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Identifying the important structural elements of brominated furanones for inhibiting biofilm formation by *Escherichia coli*

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Abstract—A collection of structurally closely related furanones was synthesized to identify the most important structural elements in brominated furanones for inhibiting the formation of bacterial biofilms. The results suggest that a conjugated exocyclic vinyl bromide on the furanone ring is the most important structural element for the non-toxic but inhibition activity for *Escherichia coli* biofilm formation. Furanones bearing monosubstituted bromide groups on saturated carbons were found to have a toxic effect that attenuates the bacterial growth.
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Bacterial biofilms are highly hydrated structures comprised of a polysaccharide matrix secreted by the bound bacteria. Biofilm formation involves significant changes in gene and protein expression and has been related to bacterial cell–cell signaling known as quorum sensing.[1–4] Due to extremely enhanced resistance to antibiotics and disinfection treatments, biofilms cause persistent infections in humans and serious corrosion and equipment failure in industrial settings.[5] Both surface modification[6,7] and new inhibitor design[8–13] have been sought after to control the formation of biofilms. Recent studies have found that a number of brominated furanones from marine algae inhibit the multicellular behaviors of Gram-negative bacteria, such as biofilm formation and quorum sensing, at concentrations that do not repress bacterial growth.[14–17] Furanone, in general, is an important class of structural moieties occurring in medicinal products.[14,15] With the ability to inhibit biofilm formation rather than to repress bacterial growth, brominated furanones are important agents for both fundamental studies and for application developments. Both the natural[16] and synthetic brominated furanones[17–23] reported to date vary widely in the types and the regiochemistry of the substituents. Because these differences are not systematic, it remains unclear what structural elements in these furanones are responsible for inhibiting the formation of biofilms. This structural information of the brominated furanones is critically important for developing biofilm control strategies and for molecular-level understanding of the inhibition mechanism, in the following three aspects. First, there is a keen interest in immobilizing brominated furanones onto surface as a strategy to resist the microbial biofilm formation.[24] However, without knowing which functional groups on the furanones should be retained for activity, the existing covalent methods[25] for incorporating brominated furanones into polymer coating or materials may compromise the activities of brominated furanones. Second, comparing the brominated furanones with the natural quorum sensing signals, N-acylated homoserine lactones (AHLs)[26] and autoinducer 2 (AI-2),[1] the structures and functional groups among these classes of compounds are vastly different. Thus, the inhibition mechanism (for instance covalent versus non-covalent binding to the target protein) for brominated furanones remains unclear, although it was recently reported that brominated furanones displace AHL from its receptor protein.[27] Third, knowing the essential structural element for biofilm inhibition activity will guide the rational design for developing non-toxic therapeutic agents for potential in vivo applications.

In light of these needs, we report here the use of efficient organic syntheses to obtain a collection of brominated furanones with closely related structural variation (Scheme 1). By studying their effects on the growth

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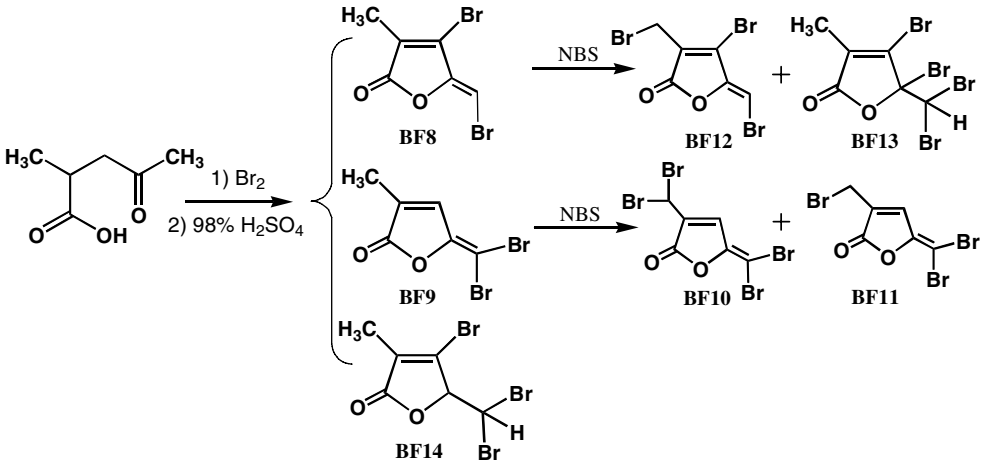

Scheme 1.

and biofilm formation of *Escherichia coli*, we identified the most important structural elements of brominated furanones for achieving biofilm inhibition.

Syntheses of brominated furanones for antimicrobial activities have been reported previously.[21–23] But a detailed understanding of structure–activity relationship is still lacking. Here, we synthesized a new class of brominated furanones starting from α-methyllevulic acid that affords a collection of furanone structures in 2–3 steps of organic synthesis (Scheme 1).[28] Brominated furanones **BF8**, **BF9**, and **BF14** were synthesized via bromination of α-methyllevulic acid followed by oxidative ring closure under acidic condition. By using Wohl Ziegler reaction,[29] furanones **BF12** and **BF13** were obtained from **BF8**; furanones **BF10** and **BF11** are obtained from **BF9**. Perhaps because there is only one methyl group instead of an aliphatic chain attached to the α-carbon relative to the carbonyl group, all these molecules were separated and purified by column chromatography. The vinyl bromide on furanone **BF8** is of Z-conformation as evidenced by proton NMR spectroscopy and by comparing with similar structures reported previously.[20,30,31] Structure **BF13** was confirmed by NMR spectroscopy (heteronuclear multiple-quantum coherence experiment—HMQC). We also included three bromine-free structures **NF1**, **NF2**, and **NF3** (Fig. 1) as negative controls for studying the effects of bromide on the activities of furanones. Furanones **NF1** and **NF2** are commercially available, furanone **NF3** was prepared by direct oxidative cyclization of α-methyllevulic acid without bromination (see supporting information).[31,32]

While furanones **BF8** through **BF14** were synthesized by different chemical transformations, they can be grouped into sub-collections of molecules according to the types of functional groups on the structures, such as those that have a monosubstituted bromide on a saturated carbon versus those that do not, or those with vinyl bromide external to the furanone ring versus those that bear substituted bromides on saturated exocyclic carbons. The furanones were then tested for their effects on growth and biofilm formation of *E. coli* through single-blinded (during data collection) experiments. The results indicate that there is a strong correlation between the structure and the activity (both potency and toxicity) of this class of brominated furanones (see below).

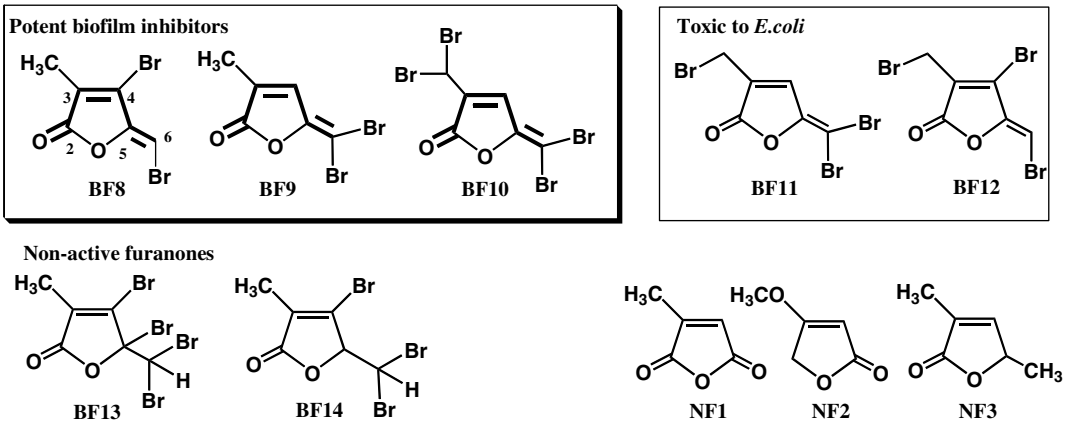

Figure 1. Structures of the brominated furanones grouped with their inhibition activity on biofilm formation and toxic effect on bacterial growth. The important structure element for inhibition is highlighted.

Toxicity of brominated furanones. To compare the biofilm inhibition by this class of synthetic furanones at concentrations non-toxic to bacteria, we first evaluated the effects of brominated furanones on the growth of *E. coli*. The same strain was used in both the toxicity test (effect on *E. coli* growth) and in biofilm study. *E. coli* RP437 was labeled with a plasmid pRSH103 that expresses red fluorescence proteins constitutively so that the biofilms can be visualized by confocal microscopy. The plasmid pRSH103 was constructed by replacing the ampicillin resistant marker (Amp^R) of the prokaryotic expression vector pDsRed-Express (Clontech Laboratories, Inc, Mountain View, CA) with a tetracycline resistant marker (Tet^R). The toxicity was studied by monitoring the optical density at 600 nm (OD_{600}) of *E. coli* cultures with different concentrations of furanones in Luria–Bertani (LB) medium[33] supplemented with 10 μg/mL tetracycline. Figures 2 and 3 show the representative growth curves (OD_{600} versus time) in the presence of furanones **BF8** and **BF12**, respectively. **BF8** with concentrations up to 60 μg/mL did not show any significant effect on *E. coli* growth as compared to when there was no furanone added (Fig. 2). However, addition of **BF12** at 5–10 μg/mL caused significant attenuation of *E. coli* growth (Fig. 3). Examining the toxicity of all of the furanones (see supporting materials) indicated that **BF11** and **BF12** are most toxic to *E. coli* while other furanones did not exhibit any significant impact up to 60 μg/mL. Examining the structures of the furanones indicated that **BF11** and **BF12** bear monosubstituted bromides on an exocyclic methyl group.

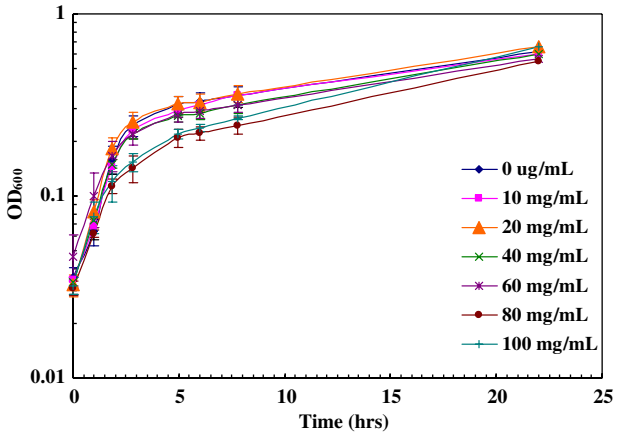

Figure 2. *Escherichia coli* growth curves with furanone **BF8**.

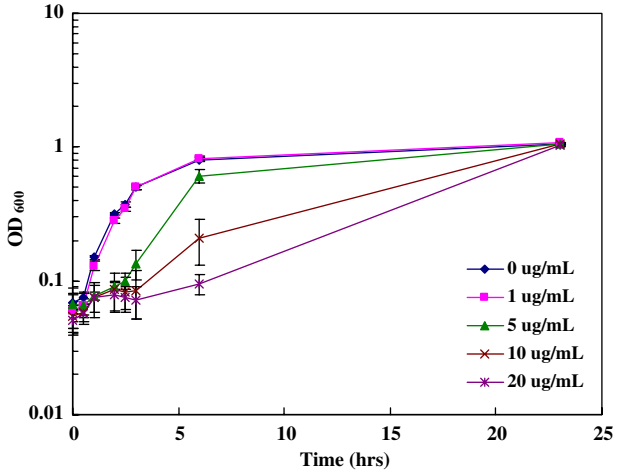

Figure 3. *Escherichia coli* growth curves with furanone **BF12**.

To determine the toxic effect of brominated furanones **BF11** and **BF12** being bactericidal or bacteriostatic, brominated furanones **BF11**or **BF12** (40 μg/mL) were added to *E. coli* RP437/pRSH103 culture in LB supplemented with 10 μg/mL tetracycline with an inoculation OD_{600} of 0.05. Same amount of ethanol (0.2%) was added to another *E. coli* culture as a negative control. After 3 h of incubation at 37 °C, **BF11** reduced the colony forming unit (CFU) by 2.4×10^4-fold ($9.2 \pm 1.8 \times 10^6$/mL before incubation and $3.9 \pm 4.8 \times 10^2$/mL after incubation), and no viable cells were found after incubation with **BF12**. These data suggest that **BF11** and **BF12** are bactericidal to *E. coli*.

Inhibition of biofilm formation by brominated furanones. Since the toxicity test indicated that furanones **BF8**, **BF9**, **BF10**, **BF13**, **BF14**, **NF1**, **NF2**, and **NF3** at 60 μg/mL had no effects on *E. coli* growth, this concentration was chosen to compare the biofilm inhibition by furanones. The biofilms consisting of DsRed-express-labeled *E. coli* were formed on stainless steel coupons (316 L, 1 in. × 1 in.) for 24 h at 37 °C. The samples were analyzed with confocal microscopy. The biomass, surface coverage, and mean thickness were calculated using COMSTAT software.[34] Statistical analysis indicated that the furanones have significantly different effects on *E. coli* biofilm formation (ANOVA, $p < 0.0001$), Tukey test was then used to further compare the effects of each pair of furanones. All the statistical analysis was performed by using SAS 9.1.3, windows version (SAS, Cary, NC). Figure 4 shows the representative images of biofilms formed in the presence of furanone **BF8**, **BF9**, and **BF13**. These experiments were conducted with four replicates.

Figure 5 shows the quantitative analysis of the biomass, surface coverage, and mean thickness of the biofilms formed with and without (control) addition of 60 μg/mL brominated furanones in the growth medium. Consistent results were obtained for the biofilm parameters of biomass, surface coverage, and thickness. Note that the results are normalized by the sample with the largest amount of biofilm formation (*E. coli* treated with non-brominated furanone **NF2**). Analysis using COMSTAT indicated that furanones **BF8**, **BF9**, **BF10**, and **BF14** exhibited significant reduction of biofilm formation compared to the control experiment where there were no furanones added ($p < 0.05$).

BF8, **BF9**, and **BF10** exhibited the strongest inhibition as they reduced the biofilm formation by 75%, 63%, and 80%, respectively (Fig. 5). In comparison, **BF14** exhibited significant but rather minor inhibition (19%, Fig. 5). Furanone **BF13** did not inhibit biofilm formation significantly ($p > 0.05$); and non-brominated furanones did not exhibit any significant inhibition of

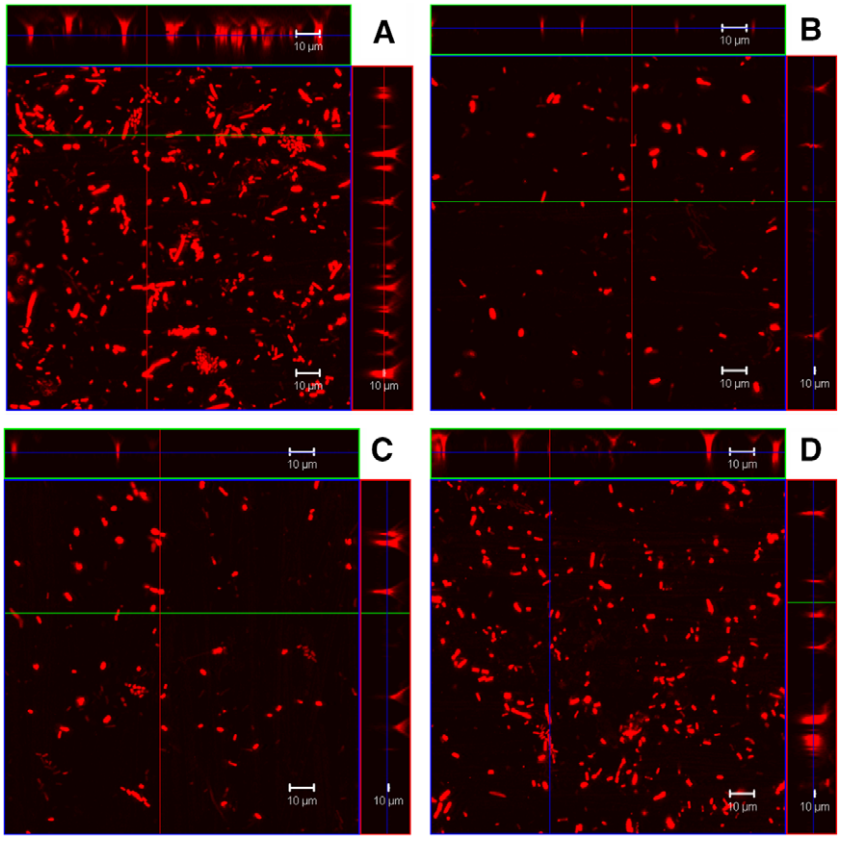

Figure 4. Representative confocal images of biofilms formed by DsRed-express-labeled *E. coli* treated without furanone (A); with 60 μg/mL **BF8** (B), **BF9** (C), and **BF13** (D). Scale bar = 10 μm.

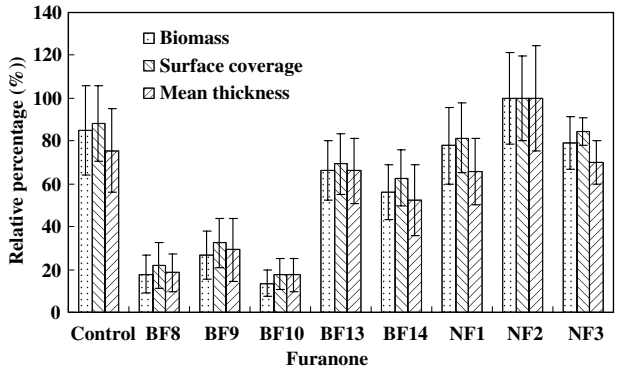

Figure 5. The biomass, surface coverage, and average thickness of *E. coli* biofilms formed in the presence of 60 μg/mL furanones. The parameters of *E. coli* biofilms formed with furanone **NF2** were normalized as 100%.

biofilm formation as compared to the control experiment ($p > 0.05$). Non-brominated furanones did not exhibit either toxic effect on bacterial growth or inhibition activity of biofilm formation.[35] Examining the structures of furanones, we note that **BF8**, **BF9**, and **BF10** all bear an exocyclic vinyl bromide conjugated with the carbonyl group, but do not bear a monosubstituted bromide on saturated carbon. Together with the toxicity data, these results reveal important structural elements for both the toxicity and biofilm inhibition activity (Fig. 1). Group 1 shows the potent inhibitor for biofilm formation at non-toxic concentrations, group 2 shows the toxic furanones, group 3 shows the mildly-inhibiting and non-toxic furanones, and group 4 shows the non-toxic and non-inhibiting furanones. Common to the structures of furanones in group 1 is an exocyclic vinyl bromide, common to structure in group 2 is a monosubstituted bromide on a saturated carbon. Together, these results allow us to propose that the most important structural element for inhibiting biofilm formation is the exocyclic vinyl conjugated with the carbonyl group in the furanone structures (highlighted in Fig. 2), whereas a monosubstituted bromide group on the saturated carbon contributes most to the toxic effect on *E. coli* growth.

We note that further derivatization of furanones using **BF11** or **BF12** under either acidic or basic conditions, with even mild base such as triethyl amine, results in decomposition of the furanones (result not included). Furthermore, we have identified the products of brominated furanones treated with radical species such as those generated in the Wohl–Zieglar reactions.[29] These products grossly differ in the substitution and regiochemistry of brominated furanones, which appears to significantly attenuate the biofilm inhibition activity. Overall, brominated furanones are reactive chemicals under acidic and basic conditions, as well as when there are radicals present. As such, we believe that direct covalent immobilization of brominated furanones to a surface may not be the best strategy for biofilm control in ex situ environments because such reaction likely will compromise or attenuate the activity of brominated furanones.

In the light of the reactivity of brominated furanones, we believe that the mechanism of inhibiting biofilm formation may proceed via covalent linkage between the brominated furanone and its target protein(s), or through a generated reactive species that damages the target protein(s). Strategies that use non-covalent immobilization of structurally intact brominated furanones for controlling biofilm formation, and for isolating protein receptors, as well as the evaluation of this class of brominated furanones on inhibiting the biofilm formation by other bacterial species are the ongoing subjects of research in our laboratories.

To conclude, by synthesizing and studying a series of structurally closely related brominated furanones, the most significant result of this work is the identification of the most important structural element of brominated furanone that is critical for the activity of biofilm inhibition. This structural element consists of a vinyl bromide at the δ-position of the extended conjugation of the furanone ring. The vinyl bromide on the furanone ring does not appear to be critical and the monosubstitution of bromide on saturated carbon appears to play a negative role in biofilm inhibition. We believe this information is critically important for controlling biofilm formation via materials design and surface modifications with brominated furanones. This structural information will also assist mechanistic studies of molecular interactions between relevant proteins and brominated furanones, which is also part of our ongoing research efforts.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.12.032.

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