] using a range of synthetic  
277 brominated furanones. Four of these in particular were found to be effective in reducing  
278 either *E. coli* or *P. aeruginosa* biofilm formation. Despite their apparent efficacy, they have  
279 not been characterised as thoroughly as C-30 and C-56.

280 Furanone compounds have another important effect on *P. aeruginosa*, specifically on so–  
281 called “persister cells”–a type of largely dormant and consequently less antibiotic–sensitive  
282 cell found in biofilms. Persister cells are believed to be involved in maintaining chronic and  
283 recurrent infections, particularly in conditions such as cystic fibrosis [63, 64]. Certain  
284 furanones, however, can sensitise PAO1 persister cells to antibiotics, reducing their numbers  
285 and making biofilm eradication more likely. Treatment with between 5  $\mu\text{g mL}^{-1}$  and 100  $\mu\text{g}$   
286  $\text{mL}^{-1}$  of a synthetic brominated furanone, (Z-4-bromo-5-(bromoethylene-3-methylfurn-2(5H)-  
287 one, resulted in a dose–dependent reduction in planktonic PAO1 persister cell numbers from  
288 over  $1 \times 10^5$  CFU  $\text{mL}^{-1}$  to approximately  $1 \times 10^4$  CFU  $\text{mL}^{-1}$  [65]. Interestingly, while the number

289 of persister cells was reduced, total cell numbers were not affected by the furanone  
290 treatment. Importantly, this reduction in persister cells also occurred in surface-attached  
291 biofilms, and here, a 2 log<sub>10</sub> reduction in numbers from 1 × 10<sup>4</sup> CFU mL<sup>-1</sup> to 1 × 10<sup>2</sup> CFU mL<sup>-1</sup>  
292 was observed following treatment with 60 µg mL<sup>-1</sup> of the synthetic brominated furanone. In  
293 addition, a relatively low dose of furanone also increased the efficacy of tobramycin and  
294 ciprofloxacin in killing persister cells: 5 µg mL<sup>-1</sup> of synthetic brominated furanone followed by  
295 tobramycin treatment reduced persister cell numbers by over 99.9 %, an order of magnitude  
296 greater than antibiotic alone, while treatment with ciprofloxacin reduced persister cell  
297 numbers by approximately 90 % compared to a 50 % reduction with antibiotic only. Thus,  
298 furanone addition potentiated biofilm persister cell killing by reducing the antibiotic tolerance  
299 of the cells. Two further, structurally distinct, furanone compounds also led to a reduction in  
300 persister cell numbers. However, it was also observed that several other distinct furanones,  
301 while capable QSI, had no effect on persister cells [65, 66]. As these furanones were all  
302 structurally related, it seems likely that differences in activity were dictated by small changes  
303 in chemical structure. The observation that two non-brominated compounds (named NF1 and  
304 NF2), which despite having structural similarity to (Z-4-bromo-5-(bromoethylene-3-  
305 methylfurn-2(5H)-one had no effect on either QSI activity or persister cells, suggests that  
306 bromine, specifically, is important for activity against persister cells. However, it should be  
307 noted that these non-brominated furanones did not only differ from the brominated  
308 furanone in their lack of halogen groups, but also in the abundance and position of other side  
309 groups. For example, the non-brominated furanone NF2 contained a methoxy group, which  
310 was not present in the brominated furanone. Additionally, furanone NF1 had an additional  
311 carbonyl group, which is not present on the brominated furanone. These changes will have  
312 undoubtedly affected the furanones reactivity and played a role in the difference in bioactivity

313 seen by Pan *et al.* (2013). Due to these differences, firm conclusions cannot be made  
314 regarding the effect of bromination and furanone activity against persister cells [66].

#### 315 Mechanism of furanone action

316 Furanone concentrations of up to 10  $\mu\text{g mL}^{-1}$  have been demonstrated to reduce expression  
317 of the *lasB* gene that encodes the LasB elastase, resulting in an approximately 50 % reduction  
318 in relative fluorescence of a *PlasB-GFP* reporter [54, 57]. This suppression was non-reversible,  
319 even following addition of moderate levels of exogenous autoinducer. Addition of 80 nM  
320 OdDHL and 2  $\mu\text{g mL}^{-1}$  of C-56 activated *lasB* expression, while the addition of 40 nM OdDHL  
321 and 2  $\mu\text{g mL}^{-1}$  C-56 did not. This elegantly demonstrates that the furanone binds competitively  
322 to the native AHL receptor. The possibility of C-56 acting against any other part of the QS  
323 network was ruled out by use of *E. coli* MT102, which lacks any AHL-mediated QS system  
324 other than the recombinant *PlasB-GFP* fusion [57]. Reductions in C-56-mediated *lasB*  
325 expression were also observed in established biofilm as well as in planktonic cultures. Overall,  
326 the experiments suggested that the furanone's antagonistic activity stems from competition  
327 with the native autoinducer to bind to, and subsequently block, the AHL receptors.

328 Gene expression studies carried out by Hentzer *et al.* [58] in PAO1 showed that expression of  
329 85 out of 93 differentially expressed genes was reduced following treatment with furanone  
330 C-30. A large proportion of these genes had previously been described as QS-controlled,  
331 including *lasA*, *lasB* and several other genes involved in virulence factor production.  
332 Interestingly, it was shown that expression of *fabH1* and *fabH2*, encoding subunits of the 3-  
333 oxo-acyl carrier protein (ACP) synthase III which has been suggested to be involved in the  
334 production of AHL [10, 67], was considerably reduced (~50 and 80-fold, respectively). This  
335 suggested that furanones could inhibit QS by reducing the quantity of ACP transcripts, leading

336 to decreased synthesis of AHL molecules. Genes exhibiting increased expression following C-  
337 30 treatment included those of the *mexEF-oprN* operon that encodes components of a  
338 multidrug efflux pump [68] and genes encoding ATP-binding cassette (ABC) transporters and  
339 major facilitator superfamily (MFS) transporters. These transporter proteins enable both  
340 influx of nutrients and the active removal of toxins from bacterial cells [69, 70] and their  
341 increased expression may be due to the tendency of bacteria to increase efflux activity in the  
342 presence of antimicrobial compounds [71]. Interestingly, expression of *mexR*, the repressor  
343 for the multidrug resistance efflux pump encoded by *mexAB-oprM*, exhibited increased  
344 expression (~5-fold) suggesting that, in the presence of the furanone, *P. aeruginosa* could be  
345 resensitised to certain antibiotics normally excreted by this system. The expression of the  
346 other *mex* efflux operon, *mexEF-oprN* (induced by nitrosative stress and chloramphenicol  
347 [68]) was, however, increased by furanone treatment (>5-fold) and may be consistent with  
348 the cells attempting to actively remove the intracellular furanone. Hentzer *et al.* also showed  
349 that although C-30 did not prevent PAO1 biofilm formation, it exerted a significant effect on  
350 the sensitivity of 72 hour biofilms to 10  $\mu$ M tobramycin as demonstrated *via* BacLight staining  
351 [58]. The possibility of furanone-mediated re-sensitisation of PAO1 to antibiotics was not,  
352 however, further investigated by the authors.

353 This reduction in QS-associated gene expression does not appear to be universal for all QS  
354 systems. The work of Shetye *et al.* on the *P. aeruginosa* Rhl QS system utilised *E. coli*  
355 containing a *PrhlI-LVA-GFP* reporter plasmid. Treatment with 100  $\mu$ M to 200  $\mu$ M of either of  
356 two synthetic brominated furanones caused an increase in fluorescence intensity (caused by  
357 increased transcription of the *rhII-LVA-GFP* genes) rather than an antagonistic effect. Thus,  
358 treatment with 200  $\mu$ M furanone BF15 more than doubled transcription of the *PrhlI-LVA-GFP*  
359 fusion, in contrast to the effect on a *PlasI-LVA-GFP* reporter for which fluorescence was



360 reduced by approximately 50 % by the same treatment [54]. This observation provides further  
361 evidence that furanone action on QS systems is highly variable, reinforcing the idea that their  
362 effects may not always be antagonistic. The authors hypothesised that, in the light of similar  
363 effects caused by minor structural changes to native signalling molecules, a low binding  
364 constant between the synthetic furanones and their cognate receptors could explain their  
365 observations. This hypothesis is further supported by data from molecular docking studies—  
366 an important tool in the process of novel drug discovery and characterisation [72] and which  
367 have been used to good effect in the search for bioactive furanone compounds. Liu *et al.*  
368 (2012) demonstrated that furanones associate with the LasR receptor protein in a number of  
369 ways depending on the structure of the given furanone. Synthetic furanones generally  
370 associate with the LasR protein *via* hydrogen bonding between the carbonyl group on the  
371 furan ring and the amine functional group of Arg 61 in the LasR receptor protein. Several  
372 other potential furanone–receptor binding sites were identified *in silico* within both the  
373 receptor protein itself and also in furanone molecules. However, these were unique to the  
374 furanones tested [73]. This work clearly shows that the affinity of a furanone for a receptor  
375 protein is likely to be highly dependent on furanone structure. This further supports the  
376 hypothesis put forward by Shetye *et al.* [54] that reduced affinity for the receptor may have  
377 caused the agonistic activity of their synthetic furanone lacking a hydrocarbon chain. These  
378 studies provide evidence that while many furanones may show antagonistic behaviour  
379 towards *P. aeruginosa* QS systems, it is by no means a universal rule.

380 Further evidence for the importance of furanone structure and its relationship to biological  
381 effect was provided by Brackman *et al.* in 2012 [74]. They demonstrated that a group of  
382 synthetic AHL derivatives known as triazolylidihydrofuranones, in which the amide function is  
383 replaced by a triazole group, displayed a degree of anti–QS activity as measured using a QSI2

384 biosensor strain of *P. aeruginosa*. The furanones' activity was dependent on the length of the  
385 carbon chain bound to the furan ring and compounds with C<sub>10</sub> or C<sub>12</sub> chains inhibited OdDHL–  
386 mediated QS most effectively. It was suggested that this was due to the similar side chain  
387 length (C<sub>12</sub>) found on the native signalling molecule and indeed the effect of chain length has  
388 also been highlighted in examinations of QSI with other AHL derivatives [75, 76]. It is  
389 important to note that the primary AHL molecules used in Lux, Las and Rhl QS systems all  
390 have carbon chains attached to their five–membered ring and it may follow that the closer  
391 the furanone structure is to the native AHL for the system, the more effectively it can inhibit  
392 that particular QS system. This hypothesis is supported by the work of Ahumendo  
393 Monterrossa *et al.* [77] who showed that the location and orientation of the AHL side chain  
394 within the LasR receptor is correlated with the biological activity of the ligand. Changes in  
395 ligand functional groups result in agonistic or antagonistic changes in ligand effect, with the  
396 interaction between the AHL acyl side chain and Tyr 47 in LasR dictating the stability of the  
397 resultant AHL–LasR complex. The lack of a side chain similar to that of the native AHL (for  
398 example, a shorter, or absent carbon chain) may result in a furanone not complexing as well  
399 with the LasR receptor, leading to a less potent effect [77].

400 While many studies have focused on the synthesis and testing of furanone compounds with  
401 different structures, such as the work of Chang *et al.* (2019) [78], the compounds in these  
402 studies often differ in more than one structure and it is difficult to ascertain which structural  
403 moiety is impacting on the QSI capability of the molecule. It is also often the case that studies  
404 in which large numbers of furanone derivatives do not extensively test the QSI potential of  
405 the derivatives with many using decreases in biofilm formation (as measured by a simple  
406 crystal violet assay) as a proxy for QSI. When these derivatives are tested more thoroughly it

407 is most often the most effective compounds and no direct comparison can be made between  
408 those moieties that increase efficacy and those that do not.

409 It is clear that the structure of a furanone derivative has an impact on the QSI efficacy of the  
410 compound. Unfortunately, no concerted effort to elucidate the exact structure function  
411 relationship of various side groups and, thus, further research is needed.

412 Although, the structure of a given furanone undoubtedly has a significant effect on the  
413 bioactivity of the compound, the structure of the target receptor protein will also have an  
414 impact on the QSI ability. Gram negative bacteria typically employ two types of QS receptor  
415 proteins, namely transmembrane, two component, LuxN type receptors and the cytoplasmic  
416 LuxR family of receptors, which typically consist of an AHL binding domain and a DNA binding  
417 domain [79, 80]. There is a high degree of variability between the receptor proteins of  
418 different QS systems and even greater variability between receptors of different species. For  
419 example, while the LasR receptor protein of *P. aeruginosa* has a high structural similarity to  
420 the TraR receptor of *Agroacterium tumefaciens* and the SdiA protein from *E.coli* the amino  
421 acid sequence only shows a 10-16% similarity [81]. It is likely that although these receptor  
422 proteins share similar topology the way in which they bind AHL and, therefore furanones, is  
423 likely quite different.

424 This variability in protein structure is also seen between receptor proteins from different QS  
425 systems from the same organisms. For example, despite both LasR and RhIR being from *P.*  
426 *aeruginosa*, both being activated by structurally similar AHL and thus being closely linked,  
427 RhIR (of which no structure and therefore no specific ligand binding data, has been  
428 elucidated) actually shares a higher sequence homology with the more distant SdiA receptor  
429 protein of *E.coli* [82]. Although their cognate receptors are similar, it appears that the

430 mechanisms of ligand binding are quite distinct. Taken together, data from a variety of  
431 molecular investigations suggest that the effects of furanones on QS systems is variable. It is  
432 clear that not only can furanones interact with a number of different QS systems, but also  
433 that they may do so with varying degrees of success and that, furthermore, this efficacy is  
434 influenced by a range of structural factors both in the structure of the furanone, including  
435 carbon chain length and the type of functional side groups found on the lactone ring and  
436 structural differences in the target receptor proteins.

#### 437 Efficacy of furanones *in vivo*.

438 Considering the useful biological effects of furanone QS antagonists on *in vitro* systems, there  
439 is clear potential for their application to *in vivo* systems. However, there is a lack of data  
440 regarding their efficacy or safety *in vivo*, in addition to a certain amount of contradiction  
441 amongst the reports published to date. In 2003, Hentzer *et al.* showed that, in a mouse model  
442 of 48 h *P. aeruginosa* lung infection, the intravenous administration of the synthetic furanone  
443 C-30 at a concentration of  $1.7 \mu\text{g g}^{-1}$  body weight resulted in a reduction in bacterial QS in the  
444 lung tissue, as measured *via* a *lasB-GFP* reporter. However, this inhibition was not permanent  
445 and QS recovered to pre-treatment levels after approximately 6 hours. Given the transient  
446 nature of the effect, further experiments showed that a subcutaneous dose of C-30 ( $0.7 \mu\text{g g}^{-1}$   
447 body weight) administered every 8 hours for 3 days immediately following infection with  
448 PAO1 enabled the mice to better clear PAO1 from the lungs when compared to a vehicle  
449 control [58]. This was later confirmed by Wu *et al.* in 2004 [83] who demonstrated that  
450 intravenous administration of  $17 \mu\text{g g}^{-1}$  body weight of the synthetic furanone C-56, 24 hours  
451 post-PAO1 infection, resulted in a significant reduction in QS in murine lung tissue as  
452 measured using a PAO1 *dsred-lasR-PlasB-GFP* reporter strain . As with C-30, this C-56-

453 mediated reduction was only temporary and QS levels returned to baseline values after  
454 approximately 8 hours. Additionally, Wu *et al.* (2004) showed that oral—as opposed to  
455 subcutaneous—administration of  $5 \mu\text{g g}^{-1}$  body weight of C-56 resulted in a significant decrease  
456 in both QS in the lung and in mouse mortality. Following infection with a lethal dose of PAO1  
457 and treatment with  $5 \mu\text{g g}^{-1}$  C-56 three times a day for 2 days, mouse survival increased from  
458 12 % to 45 % at 48 hours post—infection. In contrast to the intravenous administration of C-  
459 30 in the work of Hentzer *et al.* [58], Wu *et al.* used subcutaneous administration of between  
460  $0.25 \mu\text{g g}^{-1}$  and  $0.7 \mu\text{g g}^{-1}$  C-30 three times a day for 3 days. This resulted in significantly better  
461 bacterial clearance from the lung at 7 days post—infection in mice infected with sub-lethal  
462 doses of PAO1, where 1000—fold lower lung CFU numbers resulted following C-30 treatment.  
463 Although no survival rate data was provided for C-30 treated animals, this furanone was more  
464 effective in assisting with bacterial lung clearance than furanone C-56. Furanone—treated  
465 mice also exhibited less severe lung pathology, with fewer abscesses forming in the C-30  
466 treated mice and less tissue damage in the C-56 group. These studies show that not only were  
467 C-30 and C-56 effectively carried to the lungs following oral, intravenous and subcutaneous  
468 administration, but also that they retained their biological activity. Thus, it is clear that both  
469 C-30 and C-56 may be highly beneficial in combatting *P. aeruginosa* lung infections *in vivo*.

470 There is evidence that, in a primary human airway epithelial cell infection model, the natural  
471 furanone, HDMF, may be beneficial. It is known that *P. aeruginosa* diffusible virulence factors  
472 (e.g. LasA protease, LasB elastase) impair several important functions in airway epithelial cells  
473 including wound healing and cell proliferation and migration [84]. However, in 2016 Ruffin *et*  
474 *al.* [85] showed that growth of PAO1 in media supplemented with  $125 \mu\text{g mL}^{-1}$  HDMF led to  
475 reduced secretion of virulence factors, highlighted by a 70 % decrease in elastase activity  
476 measured using an elastin—congo red assay. It was also observed that wound healing rates in

477 epithelial cell scratch assays were significantly better when exposed to diffusible materials  
478 from HDMF-treated PAO1 cells than when exposed to the secretions from untreated PAO1  
479 cells. Diffusible materials from non-furanone-treated PAO1 reduced the wound repair rate by  
480 ~50 %, while repair rates for cells exposed to diffusible material from HDMF-treated PAO1  
481 cells was not significantly different from the controls. This would suggest that HDMF  
482 treatment decreased QS, resulting in a decrease in virulence factor production leading to  
483 better outcomes in wound repair. A similar result was obtained with highly differentiated  
484 airway epithelial cells, indicating the possible utility of HDMF in the treatment of lung  
485 infections, especially given that exposure to HDMF alone did not have a negative impact on  
486 the wound healing capacity of airway epithelial cells [85]. While HDMF apparently reduces  
487 bacterial virulence factor secretion, it is unfortunate that, to date, no data have been  
488 published regarding the cellular toxicity of HDMF. However, it could be hypothesised that  
489 because wound repair rates were not negatively impacted by HDMF, that cell viability also  
490 was not significantly impacted. Nonetheless, further investigation into the cytotoxicity of  
491 HDMF is needed to confirm this.

#### 492 [Furanones as potential therapeutics](#)

493 The published evidence for the efficacy of furanones in murine models is somewhat limited,  
494 but together with the *in vitro* human cell data, it suggests that these compounds have  
495 potential as effective human therapeutics. A number of studies have attempted to use  
496 furanones in a clinically relevant way. For example, in 2012 Kim *et al.* demonstrated that co-  
497 administration of furanone with ciprofloxacin significantly reduced *in vitro* biofilm formation  
498 using a medical implant as a substratum. The dual administration of 50  $\mu\text{g mL}^{-1}$  5-hydroxy-  
499 2(3H)-benzofuranone and 1  $\mu\text{g mL}^{-1}$  ciprofloxacin significantly reduced numbers of planktonic

500 cells and also effectively prevented *P. aeruginosa* biofilms from forming on silicone  
501 tympanostomy tubes. While the combination therapy prevented biofilm formation,  
502 treatment with the furanone only led to a reduced level of biofilm formation without a  
503 simultaneous reduction in planktonic cell numbers[26]. Thus, the presence of the furanone  
504 appeared to inhibit biofilm effectively—most likely by interfering with QS signalling—allowing  
505 the antibiotic unrestricted access to planktonic cells. It is likely that when furanone treatment  
506 is stopped, however, that planktonic cells will then begin to form biofilms once again, making  
507 dual treatment necessary.

508 Siebert *et al.* (2016) recently suggested that chemical conjugation of furanones to other drugs  
509 currently in use might facilitate development of single molecule, multiple effect treatments.  
510 For example, a synthetic brominated 2(5H) furanone was joined with rosiglitazone, a common  
511 antidiabetic drug also known to have anti-inflammatory properties [86]. The furanone was  
512 linked to a rosiglitazone skeleton in an attempt to combine the QSI properties of the furanone  
513 with the anti-inflammatory properties of rosiglitazone. This combination of effects could be  
514 very useful in treating chronic infections, such as those found in wounds, where biofilm  
515 formation often causes a prolonged inflammatory state [87]. Xu *et al.* (2018) showed that  
516 treatment with 10  $\mu$ M rosiglitazone–furanone fusion inhibited QS by up to 50% across the  
517 Las, Rhl and PQS quorum sensing systems as shown by a reduction in fluorescence of PAO1  
518 *lasB-GFP*, *rhlA-GFP* and *pqsA-GFP* reporter strains, respectively. In addition they observed a  
519 reduction in the pro-inflammatory cytokines Tissue Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) and  
520 Interleukin-6 (IL-6) in RAW264.7 murine macrophages, showing that the rosiglitazone–  
521 furanone compound simultaneously inhibited QS and achieved anti-inflammatory effects  
522 [88]. Thus, while effective on their own, furanones have the potential to be combined with  
523 various other compounds to produce new, more effective therapies.

## 524 Bacterial resistance to furanones

525 One of the main reasons furanones are so attractive to researchers investigating quorum  
526 sensing inhibition is the fact that furanones do not significantly affect bacterial numbers and  
527 show no significant impact on bacterial growth kinetics when used in low doses [58, 89].

528 As with antibiotics, the issue of resistance to furanones, and quorum sensing inhibitors in  
529 general must always be considered. It has often been stated, when discussing the use of QSI  
530 compounds, that as inhibition of bacterial signalling may constitute a reduced evolutionary  
531 pressure and present less risk of resistance developing [58, 90, 91]. However, it has become  
532 apparent in recent years that this may not be the case. In 2012 Maeda *et al.* demonstrated  
533 that *P. aeruginosa* PA14 transposon mutants with a high resistance to furanone C-30 had  
534 disruptions in the in the *mexR* gene, which acts as a repressor of the *mexAB-OprM* operon.  
535 This mutation causes an overproduction of the MexAB-OprM efflux pump, suggesting a role  
536 for heightened efflux in resistance against this furanone [92]. This work was further supported  
537 by the work of García-Contreras *et al.* who, in 2013, assessed the effect of C-30 on virulence  
538 factor production in 50 clinical strains of *P. aeruginosa* isolated from cystic fibrosis patients.  
539 They found that while many clinical isolates responded to C-30 with greatly reduced elastase  
540 and pyocyanin production, several strains showed either no decrease in virulence factor  
541 production, or exhibited an increase in production of greater than 100 %. One strain in  
542 particular IP-42 exhibited significantly higher C-30 efflux capacity, while two other strains had  
543 significantly decreased C-30 uptake. While the reason for this decreased uptake is not known,  
544 the authors suggested a possible mutation in a transporter protein which they say “have not  
545 yet been identified” [93]. Despite many strains responding to C-30 as expected, with  
546 reductions in virulence factor expression, the authors suggested that furanones should be



547 used with caution in the clinical setting as many resistant strains may already exist, with—in  
548 extreme cases—the furanone compound exacerbating the infection.

549 Resistance may also arise through mutations and changes that do not occur in the quorum  
550 sensing system “circuit”. It has been previously reported by Feltner *et al.* in 2016 that some  
551 clinical isolates of *P. aeruginosa* show variations in nucleotide sequence of the *lasR* gene of  
552 the Las QS system [94]. These isolates were obtained from cystic fibrosis respiratory cultures  
553 during the EPIC study [95], and when their *lasR* genes sequenced, 22% of the genes encoded  
554 a LasR protein that was significantly different to that of laboratory strains of *P. aeruginosa*  
555 also analysed. The mutations included insertions, deletions and single nucleotide  
556 polymorphisms (resulting in either amino acid changes or stop codons leading to truncated  
557 proteins). These LasR variants were either functionally different, or non-functional (LasR  
558 deficient). However, the most interesting finding of this study was that in the LasR deficient  
559 variants there was no significant change in a number of QS related phenotypes, such as  
560 motility or pyocyanin production. The authors then showed that the expression of some QS  
561 related phenotypes was maintained by the Rhl QS system in the absence of a functional LasR  
562 protein. This study demonstrated that clinical isolates can possess mutations that cause a  
563 ‘rewiring’ of the QS systems and their regulation. This presents a significant problem for the  
564 use of furanones, and other QSI, as a potential therapeutic as targeting a specific QS system  
565 may not be an effective treatment in such QS variants.

566 While resistance to furanones and QSI in general may be the result of repeated exposure,  
567 altered compound transport into and out of bacterial cells or mutations in the QS system  
568 genes themselves it is clear that the use of furanones may not be a universally effective  
569 treatment.

570

571 [Outstanding questions](#)

572 While many advances have been made in the use of furanones as QSIs and potential  
573 therapeutics several important questions remain unanswered. Firstly, do all furanones exert  
574 their QSI effects *via* the same mechanism and what effect does furanone structure have on  
575 the mode of action? Characterisation of structural indicators of efficacy using a furanone  
576 compound library and fluorescent QS reporter strains may help to develop a method for rapid  
577 identification of potentially useful furanone compounds or provide opportunities to modify  
578 current compounds to increase efficacy.

579 If furanones are to be used in humans as a therapeutic agent the effect of therapeutic  
580 concentrations of furanones on mammalian cells must be investigated. Further  
581 experimentation is needed to elucidate any deleterious effects on human cells in relation to  
582 protein production, cell proliferation and release of inflammatory mediators. Additionally,  
583 will the positive effects seen with *in vitro* studies and in the small number of mouse models  
584 translate to use in humans? If furanones are not shown to be detrimental to human cell lines,  
585 will their efficacy translate to systemic use or will drug activity be lost due to their metabolism  
586 in the liver or clearance by the kidneys?

587 While furanones are thought to be effective against pathogens at non-lethal concentrations,  
588 it is unclear if resistance to furanones can be avoided by using sub inhibitory concentrations?  
589 What are the potential mechanisms of furanone resistance? For example, would mutations  
590 in Las and Rhl receptors result in furanones not being able to bind effectively, or does  
591 increased efflux capacity allow the cells to better remove furanones? If resistance is  
592 unavoidable, are there methods by which the mechanisms of resistance could be subverted?

593 Another important consideration is the route of administration of these compounds. How  
594 could furanones be administered effectively to combat infections in which quorum sensing  
595 and quorum sensing controlled behaviours are a significant problem? Does method of  
596 delivery significantly impact the efficacy of the compounds?

597 Finally, there is the issue of off target effects arising from furanone therapy. What, if any, are  
598 the effects of topical and systemic use of quorum sensing inhibitors on the microbiome? It is  
599 known that many microbial communities, such as those found on the skin and in the gut, are  
600 dependent on effective quorum sensing and, to date, it is unclear what the effect of using  
601 quorum sensing inhibitors such as furanones on these communities would be.

602

### 603 [Concluding remarks](#)

604 Quorum sensing is a method of cell–cell communication responsible for the production of  
605 numerous virulence factors and the coordination of complex bacterial behaviours such as  
606 biofilm formation. Quorum sensing and its related processes have been shown to be involved  
607 in the formation and maintenance of a range of clinically relevant infections. In recent years  
608 numerous compounds, including furanones, have been investigated for their ability to  
609 interfere or inhibit the process of quorum sensing. Furanones have been demonstrated to  
610 significantly decrease virulence factor production and biofilm formation in a range of  
611 environmental and clinical pathogens. However, concerns have been raised surrounding both  
612 the potentially negative effects of furanones on human cells and the possibility of developing  
613 resistance. The material considered in this review clearly demonstrates that while both  
614 natural and synthetic furanones have great potential as novel antimicrobial therapeutics,  
615 there are a number of questions which still need to be addressed. More focused studies on

616 the efficacy of furanones as therapeutic agents in animal models are required as well as  
617 research into novel delivery methods for the compounds themselves. Critically, investigations  
618 are also needed regarding possible adverse effects on mammalian cells. If these issues are  
619 addressed it is clear that furanones may prove to have excellent potential in combatting  
620 bacterial infections in an age of widespread antimicrobial resistance.

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622 Statement of Conflict

623 The authors note no conflict of interest.

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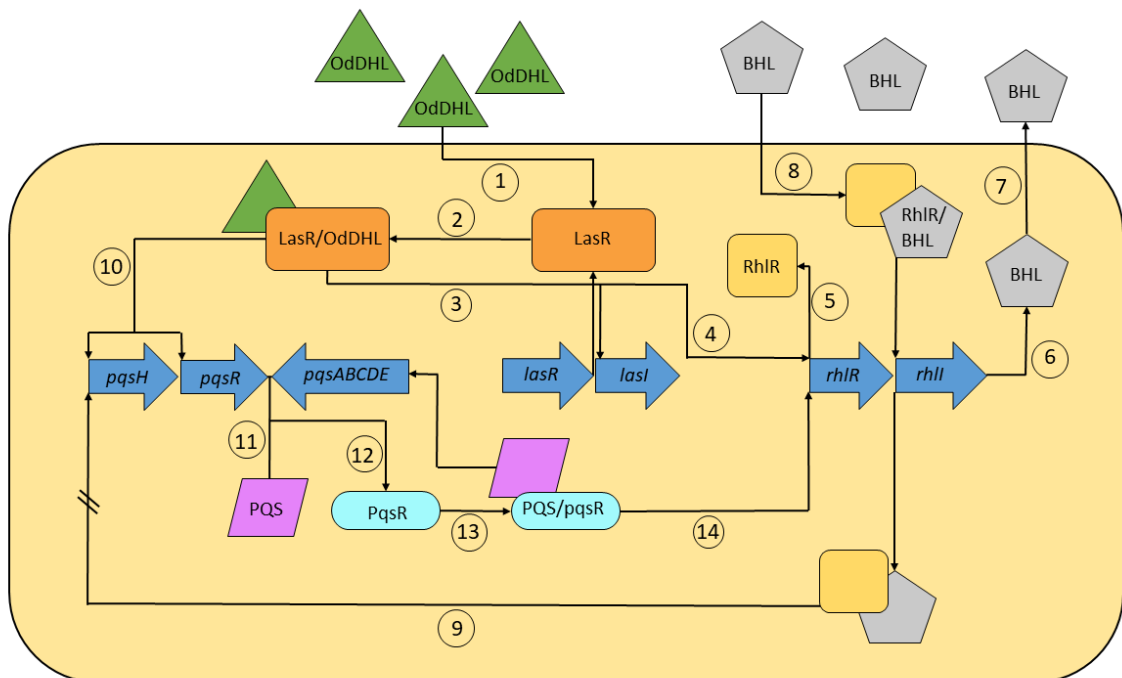
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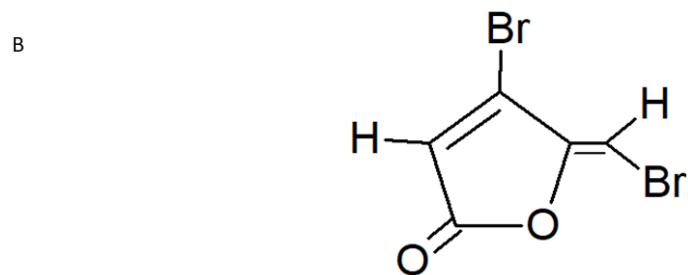
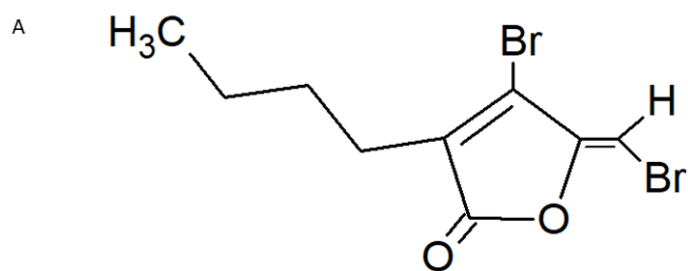
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914 Figure 1



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920 Figure 2



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