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Structure–activity relationship of brominated 3-alkyl-5-methylene-2(5*H*)-furanones and alkylmaleic anhydrides as inhibitors of *Salmonella* biofilm formation and quorum sensing regulated bioluminescence in *Vibrio harveyi*

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ABSTRACT

A library of 25 1′-unsubstituted and 1′-bromo or 1′-acetoxy 3-alkyl-5-methylene-2(5*H*)-furanones and two 3-alkylmaleic anhydrides was synthesized using existing and new methods. This library was tested for the antagonistic effect against the biofilm formation by *Salmonella* Typhimurium and the quorum sensing regulated bioluminescence of *Vibrio harveyi*. The length of the 3-alkyl chain and the bromination pattern of the ring structure were found to have a major effect on the biological activity of the 1′-unsubstituted furanones. Remarkably, the introduction of a bromine atom on the 1′ position of the 3-alkyl chain did drastically enhance the activity of the furanones in both biological test systems. The introduction of an acetoxy function in this position did in general not improve the activity. Finally, the potential of the (bromo)alkylmaleic anhydrides as a new and chemically easily accessible class of biofilm and quorum sensing inhibitors was demonstrated.

1. Introduction

In the last decade, we faced a rapid increase in pathogenic bacteria that were resistant to antibiotics. This resistance was a result of two factors. First, by blocking the growth, antibiotics placed the bacteria under harsh selective pressure to develop resistance. Second, bacteria that grew in specialized surface-attached communities, called biofilms,[1] had an innate tolerance to antibiotics. Bacteria in biofilms were shown to be up to 1000-fold more resistant to antibiotics than their planktonic counterparts.[2] This is of great concern since, according to the National Institutes of Health, approximately 80% of persistent bacterial infections in the US are associated with biofilms.[3]

In an attempt to tackle this problem, strategies have been pursued to find alternative drugs, referred to as 'anti-pathogenic' drugs,[4] that selectively block virulence and/or biofilm formation, without affecting the planktonic growth of the bacteria. The halogenated furanones isolated from the macro-algae *Delisea pulchra* and their synthetic analogues are providing to be promising candidates in the search for anti-pathogenic drugs as they have been shown to act as inhibitors of biofilm formation for several bacterial species.[5–11] Interference with the *N*-acyl-L-homoserinelactone (AHL) and autoinducer 2 (AI-2) mediated quorum sensing systems (cell–cell-communication systems) has been proven as a mechanism for this reduced biofilm formation, at least in a subset of the bacterial species tested.[8,12–14] As in some species virulence is also mediated by quorum sensing, halogenated furanones have also been shown to block virulence in these species.[15–21] Despite the fact that considerable effort has been made to study the biological effects of a small group of halogenated furanones (especially (*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone and (*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone), only a few studies have focused on determining elaborate structure–activity relationships.[9,10,22–25] Thus, for this study we focused on determining elaborate structure–activity relationships by synthesizing a broad range of 25 1′-unsubstituted and 1′-subsituted 3-alkyl-5-methylene-2(5*H*)-furanones. All of these furanones have been previously described, except for the mono-brominated furanones 3-hexyl-5-bromomethylene-2(5*H*)-furanone **11c** and

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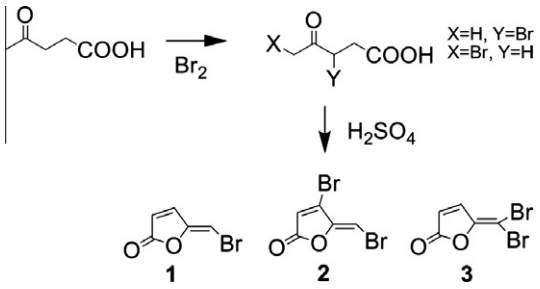
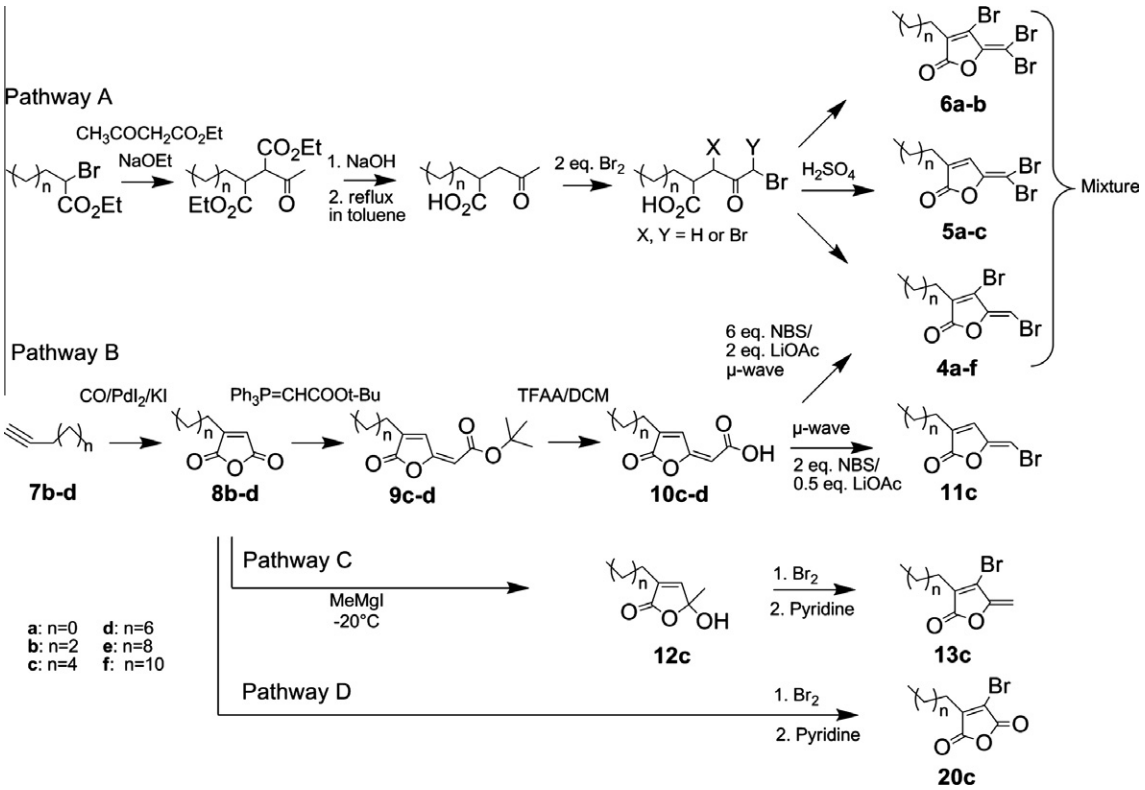


Scheme 1. Reaction scheme for the synthesis of furanones **1**–**3**, adapted from Kumar and Read (2002).

3-hexyl-4-bromo-5-methylene-2(5*H*)-furanone **13c** (Scheme 2). These mono-brominated compounds could not be synthesized by using existing procedures. Furthermore the known synthesis pathways towards halogenated furanones have some important drawbacks regarding environmental friendliness and selectivity. These shortcomings prompted us to develop a new, more versatile, synthesis procedure. We evaluated the biological activity of the halogenated furanones and some 3-alkylmaleic anhydrides, which are intermediates in the new synthesis pathway, in two model systems: biofilm formation by *Salmonella enterica* Typhimurium and quorum sensing by *Vibro harveyi*. In an iterative process of synthesis and biological testing, we tried to identify the features of the molecules mediating their biological response.

As a first biological model system, we used the biofilm formation by *Salmonella* Typhimurium. *Salmonella enterica* is worldwide one of the most prevalent food borne pathogens. Most patients infected develop a self-limiting gastroenteritis, however, in severe cases the infection may spread to the blood stream and can cause death unless the patient is treated with antibiotics. While non-typhoid *Salmonella* infections are commonly treated with fluoroquinolones such as ciprofloxacin and third-generation cephalosporins (such as ceftriaxone), there are alarming reports concerning the development of resistance against these antibiotics.[26] In addition, *Salmonella* has been able to form biofilms on different surfaces, ranging from abiotic surfaces (e.g., concrete, plastics, glass, polystyrene,...),[27] to biotic surfaces (gallstones,[28] plant surfaces,[29] and epithelial cell layers[30]). These biofilms are an important survival strategy in all stages of infection, from transmission to chronic infection. Alternative anti-*Salmonella* strategies are urgently needed. Previously, we reported that several furanones were able to inhibit *Salmonella* biofilm formation at non-growth-inhibiting concentrations and that pre-treatment with furanones rendered the biofilms more susceptible to antibiotics.[10] Others also recently reported that the effect of disinfectants on *Salmonella* in biofilms is significantly enhanced in the presence of furanones.[31] Although halogenated furanones are generally considered to interfere with the quorum sensing systems of gram-negative bacteria, no evidence was found that furanones act on the currently known quorum sensing systems of *Salmonella*.[10] Since (*Z*)-4-bromo-5-bromomethylene-3-alkyl-2(*5H*)-furanones with chain lengths of two to six carbon atoms were found to be the most interesting compounds in our previous study, these furanones served as structural templates for the development of more potent analogues in this study.

We also wanted to test the influence of our library of halogenated furanones on a test system, which has been shown to be regulated by quorum sensing. Therefore, as a second model system we made use of the quorum sensing regulated bioluminescence of *V. harveyi*. This marine bacterium and closely related species, such



Scheme 2. Reaction schemes for the synthesis of 3-alkyl-5-methylene-2(*5H*)-furanones **4a**–**f**, **5a**–**c**, **6a**–**b**, **11c** and **13c** and 3-alkyl-4-bromo-maleic anhydride **20c**. Pathway A: procedure developed by Manny et al. (1997). Pathway B: new procedure for the synthesis of (*Z*)-3-alkyl-4-bromo-5-bromomethylene-2(*5H*)-furanones and (*Z*)-3-alkyl-5-bromomethylene-2(*5H*)-furanones. Pathway C: pathway for the synthesis of 3-alkyl-4-bromo-5-methylene-2(*5H*)-furanones. Pathway D: pathway for the synthesis of 4-bromo-3-alkylmaleic anhydrides.

as *Vibrio campbellii*, are important pathogens in the intensive rearing of marine fish and invertebrates like penaeid shrimp. Due to large-scale (mis)use of antibiotics in aquaculture, multi-resistant *Vibrio* strains have emerged and antibiotics are no longer effective in the treatment of luminescent vibriosis.[32] Therefore, alternative methods will need to be developed to prevent the outbreak of vibriosis in aquaculture.[21] One possible strategy could be interference with quorum sensing. *V. harveyi* makes use of three parallel quorum sensing systems (resp. HAI-1, CAI-1 and AI-2 system) that converge to control the production of the master regulator LuxR,[33] which regulates the expression of phenotypes such as bioluminescence, siderophore production, biofilm formation, and virulence. A natural furanone as well as several synthetic analogues have been shown to inhibit luminescence and virulence of *Vibrio* species.[5,8,19,20,24,34–37] Recently, the natural furanone was shown to decrease the DNA-binding capacity of the master regulator LuxR.[36] However, in vivo experiments revealed toxic effects of this natural furanone and the synthetic furanone C-30 at low concentrations against the rotifer *Brachionus plicatilis*, the brine shrimp *Artemia franciscana,* and the rainbow trout.[19,20,35] Therefore, we screened our library of furanones for their ability to inhibit luminescence production of *V. harveyi*, in an attempt to find analogues with a higher anti-quorum sensing activity and possibly a higher therapeutic index.

2. 3-Alkyl-5-methylene-2(*5H*)-furanones

2.1. Chemistry

The brominated furanones **1**–**3** without a 3-alkyl chain were synthesized by using an adaptation of the protocol of Kumar and Read (2002), as shown in Scheme 1. The brominated 3-alkyl-5-methylene-2(*5H*)-furanones **4a**–**f**, **5a**–**c** and **6a**–**b**, shown in Scheme 2, were initially synthesized by the method developed by Beechan and Sims[38] and reinvestigated by Manny et al.[39] Although this pathway, depicted in Scheme 2 (pathway A), is the most versatile procedure available, it has some major drawbacks. First, the bromination step (step 3) and the acid-catalyzed oxidative cyclodehydration step (step 4) make use of harsh, environmentally unfriendly conditions. Second, the cyclodehydration step is not selective, as mixtures of different di- and tribrominated compounds are formed, resulting in ratios that are difficult to control. This means that extensive purification and separation of compounds are required. Finally, mono-brominated compounds that are specifically brominated at the 4-position of the ring or the methylene position are not (or only in trace amounts) observed in the reaction mixture. The latter is especially problematic in the context of this study, since our previous results indicate a drastic influence of the bromination pattern of the ring structure on the biological activity of the compounds.[10] To study the influence of the bromination pattern more in detail, a selective synthesis of these mono-brominated furanones was needed. Taking these shortcomings of the Manny synthesis into account, it was decided to develop a new synthesis procedure.

This new synthesis pathway, shown in Scheme 2 (pathway B), is built around the Wittig reaction on maleic anhydride derivatives. The first step consisted of the synthesis of an alkylmaleic anhydride **8b**–**d** starting from the corresponding commercially available alkyne **7b**–**d**. The reaction was performed in an atmosphere composed by CO/CO_2/air with the following proportions: 3/8/1.[40] The couple PdI_2/KI was used as catalytic system. Maleic anhydrides with alkyl chain lengths of four (**8b**), six (**8c**) and eight (**8d**) carbon atoms have been synthesized, with yields around 60–70%. The second step, the Wittig reaction,[24,41] required the use of a protected ylide.[42] The coupling reaction with anhydrides **8c**–**d** gave a conversion yield of 95% with a selectivity of 90% of the desired isomers **9c**–**d**, according to the literature.[42] The crude product was directly used in the deprotection step, which employs trifluoroacetic acid in dry chloroform. The final step consists of a one-pot Hunsdiecker bromination/debromocarboxylation and a bromination/dehydrobromination promoted by microwave, adapted from a simple bromination/dehydrobromination procedure.[43] The brominating agent in the reaction is *N*-bromosuccinimide (NBS) and the base used for dehydrobromination is lithium acetate (LiOAc). When we used an excess of NBS of 6 equiv and an excess of lithium acetate of 2 equiv, this reaction permitted us to obtain up to 30% yield of the dibrominated compounds **4c**–**d**. Because of the difference in reactivity of the two double bonds, we could easily perform the selective bromination of the methylene position. In the microwave promoted reaction, we simply decreased the proportion of NBS to 2 equiv and LiOAc to 0.5 equiv, leading to 45% yield of product **11c**. With this synthesis pathway, we can produce compounds **4** and **11** with any 3-alkyl side chain, except for a methyl group because of possible bromination of the methyl group.[9] Conclusively, these four steps gave stable products, with good yields and predictable results. Moreover, the last step is relatively selective and permits some adaptations. Full details and insights in this new route will be published in an upcoming paper.

In order to synthesize mono-brominated 3-alkyl-2(5*H*)-furanones that are selectively brominated at the 4-position of the ring, we followed an alteration of an existing synthesis pathway,[44] also starting from the anhydride. As depicted in Scheme 2 (pathway C), the first reaction is a classical Grignard reaction implying an anhydride with methylmagnesium iodide as Grignard agent. Yields of compound **12c** evolved around 50%, as expected from the literature.[44] The second reaction is the bromination of the double bound using an excess of Br_2. After the reaction, we surprisingly found that a dehydrobromination and a dehydration of the molecule occurred in the same reaction mixture which had been treated with Br_2. This unexpected behaviour gave us directly the desired compound **13c**, with 24% yield. This allowed us to avoid the dehydrobromination and the dehydration steps.

Finally, it was attempted to simplify the structure of the active molecules. Considering all efforts required to prepare furanones starting from anhydrides, it was decided to test the unbrominated alkylmaleic anhydride **8c** and the brominated alkylmaleic anhydride **20c**, which has, to the best of our knowledge, never been described before. The synthesis pathway used for **20c** is based on two reactions already described, as shown in Scheme 2 (pathway D).

2.2. Biological results

Firstly, the activity of the furanones **4a**–**c**, **5a**–**c**, **6a, 11c** and **13c** on biofilm formation of *S.* Typhimurium ATCC14028 was tested, using a 96-well microtiter plate assay with polystyrene pegs and crystal violet staining. In our previous study, which was focused on the effect of the length of the 3-alkyl chain, we found that compounds with ethyl, butyl, and hexyl side chains have the most interesting activity as they show a broad concentration range in which they inhibit biofilm formation without affecting the planktonic growth. Compounds without a 3-alkyl chain were found to be toxic to the planktonic cells, while compounds with a longer side chain were shown not to reduce biofilm formation.[10]

In this study, focus is on the effect of the bromination pattern of the ring structure on the activity of the furanones with the most interesting side chain lengths (ethyl, butyl and hexyl). Table 1 lists the concentrations of furanones needed to inhibit *Salmonella* biofilm formation by 50% (IC_{50}s). Within this range from ethyl to hexyl, the length of the alkyl chain does not have a major effect on the antagonistic activity. However, the bromination pattern of the ring has a large impact on the biofilm inhibition. Dibrominated

Table 1
Antagonistic effect of compounds **4**–**6**, **11c**, **13c**, **8c** and **20c** against *S.* Typhimurium biofilm formation and planktonic growth

Compound	IC_{50}[a] (μM)	95% Confidence interval for IC_{50}	Effect on growth at 250 μM	Effect on growth at 100 μM	Effect on growth at 10 μM
4a	17.91	12.57–25.54	+[b]	+–[c]	–[d]
5a	199.9	126.6–315.6	–	–	–
6a	57.46	36.17–91.29	+–	–	–
4b	23.12	14.52–36.80	+	–	–
5b	148	64.76–338.3	nd[e]	nd	nd
4c	10.74	8.056–14.33	+	+–	–
5c	160.1	103.4–247.8	nd	nd	nd
11c	>1000		nd	nd	nd
13c	19.42	12.97–29.09	+	+	–
8c	>1000		nd	nd	nd
20c	65.89	42.90–101.2	–	–	nd

[a] IC_{50}: Concentration of inhibitor needed to inhibit biofilm formation by 50%.
[b] +: The planktonic growth is completely or almost completely inhibited when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor.
[c] +–: The planktonic growth is slightly slowed down when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor.
[d] –: The planktonic growth is not affected when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor.
[e] nd: Not determined.

compounds **4a**–**c** with one bromine atom on the 4-position of the ring and one bromine atom on the methylene group (di1-configuration) are much more active than, respectively compounds **5a**–**c** with a dibrominated methylene group (di2-configuration). Moreover, the mono-brominated compound **13c** with its bromine atom on the 4-position had a similar activity as compound **4c**, while the mono-brominated compound **11c** with its bromine atom on the methylene group was not active at the highest concentration tested. This indicates that the bromine at position 4 is crucial for the biofilm inhibitory activity. To validate that the compounds are true inhibitors of biofilm formation and not acting as bactericidal agents, growth curves were determined in the presence of different concentrations of the most active furanones by measuring the OD_{600} in function of time using an automated system (Bioscreen, Oy Growth Curves Ab Ltd). All the furanones tested inhibited biofilm formation by 50% at a concentration that did not affect planktonic growth (Table 1).

The unbrominated maleic anhydride **8c**, which is an intermediate in the new synthesis pathway towards halogenated furanones, did not inhibit biofilm formation at the highest concentration tested. However, the C4-brominated maleic anhydride **20c,** inhibited *Salmonella* biofilm formation with an IC_{50} of 65.89 μM (Table 1), which is about six times higher than the IC_{50} of the corresponding 3-hexyl-2(5*H*)furanone **4c**. Nevertheless, this is a very interesting result as **20c** is chemically much more easily available. Moreover, this compound did not affect the planktonic growth of *Salmonella* at 250 μM, which was the highest concentration tested.

The ability of furanones **1**–**3**, **4a**–**f**, **5a**–**c**, **6a**–**b** and **13c** to inhibit the quorum sensing regulated bioluminescence of *V. harveyi* BB120 was also investigated. Both the IC_{50} values (concentrations inhibiting the luminescence expression by 50%) and the GIC_{50} values (concentrations reducing the growth (OD_{600}) of the *V. harveyi* cultures by 50%) are listed in Table 2. The compounds **1**–**3** without side chain and the compounds **4a** and **6a** with an ethyl side chain inhibited the bioluminescence expression at low concentrations, but also inhibited the growth at low concentrations. Also the tribrominated compound **6b** with a butyl side chain was highly toxic for the bacteria. For these compounds, inhibition of the luminescence could at least be partly due to a reduction of the cell number. However, all mono- and dibrominated compounds with alkyl chains of 4 to 10 carbon atoms showed a clear concentration range with only inhibition of bioluminescence and no effect on the growth. Only compound **4f**, with a dodecyl side chain, did not affect the bioluminescence, which could be explained by a low solubility in the growth medium and/or difficult access to the cell. In accordance with the *Salmonella* biofilm inhibitory activity, the effect on the bioluminescence was clearly influenced by the bromin-

Table 2
Antagonistic effect of compounds **1**–**6**, **8c**, **13c** and **20c** against *V. harveyi* bioluminescence

Compound	IC_{50}[a]	95% Confidence interval	GIC_{50}[b]	95% Confidence interval
1	4.071	3.207–5.168	15.7	13.22–18.65
2	2.708	1.687–4.348	3.155	2.854–3.489
3	2.878	1.640–5.051	25.51	15.99–40.68
4a	9.268	8.686–9.889	10.79	10.46–11.12
5a	104.6	81.88–133.6	325	232.8–453.6
6a	1.152	1.000–1.328	2.264	1.991–2.574
4b	1.362	1.167–1.589	65.27	56.44–75.48
5b	18.9	17.18–20.80	140.9	124.2–159.8
6b	14.36	13.38–15.41	20.5	19.31–21.77
4c	11.91	10.35–13.69	34.04	28.66–40.44
5c	84.05	47.98–147.2	197.4	164.3–237.1
4d	3.414	2.522–4.623	267	86.85–820.6
4e	26.62	19.81–35.78	>300	
4f	–[c]	–	–	–
13c	1.357	1.046–1.761	36.30	30.49–43.22
8c	97.77	89.94–106.3	639.5	538.9–759.0
20c	10.56	8.257–13.51	208.3	185.9–233.5

[a] IC_{50}: Concentration of inhibitor needed to inhibit luminescence by 50%.
[b] GIC_{50}: Concentration of inhibitor needed to inhibit bacterial growth by 50%.
[c] –: No effect observed at the highest concentration tested (1000 μM).

ation pattern of the ring structure. Dibrominated molecules **4a–c** with a di1-configuration showed a higher activity than, respectively compounds **5a–c** with a di2-configuration. Interestingly, the chemically easily accessible 3-bromo-4-hexylmaleic anhydride **20c** also inhibited the bioluminescence of *Vibrio* at low concentrations (IC_{50} = 10.56 μM).

Compound **4b** has previously been shown to have a similar impact on the bioluminescence of wildtype *V. harveyi* BB120 and the constitutively luminescent mutant strains JAF553,[45] JAF483,[45] and BNL258,[46] which are affected in the genes encoding, respectively LuxU, LuxO and Hfq.[36] LuxU is the most upstream protein in the common part of the signal transduction pathway that is shared by the 3 quorum sensing systems, while LuxO is located immediately downstream of LuxU. Hfq is a chaperone, which plays a role at the end of the pathway, immediately upstream of the response regulator LuxR. The fact that compound **4b** still blocked bioluminescence in the Δ*hfq* mutant was given as evidence by Defoirdt et al. that this compound acts downstream of Hfq, that is, at the level of the *luxR* mRNA and/or the LuxR protein.[36] In order to get a tentative idea whether the mono-brominated furanone **13c** and the brominated anhydride **20c** act on the same target as the dibrominated furanone **4b**, we compared the influence of these 3 compounds on the bioluminescence of wildtype *V. harveyi* BB120 and the mutant strains JAF553, JAF483 and BNL258. As depicted in Figure 1, compounds **13c** and **20c** had a similar effect on the bioluminescence of the different strains as observed for compound **4b**. From this we may conclude that compounds **13c** and **20c** like compound **4b** act downstream of Hfq.

The potential application of the compounds as anti-pathogenic drugs depends on the therapeutic window between the IC_{50} for biofilm or quorum sensing inhibition and the toxicity in eukaryotic cells. To compare the cytotoxicity of the new compounds **13**c and **20c** to the previously described compound **4a**, we determined the influence of the three compounds on the proliferation of five eukaryotic cell lines. As shown in Table 3, the new compounds do not seem to have an improved therapeutic index as compared to compound **4a.**

3. 1′-Substituted 3-alkyl-5-methylene-2(*5H*)-furanones

3.1. Chemistry

To study the influence of functionalization of the alkyl chain, compounds **14a–c**, **15a–b**, **16a** and **17–19** with a bromine atom or acetoxy group on the 1′ position of the side chain were synthesized using methods developed by Kumar and Read,[22] as shown in Scheme 3. Since the furanones with an ethyl, butyl and hexyl side chain showed the most interesting activity, the functionalization was only performed on furanones with these particular side chain lengths. Briefly, bromination of the side chain was performed via a radical reaction with NBS in CCl_4 in the presence of small amounts of the initiator benzoyl peroxide. The reaction mixture was irradiated with light and refluxed during an 18 h period. After purification, some of the 3-(1′-bromoalkyl)-5-methylene-2(*5H*)-furanones were converted into 3-(1′-acetoxyalkyl)-5-methylene-2(*5H*)-furanones via a nucleophilic substitution with sodium acetate in acetic acid.

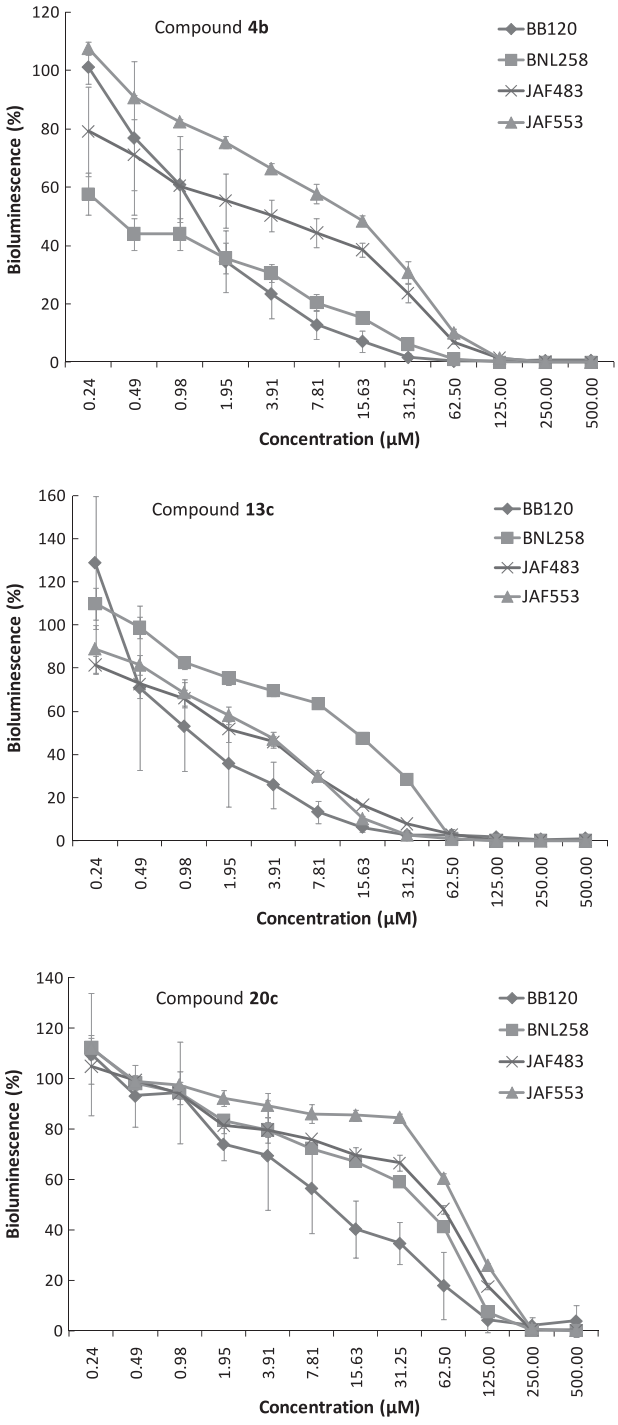


Figure 1. Influence of a dilution series of compounds **4b**, **13c** and **20c** on the bioluminescence of wildtype *V. harveyi* BB120 and the mutant strains BNL258 (*hfq*::Tn*5lacZ*), JAF483 (*luxO* D47A linked to Kan^R) and JAF553 (*luxU* H58A linked to Kan^R). The bioluminescence of each strain in the presence of a compound is expressed as a percentage compared to an untreated control for that strain.

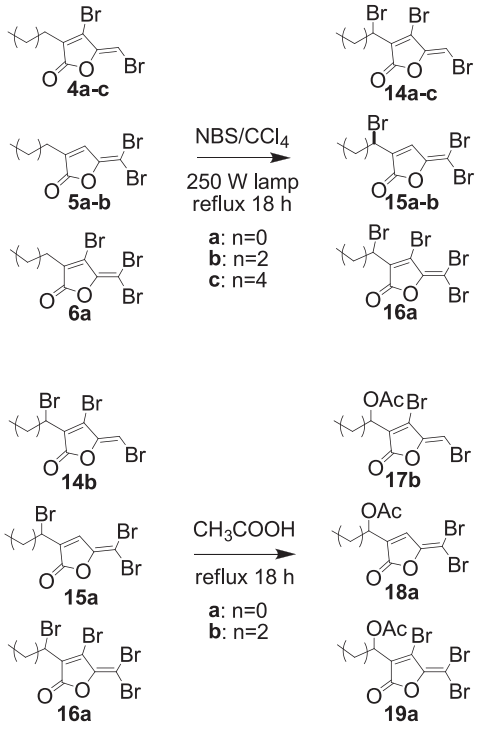
3.2. Biological results

As shown in Table 4 the molecules with a bromine atom on the side chain were remarkably more active in the *Salmonella* biofilm inhibition assay than the molecules without this substitution. Surprisingly, there was no clear difference in activity between the molecules with a di1- or di2-bromination pattern of the ring structure, which indicates that the presence of a bromine atom on the alkyl chain overrules the necessity of a bromine atom on the 4-position of the ring. The increased activity could be explained by the hypothesis that the target receptor binds covalently to the halogenated furanones via a nucleophilic substitution of a bromine atom. The bromine atom on the side chain of the furanone is much more prone to nucleophilic substitution than the other bromine atoms. Indirect support for this hypothesis is provided by Hjelmgaard et al., who tested an array of brominated and non-brominated furanones for

Table 3
Inhibitory effect of compounds **4a**, **13c** and **20c** on the proliferation of murine leukemia cells (L1210/0), murine mammary carcinoma cells (FM3A) and human T-lymphocyte cells (CEM/0), human cervix carcinoma cells (HeLa) and human osteosarcoma cells (OST TK$^-$)

Compound	IC_{50}[a] (μM)				
	L1210/0	FM3A/0	CEM/0	Hela	OST TK$^-$
4a	284 ± 9	>500	208 ± 191	39 ± 0	6.3 ± 3.2
13c	8.5 ± 0.4	16 ± 2	7.5 ± 2.3	48 ± 20	2.0 ± 0.9
20c	208 ± 13	243 ± 17	90 ± 49	156 ± 31	43 ± 7

[a] IC_{50}: Concentration of compound needed to inhibit the proliferation by 50%.



Scheme 3. Synthesis of 1′-bromo- and 1′-acetoxy-3-alkyl-2(5*H*)-furanones with different bromination patterns of the ring structure.

quorum sensing antagonism and found that the brominated compounds were much more efficient.[23] This hypothesis is further supported by the finding of Zang et al. that furanone **4b** is able to covalently modify purified recombinant *Bacillus subtilis* LuxS enzymes (which is the synthase of AI-2).[14] However, in *Salmonella* the target is probably not LuxS, as our previous experiments showed that the furanones do not affect the expression of AI-2 regulated genes in *Salmonella*.[10] Bioscreen analysis revealed that the molecules **14a** and **15a**–**b** retarded the planktonic growth of the bacteria slightly at concentrations near the IC_{50} value for biofilm inhibition, which means that it cannot be excluded that biofilm formation is (at least partly) inhibited by reducing the number of planktonic cells before the biofilms are established. However, molecules **14b**–**c** are clearly selective biofilm inhibitors as they showed a wide concentration window with only biofilm inhibition and no effect on the planktonic growth. Molecules **17b** and **18a** with an acetoxy function at the first carbon atom of the side chain did not show an improved activity as compared to their unsubstituted counterparts. Compound **19a** did have a slightly improved activity.

Similar structure–activity relationships were found in the case of the inhibition of the quorum sensing regulated bioluminescence of *V. harveyi* (Table 5). In general introduction of a bromine atom on the side chain improved the anti-bioluminescence activity. This effect was most pronounced for the compounds **15a**–**b** with a di2-configuration. However, all compounds with a bromine on the side chain also inhibited the growth of the bacteria, starting from concentrations that are similar or only slightly higher than the concentrations at which inhibition of bioluminescence occurred (the GIC_{50} is only slightly higher than the IC_{50}). This means that the bioluminescence inhibition is partly due to a reduction of the cell number. Nevertheless, we continue to observe a dose-dependent inhibition when the output is expressed as relative light units (RLU) that is, light intensity divided by OD_{600} (data not shown). This could suggest that these 1′-brominated furanones have a specific, non-growth related effect on the bioluminescence. As in the case of *Salmonella* biofilm inhibition, substitution with an acetoxy group did not improve the activity.

In conclusion, we synthesized a library of 25 1′-unsubstituted and 1′-subsituted 3-alkyl-5-methylene-2(*5H*)-furanones and two 3-alkylmaleic anhydrides and tested their antagonistic effect against the biofilm formation by *S.* Typhimurium and the quorum sensing regulated bioluminescence of *V. harveyi*. Firstly, we found that the length of the 3-alkyl chain plays an important role in the antagonistic activity.[10] The molecules without a 3-alkyl chain were shown to be highly toxic for both *Salmonella* and *Vibrio*, while the 1′-unsubstituted furanones with a long 3-alkyl chain did not reduce biofilm formation (octyl chain and longer) nor bioluminescence (dodecyl chain). However, the 1′-unsubstituted furanones with ethyl, butyl or hexyl side chains inhibited biofilm formation at low concentrations, without affecting the planktonic growth at these concentrations. Similarly, the 1′-unsubstituted furanones with a butyl to decyl side chain inhibited bioluminescence without affecting the planktonic growth of the bacteria at the same concentrations. Secondly, the bromination pattern of the ring structure was shown to have a large influence on the antagonistic activity

Table 4
Antagonistic effect of compounds **14**–**19** against *S.* Typhimurium biofilm formation and planktonic growth

Compound	IC_{50}[a] (μM)	95% Confidence interval for IC_{50}	Effect on growth at 250 μM	Effect on growth at 100 μM	Effect on growth at 10 μM	Effect on growth at 1 μM
14a	4.505	3.170–6.401	+[b]	+	+–	–
15a	1.166	0.8035–1.693	+	+	+	–
16a	4.094	2.679–6.256	+–[c]	+–	+–	–
14b	5.714	4.503–7.251	+	+	–	–
15b	1.31	1.115–1.541	+	+	+–	–
14c	3.235	2.389–4.379	+–	–	–	–
17b	131.6	96.03–180.5	–[d]	–	–	–
18a	161.6	124.4–209.8	+–	–	–	–
19a	7.295	3.375–15.77	+	+–	–	–

[a] IC_{50}: Concentration of inhibitor needed to inhibit biofilm formation by 50%.
[b] +: The planktonic growth is completely or almost completely prevented when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor.
[c] +–: The planktonic growth is slightly slowed down when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor.
[d] –: The planktonic growth is not affected when the bacteria are grown in the presence of the indicated concentration of biofilm inhibitor.

Table 5
Antagonistic effect of compounds **14**–**19** against *V. harveyi* bioluminescence

Compound	IC_{50}[a]	95% Confidence interval	GIC_{50}[b]	95% Confidence interval
14a	6.339	5.700–7.050	16.14	14.49–17.97
15a	0.7742	0.6119–0.9795	3.073	2.639–3.579
16a	2.761	2.379–3.205	9.738	8.571–11.07
14b	1.180	0.9382–1.484	4.042	3.451–4.735
15b	0.1999	0.1459–0.2740	1.074	0.9306–1.240
14c	5.207	4.273–6.345	7.271	6.401–8.260
17b	50.05	36.05–69.48	147.2	136.5–158.7
19a	6.311	5.640–7.062	25.38	22.16–29.06

[a] IC_{50}: Concentration of inhibitor needed to inhibit luminescence by 50%.
[b] GIC_{50}: Concentration of inhibitor needed to inhibit bacterial growth by 50%.

of the 1′-unsubstituted furanones: the dibrominated compounds with one bromine atom on the 4-position of the ring and one bromine atom on the methylene group (di1-configuration) were much more active than the compounds with a dibrominated methylene group (di2-configuration), both in the *Salmonella* and *Vibrio* test system. Furthermore, we found that the introduction of a bromine atom on the first carbon atom of the alkyl side chain drastically improved the activity of the furanones in both test systems. This effect was most pronounced for the molecules with a di2-configuration. On the other hand, in general the placement of an acetoxy function at this position did not improve the activity of the molecules. Finally, we demonstrated the potential of the bromoalkylmaleic anhydrides as a new and easily accessible class of biofilm and quorum sensing inhibitors.

4. Experimental section

4.1. Chemistry

All reagents used for synthesis were purchased from commercial sources and used without further purification. Chromatography was performed using a Biotage flash Purification system SP1 and adapted cartridges KP-Sil. NMR solvents were obtained from Acros. ^1H NMR and ^{13}C NMR spectra were recorded at room temperature on a 300 MHz Bruker spectrometer. Chromatograms and mass spectra were obtained from an Agilent 6890N GC–MS. Microwave enhanced reactions were performed on a CEM Single-Mode Microwave oven.

4.1.1. General procedure for 5-methylene-2(*5H*)-furanones without 3-alkyl side (Scheme 1)

The molecules were prepared following a method adapted from a procedure described in the literature.[47] 4-Oxopentanoic acid was brominated with an equimolar amount of Br_2 to yield mono-brominated 4-oxopentanoic acid. Subsequently this crude product was cyclized using 100% H_2SO_4 to yield a mixture of compounds **1**–**3**. Analytical data of the compounds are provided as Supplementary data.

4.1.2. General procedure for 3-alkyl-5-methylene-2(*5H*)-furanones via protocol of Manny et al. (1999) (Scheme 2 (pathway A))

The molecules were prepared following a procedure described in the literature.[39] Analytical data of the compounds have been provided as Supplementary data.

4.1.3. General procedure for alkylmaleic anhydrides (8b–d) (Scheme 2 (pathway B))

4.1.3.1. Oxidative carbonylation procedure. 50 ml of dioxane was poured into a 300 ml stainless steel Parr reactor. Alkyne (8 mmol), H_2O (20 mmol), KI (0.4 mmol) and PdI_2 (0.04 mmol) were added to the solvent. The Parr reactor was carefully closed and filled, respectively with 5 atm of air, 40 atm of CO_2 and 16 atm of CO. The reactor was heated at 80 °C for 40 h. After degassing, the solution was filtered. The filtrate was concentrated under vacuum. The resulting mixture was filtered on silica with DCM as eluent.

4.1.4. General procedure for 3-alkyl-4-bromo-5-(bromomethylene)-2(*5H*)-furanones and 3-alkyl-5-bromomethylene-2(*5H*)-furanones starting from alkylmaleic anhydrides (Scheme 2 (pathway B))

4.1.4.1. Wittig coupling procedure. A solution of (*tert*-butoxycarbonylmethyl)-triphenylphosphonium bromide (4.5 mmol) in DCM (10 ml) was vigorously stirred with 10 ml of a solution of Na_2CO_3 (10% w/w in water) for 30 min. The phases were separated and the aqueous phase was washed twice with DCM. The combined organic phases were concentrated under vacuum. The obtained solid was poured in a solution of alkylmaleic anhydride (4.5 mmol in 30 ml benzene). This solution was flushed with argon and then stirred during 2.5 h at reflux. After the reaction was completed, the solvent was removed under vacuum. The residue was triturated by hexane and filtrated. The filtrate was concentrated under vacuum and purified by chromatography.

4.1.4.2. Deprotection procedure. The protected furanone (5 mmol) was stirred at room temperature for 30 min in a mixture of 15 ml DCM and 7.5 ml of CF_3COOH. The solution was concentrated under strong vacuum to remove the excess of trifluoroacetic acid.

4.1.4.3. Bromination/debromocarboxylation and bromination/dehydrobromination procedure. In an adapted vial we placed the (*E*/*Z*)-2-(4-alkyl-5-oxofuran-2(*5H*)-ylidene)acetic acid compound (1 mmol) with NBS and $LiOAc{\cdot}2H_2O$. The proportions were depending on the desired product: for the synthesis of 3-alkyl-4-bromo-5-(bromomethylene)-2(*5H*)-furanones we used 6 equiv NBS and 2 equiv LiOAc; for the synthesis of 3-alkyl-4-bromo-5-methylene-2(*5H*)-furanones we used 2 equiv NBS and 0.5 equiv LiOAc. We added 2 ml of acetonitrile just before the reaction. The microwave oven was limited to 100 W of power, the cut-off pressure inside the vial was 280 psi, and the temperature was 100 °C. The program started with 2 min of gradual heating, followed by 90 s at maximum temperature. After reaction, the tube was cooled down. The solvent was removed under vacuum and the residue was purified by chromatography.

4.1.4.4. (E/Z)-2-(4-Hexyl-5-oxofuran-2(5H)-ylidene)acetic acid (10c). White solid-^1H NMR (300 MHz, $CDCl_3$): δ 8.02 (1H, s, –OH); 5.84 (1H, s, –CHCO); 2,41 (2H, t, *J* 7.5 Hz, $(H1')_2$), 1.63 (2H, q, *J* 7.4 Hz, $(H2')_2$), 1.30 (6H, m, $(H3')_2$ to $(H5')_2$), 0.88 (3H, t, *J* 6.3 Hz). ^{13}C NMR ($CDCl_3$): δ 14.2; 22.7; 25.8; 27.6; 29; 31.6; 90.8; 135.5; 136.4; 161.5; 168.6; 170.4. Mass spectrum: *m/z* 224 (M, 2.2%), 206 (5.8),180 (9.6),179 (79.7), 178 (5.6), 155 (10.6), 154 (100), 141 (13.1), 136 (52.1), 126 (20.3), 108 (16.7), 69 (32.8), 67 (12.3), 55 (16.2), 53 (13.7), 43 (46.6), 42 (10.2), 41 (35.7), 39 (21.7).

4.1.4.5. (E/Z)-2-(4-Octyl-5-oxo-furan-2(5H)-ylidene)acetic acid (10d). White solid- ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.95 (1H, s, –OH); 5.83 (1H, s, –CHCO); 2,44 (2H, td, *J* 7.3 Hz, $(H1')_2$), 1.62 (2H, q, *J* 7.4 Hz, $(H2')_2$), 1.31 (10H, m, $(H3')_2$ to $(H7')_2$), 0.90 (3H, t, *J* 6.4 Hz). ^{13}C NMR ($CDCl_3$): δ 14; 22.4; 25.8; 27.3; 28.9; 31.4; 99.7; 128.5; 134.7; 141.1; 161.5; 168.5; 170.4. Mass spectrum: *m/z* 252 (M, 2.4%), 234 (3.8), 208 (11.7), 207 (81.2), 164 (10), 155 (14.7), 154 (100), 141 (15.8), 137 (13.9), 136 (49), 126 (17.7), 108 (15.9), 69 (28.5), 67 (11.6), 57 (20.4), 55 (18.4), 43 (24.4), 41 (33.6), 39 (13.4).

4.1.4.6. 3-Hexyl-5-bromomethylene-2(5H)-furanone (11c). Light yellow solid - ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.03 (1H, s, H4); 5.95 (1H, s, 5-CHBr); 2.34 (2H, t, *J* 7.6 Hz, $(H1')_2$), 1.58 (2H, q, *J* 7.46 Hz, $(H2')_2$), 1.30 (6H, m, $(H3')_2$ to $(H5')_2$), 0.88 (3H, t, *J* 6.3 Hz). ^{13}C NMR ($CDCl_3$): δ 14.6; 22.9; 25.8; 27.7; 29.2; 31.83; 89.8; 135.3; 136.5; 151.8; 169.5. Mass spectrum: *m/z* 261 (0.4), 260 (M(^{81}Br), 2.7%), 259 (0.5), 258 (M(^{79}Br), 2.7%), 217 (0.4), 215 (0.4), 203 (2.6), 201 (2.6), 190 (18.6), 189 (4.7), 188 (20), 179 (100), 162 (5.5), 160 (5.9), 133 (4.2), 122 (7.8), 120 (7.4), 109 (12.5), 95 (3.5), 81 (5.8), 79 (3.8), 65 (5.2), 53 (5.8), 43 (11.6), 41 (10.5).

4.1.5. General procedure for 3-alkyl-4-bromo-5-methylene-2(*5H*)-furanones starting from alkylmaleic anhydrides (Scheme 2 (pathway C))

4.1.5.1. Grignard methylation procedure. To a solution of 8.9 mmol of alkylmaleic anhydride in 15 ml of anhydrous diethylether, under argon, at −20 °C, a solution of methylmagnesium iodide was added dropwise (1.1 equiv). After addition, the reaction was stirred for 2 h at the same temperature. Afterwards, the reaction was quenched with a saturated NH_4Cl solution. The biphasic mixture was extracted with ethyl acetate. The organic phase was washed with brine, dried with magnesium sulfate, and concentrated under vacuum. The residue was purified by chromatography.

4.1.5.2. Bromo/dehydrobromination procedure. 3-Alkyl-5-hydroxy-5-methyl-2(*5H*)-furanone (1 mmol) and Br_2 (1 mmol) were placed in a vial. The vial was sealed and the mixture was stirred for 3–4 days. After the reaction was completed, the vial was degassed. Then 4 ml of $CHCl_3$ solvent was added. A solution of pyridine (1 mmol) in 1 ml of $CHCl_3$ was added dropwise over 20 min. After this addition, the solution was stirred for 1.5 h. The mixture was concentrated under vacuum and the residue was filtered through silica with DCM as eluent.

4.1.5.3. 3-Hexyl-5-hydroxy-5-methyl-2(5H)-furanone (12c). Pale yellow oil - ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.64 (1H, s, –CHCO–); 5.13 (1H, broad s, –OH); 2.27 (2H, m, $(H1')_2$), 1.57 (3H, s, $-CH_3$), 1.55 (2H, q, J 7.8 Hz, $(H2')_2$), 1.26 (6H, m, $(H3')_2$ to $(H5')_2$), 0.83 (3H, t, j 6.5 Hz). ^{13}C NMR ($CDCl_3$): δ 14.4; 22.9; 24.1; 26.8; 26.9; 29.3; 31.8; 107.8; 115.6; 172.1; 173.8. Mass spectrum: *m/z* 198 (0.3%), 184 (9.4), 183 (84), 180 (2.7), 156 (5.5), 155 (54.6), 153 (2.3), 152 (6.3), 138 (3.4), 137 (18.6), 124 (5.2), 123 (8.2), 113 (19.4), 111 (30.1), 110 (46.5), 109 (33.7), 97 (13.9), 95 (31.8), 94 (16.9), 82 (21.8), 81 (55.6), 69 (27.1), 68 (31.3), 67 (41), 53 (19.5), 43 (100), 41 (37.8), 39 (27.8).

4.1.5.4. 3-Hexyl-4-bromo-5-methylene-2(5H)-furanone (13c). Brown oil - ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.09 (1H, t, *J* 1.8 Hz, $-CO{=}CH_2$); 5.90 (1H, broad s, $-CO{=}CH_2$); 2.64 (1H, qd, *J* 8.2 Hz,$(H1')_1$); 2.35 (1H, qd, *J* 8.5 Hz, $(H1')_1$); 1.73 (2H, qd, J 7.5 Hz, $(H2')_2$); 1.45 (6H, m, $(H3')_2$ to $(H5')_2$); 0.92 (3H, t, *J* 6.9 Hz, $(H6')_3$). ^{13}C NMR ($CDCl_3$): δ 14.1; 22.5; 26.4; 27.3; 28.7; 31.4; 43.5; 94.6; 116.7; 167.3; 171.6. Mass spectrum: *m/z* 260,9 (M+1(^{81}Br), 0.6%), 260 (M(^{81}Br), 8.3), 258.9 (M+1(^{79}Br), 0.8), 258 (M(^{79}Br), 9.7), 216.9 (1.4), 215 (0.9), 203 (3.8), 200.9 (4.3), 190.9 (10.5), 190 (44.4), 189 (19.4), 188 (45.8), 187 (9.4), 179 (79.0), 162 (12.1), 159.9 (12.6), 151 (55.5), 137 (83.6), 135 (18,0), 133 (16.2), 123 (19.0), 121.9 (20.9), 119.9 (18.4), 111 (15.7), 110 (28.1), 109 (100.0), 107 (15.8), 95 (23.5), 93 (14.2), 91 (14.2), 81 (46.4), 79 (32.1), 69 (30.6), 67 (58.3), 65 (29.6), 55 (37.7), 53 (51.2), 51 (32.7), 43 (99.5), 41 (92.7), 39 (83.9).

4.1.6. General procedure for bromoalkylmaleic anhydrides starting from alkylmaleic anhydrides (Scheme 2 (pathway D))

The bromination of the alkylmaleic anhydrides was performed via the bromo/dehydrobromination procedure described above for the bromination of 3-alkyl-5-hydroxy-5-methyl-2(*5H*)-furanones.

4.1.6.1. Bromohexylmaleic anhydride (20c). White solid - ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.55 (2H, t, *J* 7.4 Hz, $(H1')_2$); 1.64 (2H, q, *J* 7.3 Hz, $(H2')_2$); 1.27 (6H, m, $(H3')_2$ to $(H5')_2$); 0.88 (3H, t, *J* 6.5 Hz, $(H6')_3$). ^{13}C NMR ($CDCl_3$): δ 14.5; 23; 26.4; 27.2; 29.4; 32.1; 125.4; 126.8; 149.5; 160.6. Mass spectrum: *m/z* 262 (M(^{81}Br), 0.1), 260 (M(^{79}Br),0.1), 243 (0.2), 240.9 (0.3), 233.9 (0.7), 232 (0.8), 217 (1.5), 215 (1.5), 205 (3.2), 203 (2.8), 192 (63.1), 190 (66.0), 181 (5.4), 164 (17.9), 161,9 (17.6), 161 (4.9), 159 (5.5), 153 (7.8), 147 (5.2), 145 (3.7), 135 (12.6), 125 (8.4), 118.9 (8.6), 117 (8.9), 111 (11.8), 107 (15.3), 81 (6.6), 79 (17.6), 77 (9.2), 71 (13.5), 70 (15.4), 69 (10.7), 67 (15.0), 66 (4.2), 55 (26.7), 53 (13.4), 51 (17.4), 43 (100.0), 41 (58.8), 39 (314).

4.1.7. General procedure for 3-(1′-bromoalkyl)-5-methylene-2(*5H*)-furanones (Scheme 3)

The molecules were prepared from the 1′-unsubstituted 3-alkyl-5-methylene-2(*5H*)-furanones by following a procedure described in the literature.[22] Analytical data are provided as Supplementary data.

4.1.8. General procedure for 3-(1′-acetoxalkyl)-5-methylene-2(*5H*)-furanones (Scheme 3)

The molecules were prepared from the 3-(1′-bromoalkyl)-5-methylene-2(*5H*)-furanones by following a procedure described in the literature.[22] Analytical data are provided as Supplementary data.

4.2. Biological assays

4.2.1. Static peg assay for prevention of *S.* Typhimurium biofilm formation[10]

The device used for biofilm formation is a platform carrying 96 polystyrene pegs (Nunc no. 445497) that fits as a microtiter plate lid with a peg hanging into each microtiter plate well (Nunc no. 269789).[48]

Twofold serial dilutions of the compounds in 100 µl liquid TSB 1/20 broth per well were prepared in the microtiter plate. Subsequently, an overnight culture of *S.* Typhimurium ATCC14028 (grown in Luria-Bertani medium[49]) was diluted 1:100 into Tryptic Soy Broth diluted 1/20 (TSB 1/20; BD Biosciences) and 100 µl (ca. 1×10^6 cells) was added to each well of the microtiter plate, resulting in a total amount of 200 µl medium per well. The pegged lid was placed on the microtiter plate and the plate was incubated for 48 h at 16 °C without shaking. During this incubation period, biofilms were formed on the surface of the pegs. After 24 h, the lid was transferred into a new plate containing medium and the specific molecules used for testing. For quantification of biofilm formation, the pegs were washed once in 200 µl phosphate buffered saline (PBS). The remaining attached bacteria were stained for 30 min with 200 µl 0.1% (w/v) crystal violet in an isopropanol/methanol/PBS solution (v/v 1:1:18). Excess stain was rinsed

off by placing the pegs in a 96-well plate filled with 200 μl distilled water per well. After the pegs were air dried (30 min), the dye bound to the adherent cells was extracted with 30% glacial acetic acid (200 μl). The OD_{570} of each well was measured using a VERSAmax microtiter plate reader (Molecular Devices). The IC_{50} value for each compound was determined from concentration gradients in two or three independent experiments (with two or three repeats per experiment), by using the GraphPad software of Prism.

4.2.2. Bioscreen assay for measuring *S.* Typhimurium growth inhibition

The Bioscreen device (Oy Growth Curves Ab Ltd) was used for measuring the influence of the chemical compounds on the planktonic growth of *S.* Typhimurium. The Bioscreen is a computer controlled incubator/reader/shaker that uses 10 × 10 well microtiter plates and measures light absorbance of each well at a specified wave length in function of time. An overnight culture of *S.* Typhimurium ATCC14028 (grown up in LB medium) was diluted 1:200 TSB 1/20 broth. We added 300 μl of the diluted overnight culture to each well of the 10 × 10 well microtiter plate. Subsequently, serial dilutions of the chemical compounds were prepared in EtOH. We added 3 μl of each diluted stock solution to the wells (containing the 300 μl bacterial culture) in threefold. As a control, 3 μl of the appropriate solvent was also added to the plate in three- or fourfold. The microtiter plate was incubated in the Bioscreen device at 16 °C for at least 36 h, with continuous medium shaking. The absorbance of each well was measured at 600 nm each 15 min. Excel was used to generate the growth curves for the treated wells and the untreated control wells.

4.2.3. Luminescence assay for inhibition of *V. harveyi* quorum sensing

Twofold serial dilutions of the compounds in 100 μl liquid LM broth per well were prepared in white microtiter plates (Cliniplate, Thermo Life sciences). Subsequently, an overnight culture (grown in AB medium[50]) of *V. harveyi* strain BB120 (wildtype), BNL258 (*hfq*::Tn5lacZ),[45] JAF483 (l*uxO* D47A linked to Kan^R),[46] or JAF553 (*luxU* H58A linked to Kan^R)[46] was diluted 1:100 into Luria-Marine broth[46] (after adjusting the OD_{600} to 0.4) and 100 μl was added to each well of the microtiter plates, resulting in a total amount of 200 μl medium per well. The microtiter plates were covered with Breathable Sealing Membranes (Greiner Bio-One N.V.) and incubated with aeration for 5 h at 30 °C. After incubation, the luminescence was measured using a CCD camera (PerkinElmer Life Science) and the OD_{600} of 135 μl of each well was measured with a VERSAmax microtiter plate reader. The IC_{50} and the GIC_{50} value for each compound was determined from concentration gradients in two or three independent experiments (with two or three repeats per experiment), by using the GraphPad software of Prism.

4.2.4. Determination of cytostatic activity

Murine leukemia L1210, murine mammary carcinoma FM3A, human T-lymphocyte CEM, human cervix carcinoma (HeLa) and human osteosarcoma (OST) cells were suspended at 300,000–500,000 cells/mL of culture medium, and 100 μL of a cell suspension was added to 100 μL of an appropriate dilution of the test compounds in 200 μL-wells of 96-well microtiter plates. After incubation at 37 °C for two (L1210, FM3A), three (CEM) or four (HeLa, OST) days, the cell number was determined using a Coulter counter. The IC_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.055.

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