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Research paper

New bicyclic brominated furanones as potent autoinducer-2 quorum-sensing inhibitors against bacterial biofilm formation

Ji Su Park [a, 1], Eun-Ju Ryu [b, 1], Linzi Li [a], Bong-Kyu Choi [b, **], B. Moon Kim [a, *]

[a] Department of Chemistry, College of Natural Sciences, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea
[b] Department of Oral Microbiology and Immunology and Dental Research Institute, School of Dentistry, Seoul National University, 101 Daehak-ro, Jongno-gu, Seoul 03080, Republic of Korea

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ABSTRACT

Bacterial behaviors such as virulence factor secretion and biofilm formation are critical for survival, and are effectively regulated through quorum sensing, a mechanism of intra- and interspecies communication in response to changes in cell density and species complexity. Many bacterial species colonize host tissues and form a defensive structure called a biofilm, which can be the basis of inflammatory diseases. Periodontitis, a chronic inflammatory disease affecting the periodontium, is caused by subgingival biofilms related to periodontopathogens. In particular, *Fusobacterium nucleatum* is a major co-aggregation bridge organism in the formation and growth of subgingival biofilms, linking the early and late colonizers in periodontal biofilms. According to our previous study, the intergeneric quorum-sensing signal molecule autoinducer-2 (AI-2) of *F. nucleatum* plays a key role in intra- and interspecies interactions of periodontopathogens, and may be a good target for periodontal biofilm inhibition. Recently, brominated furanones produced by the macroalga *Delisea pulchra* were shown to inhibit biofilm formation via AI-2, and have been investigated toward the goal of increasing the inhibition effect. In this study, we describe the synthesis of new bromofuranone analogs, i.e., 3-(dibromomethylene)isobenzofuran-1(3H)-one derivatives, and demonstrate their inhibitory activities against biofilm formation by periodontopathogens, including *F. nucleatum, Porphyromonas gingivalis, and Tannerella forsythia.*

1. Introduction

Quorum sensing (QS) is the process of cell-to-cell communication in microorganisms mediated by small signaling molecules referred to as autoinducers, which are secreted by bacteria and fungi [1–3]. Bacteria can effectively regulate numerous phenotypes, including virulence factor expression, bioluminescence, and biofilm formation, in response to their changes in population density through recognition of the threshold reached by autoinducers, the so-called quorum, via intracellular or intercellular communication [4]. In particular, formation of a biofilm can not only enhance the viability of bacteria in response to antibiotics but can also be the basis of the development of chronic inflammatory diseases, including endocarditis, cystic fibrosis, osteomyelitis, chronic urinary tract infections, chronic prostatitis, and periodontal diseases [5].

QS molecules can be classified into three major types: oligopeptides, acyl-homoserine lactones (AHLs, autoinducer-1), and autoinducer-2 (AI-2). Oligopeptides and AHLs are used by gram-negative and gram-positive bacteria, respectively, in intraspecies communication [6]. AI-2 is a universal QS molecule secreted by both gram-negative and gram-positive bacteria [7–9], and plays a critical role in the virulence of pathogenic bacteria and in biofilm formation [10]. AI-2 mediates both intra- and interspecies communication, and can thus be a good target for the regulation of bacterial infection. Accordingly, AI-2 inhibitors are ideal compounds for bacterial biofilm inhibition in multispecies bacterial communities.

More than 700 species of bacteria have been found in the human oral cavity, which establish mixed-species communities [11]. Among these oral bacteria, periodontopathogens, including *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Tannerella forsythia*, and spirochetes, form subgingival biofilms and cause periodontitis, which is a

* Corresponding author.
** Corresponding author.
E-mail addresses: bongchoi@snu.ac.kr (B.-K. Choi), kimbm@snu.ac.kr (B.M. Kim).
[1] These two authors contributed equally to this work.

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chronic inflammatory disease. Since AI-2 is an interspecies QS molecule that controls intergeneric signaling, it may induce oral biofilm formation and increase the bacterial virulence of periodontopathogens [12–14]. Furthermore, Kolenbrander et al. [15] suggested that, compared to commensal bacteria, periodontopathogens produce much higher levels of AI-2; such high concentrations would enhance pathogen biofilm maturation, leading to periodontitis. In particular, *F. nucleatum* is known as a major co-aggregation bridge organism that connects late pathogenic colonizers and early commensal colonizers in a periodontal biofilm [15–19]. We previously reported that the level of *F. nucleatum* AI-2 was reduced by QS inhibitors (QSIs), including (5Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone and D-ribose [20]. Because of the diversity of oral bacteria, it is hard to selectively eliminate periodontopathogens without disturbing oral commensals. Therefore, oral biofilm formation regulation using QSIs is one of the most hopeful protective means for preserving oral health [21].

Brominated furanones produced from the macroalga *Delisea pulchra* are known to prevent microbial colonization [22,23]. In addition, these compounds have been shown to inactivate LuxS, which is required for AI-2 synthesis, and to inhibit the QS activity of various bacterial species [4,24–27]. Recently, Yang et al. [28] reported a bicyclic version of brominated furanones that could potentially reduce their toxicity while retaining their biofilm inhibitory activities. However, compared with reported monocyclic brominated furanones, the inhibitory activities of the bicyclic furanones were relatively low [28]. These results suggest that modification of the exocyclic vinyl position, which is an essential structural element for the inhibition of LuxS [29], is not an appropriate strategy for the development of effective QSI's. Therefore, discovery of new potent and safer brominated furanone candidates is still desired. In this study, to improve the QS inhibitory activity and biofilm inhibition efficacy of periodontal bacteria, we report the synthesis and evaluation of 3-(dibromomethylene)isobenzofuran-1(3H)-one derivatives, which have different ring sites from the existing bicyclic compound. In particular, we investigated the structure-activity relationships of the biofilm inhibition effects of various new furanone derivatives prepared with new ring structures and possible side chains on the new ring.

2. Results and discussion

2.1. Chemistry

We focused on the design and synthesis of new bicyclic brominated furanone derivatives with reduced toxicity while retaining or enhancing their biofilm inhibitory activities. As shown in Table 1, we synthesized two kinds of bicyclic brominated furanone derivatives: one through the modification of the ring structure and the other through introduction of a side chain onto the benzene ring. The ring structural component of furanones has been reported to be important for the inhibition of biofilm formation by bacteria [28]. Thus, compounds with different ring structures (**1**–**5**) were designed for evaluation of the ring structure effect on the biofilm inhibitory activity. Another set of compounds possessing side chains on the benzene ring (**6**–**9d**) were designed with the aim of investigating the influence of the side chain length on the benzene ring, since previous reports have shown that monocyclic furanones with chain lengths of two to six atoms show good inhibitory activities [30,31].

According to the Ramirez olefination procedure [32], brominated compounds with various ring structures (**10**–**14**) shown in Table 1 were synthesized from the corresponding commercially available compounds in one step. The yields of the products ranged from low to moderate (14–65% yields). Although the reactions proceeded for cyclopropane- and cyclobutane-1,2-dicarboxylic anhydrides, the olefination products decomposed rapidly at room temperature, presumably due to the unstable nature of the anhydrides based on the angle strains of the hydrocarbon ring [33].

For the preparation of brominated furanone derivatives containing alkoxy groups on the benzene ring, such as **16a**–**16e** and **18a**–**18d**, starting materials containing the alkoxy derivatives **7a**–**7e** and **9a**–**9d** were prepared from the corresponding phenol derivatives through the use of a reported synthetic method [34]. These precursors (**7a**–**7e** and **9a**–**9d**) were obtained at moderate to good yields over four steps (45–77%). Through the Ramirez olefination with anhydrides possessing side chains on the benzene ring (**6**–**9d**), brominated compounds **15**–**18d** were produced with 70–83% regioselectivity. Similar to a study that examined the regioselectivity of Wittig reactions for mono-substituted phthalic anhydrides [35], this result showed that the electronic effects of the substituents appear to have a large influence on the regioselectivity of the olefination. Brominated compounds **15**–**18d** were obtained in overall yields ranging from 16% to 22%. Although small amounts of minor regioisomers were produced in olefination with the anhydrides **6**–**9d,** we focused on the major product because of its better inhibitory effect on biofilm formation. All target compounds were separated and purified through the use of column chromatography. The structures of the desired products were confirmed by spectroscopic analyses, including ^{1}H, ^{13}C, heteronuclear multiple bond correlation (HMBC), and mass spectrometry (MS).

2.2. Biological evaluation

In our preliminary experiments, in order for us to evaluate the inhibitory activities based on the ring structural component of the inhibitors, brominated furanone compounds with different ring structures (**10**–**14**) were screened for their inhibitory effects on the biofilm formation of *F. nucleatum* at 0.2 and 2 μM concentrations (Fig. S1). Since it was confirmed in previous studies that the reference furanone compound **R1**, (*Z*)-4-bromo-5-(bromomethylene)furan-2(5H)-one, has neither bactericidal effect nor cytotoxicity against host cells at 2 μM [20], the following experiments were performed starting at 2 μM. As shown in Fig. S1, bromofuranones containing a benzene ring (**10**) and a 5-membered ring (**13**) showed higher inhibitory activities at 0.2 and 2 μM compared to other compounds examined.

According to previous reports [30,31], the biofilm inhibitory activities of furanones were dependent on the side chain structure. Therefore, we synthesized brominated furanone derivatives (**15**–**18d**) based on compound **10**, which showed good inhibitory activity, and investigated the biofilm inhibitory effects according to the variation of the side chains. Compounds **15**–**18d** were evaluated along with compounds **10**–**14** at 0.02 and 0.2 μM to investigate the activities at lower concentrations. Compounds with 5-membered ring structure (**13**) and a short side chain on the benzene ring (**15** and **17**) showed high inhibitory effects on the biofilm formation of *F. nucleatum*. However, as shown in Fig. S2, the inhibitory activities of compounds except for **13**, **15** and **17** were worse than that of compound **R1** as the increase of the chain length rather decreased the inhibitory activity.

2.2.1. Inhibitory effect of new furanone compounds on AI-2 activity

To investigate whether the inhibitory effects of highly active compounds (**13**, **15** and **17**) on biofilm formation arise from the inhibition of AI-2 activity, these furanone compounds were evaluated for their inhibitory activities on the AI-2 activity at 2 μM. AI-2 activities were assessed using *Vibrio harveyi* BB170, an AI-2 reporter strain, by measuring the AI-2-mediated bioluminescence of the