Screening of Anti-Biofilm Compounds from Marine-Derived Fungi and the Effects of Secalonic Acid D on Staphylococcus aureus Biofilm

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Biofilm formation of Staphylococcus aureus is one of its mechanisms of drug resistance. Anti-biofilm screening of 106 compounds from marine-derived fungi displayed that 12 compounds inhibited S. aureus biofilm formation by >50% at the concentration of 100 µg/ml, and only secalonic acid D (SAD) and B inhibited by >90% at 6.25 µg/ml without inhibiting cell growth after 24-h incubation. Meanwhile, it was found that the double bond between C-1 and C-10 of citrinin derivatives and the C-C connection position of two chromone monomers may be important for their anti-biofilm activities. Moreover, SAD slightly facilitated biofilm eradication and influenced its architecture. Furthermore, SAD slowed the cell growth rate in the preceding 18-h incubation and differentially regulated transcriptional expression of several genes, such as agr, isaA, icaA, and icaD, associated with biofilm formation in planktonic and biofilm cells, which may be the reason for the anti-biofilm activity of SAD. Finally, SAD acted synergistically against S. aureus growth and biofilm formation with other antibiotics. These findings indicated that various natural products from marine-derived fungi, such as SAD, could be used as a potential biofilm inhibitor against S. aureus.

Keywords: Anti-biofilm activity, Staphylococcus aureus, marine natural product, secalonic acid D, mechanism of action

Introduction

Staphylococcus aureus, a notorious pathogen, can secrete several exotoxins, such as α-hemolysin, enterotoxins, coagulase, and protein A, all of which may be capable of causing a series of acute and chronic infections, such as infective endocarditis and osteomyelitis, and is a frequent invader in chronic lung disease [1, 2]. Furthermore, it has been reported to be a serious threat owing to the appearance of multidrug-resistant strains, such as methicillin-resistant S. aureus and vancomycin–methicillin-resistant S. aureus [3]. S. aureus attaches preferentially to a variety of surfaces, and then forms a community encased by an extracellular matrix of protein, polysaccharide, and nucleic acids, called a biofilm, which protects S. aureus from antibiotics and the host immune system [4]. Therefore, biofilms have shown to house 100–1,000-fold increases in tolerance towards antimicrobial agents compared with equivalent populations of their free-floating counterparts [5]. Once biofilms colonize the indwelling medical devices, it becomes almost impossible to clear the infections [6]. Thus, there is an urgent need to find an effective combination therapy or novel drugs to overcome the problem caused by biofilm-associated S. aureus.

To try to find feasible sources of anti-biofilm agents, many investigators have begun to extract and analyze the metabolites from a number of plants and marine organisms [7, 8]. For example, quercetin and tannic acid extracted from Alnus japonica have been reported to inhibit the formation of recalcitrant biofilm of S. aureus without disrupting cell growth [9]. An extracellular protease in actinomycete culture supernatants inhibits and detaches S. aureus biofilm formation in a non-microbialid manner [10]. Additionally, two classes of marine sponge metabolites...
are demonstrated to be non-bactericidal biofilm modulators; namely, the terpenoids and the pyrrole-imidazoles, such as (-)-ageloxime-D towards S. epidermidis, and trans-bromoageliferin and cis-bromoageliferin towards Pseudomonas aeruginosa [11–16].

The diverse mechanisms and environmental conditions that contribute to biofilm formation and disassembly of S. aureus include intercellular adhesion proteins, quorum sensing, protease, DNase, cis-2-decenoic acid, D-amino acids, phenol-soluble polypeptides, and pH change [17]. Quercetin and tannic acid repressed the transcription of two intercellular adhesion-related genes (icaA and icaD), the quorum-sensing gene agrA, and two virulence-regulatory genes (sigB and sarA), all of which are important for the formation of biofilms of S. aureus [9]. Magnolol, a major component isolated from the stem bark of Magnolia species, has anti-biofilm properties against S. aureus through reducing S. aureus autolysin activity in a dose-dependent manner and then controlling lysis and eDNA release that are essential for S. aureus biofilm formation [18–20]. The 2-aminoimidazole motif found in the sponge-derived natural products might be functioning as a biofilm inhibitor through a zinc chelation mechanism [21].

Many secondary metabolites, including chromones like quercetin [9], alkaloids such as pyrrole-imidazole alkaloids, trans-bromoageliferin and cis-bromoageliferin from marine sponges [6], antraquinones such as alizarin, purpurin, emodin, and quinalizarin [3] have been reported to inhibit the biofilm formation of Pseudomonas and/or Staphylococcus species. Meanwhile, cyclic peptides such as rhamnolipid and safractin could reduce the adhesion and remove biofilms of individual and mixed cultures of food pathogenic bacteria, including S. aureus [22]. Two hydroxyl units at the C-1 and C-2 positions of antraquinones were verified to be important for antibiofilm and anti-hemolytic activities in S. aureus [3]. Marine fungi have been considered as hotshots in recent research owing to their wide distribution and their various secondary metabolites, including chromones, alkaloids, antraquinones, etc., with wide ranges of bioactivities and remarkable safety records. In this context, the aim of this study was to screen the inhibiting activities of 106 natural products, including 9 simple phenol derivatives, 19 citrinin derivatives, 28 chromones, 6 antraquinones, 25 alkaloids, 11 esters, 4 cyclic peptides, 3 pyrroles, and 1 polyol, isolated from marine-derived fungi against the biofilm formation of S. aureus and to analyze the structural motif important for the anti-biofilm activity. Additionally, the underlying mechanisms of secalonic acid D (the most bioactive compound) in inhibiting biofilm formation were investigated through confocal microscopy, cell aggregation assay, transcriptional analysis, and synergistic effects with other important antibiotics to S. aureus (penicillin, vancomycin, and chloramphenicol). The results showed that natural products, such as SAD, from marine-derived fungi might possess potential as a non-microbialidal strategy against S. aureus.

**Materials and Methods**

**Bacterial Strains and Tested Compounds**

*S. aureus* ATCC 6538 was obtained from the American Type Culture Collection (ATCC) and routinely cultivated at 37°C in Luria Bertani (LB) medium [3]. All of the tested compounds, including phenol derivatives, citrinin derivatives, chromones, antraquinones, alkaloids, cyclic peptides, pyrroles, ethers, and polyols (see Fig. S1), were isolated from the fermentation broth of marine-derived fungi (see Table 1 and Fig. S1) in our laboratory, such as *Penicillium* sp. SCGAF 0023 (CCTCC M 2012507) [23, 24], *Aspergillus* sp. SCGAF 0076 (CCTCC M 2012397) [25, 26], *Engyodontium album* DFFSOS21 (CCTCC M 2014521) [27], and *Aspergillus westerdijkiae* DFFSOS13 (CCTCC M 2013078) [28], which were deposited in the China Center for Type Culture Collection (CCTCC). The fungal fermentation broth, after 30-day static incubation of each marine fungus, was collected and filtered through cheesecloth to separate the supernatant and mycelia. The supernatant was mixed with XAD-16 resin, and mycelia were extracted with 80% acetone. The resin and acetone extracts were evaporated and extracted with ethyl acetate to afford the brown crude gum. The crude gum was subjected to repeated column chromatography, including HLPC with an ODS column, semi-preparative HLPC, and reversed-phase-medium pressure preparative liquid chromatography, to give tested compounds with >90% purity. All of the tested compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma, USA) and kept at −20°C.

**Biofilm Formation and Destruction Assay**

*S. aureus* biofilm formation in static condition was detected in pre-sterilized polystyrene flat-bottomed 96-well plates (Corning, USA) [10, 29]. Briefly, *S. aureus* cells cultivated overnight were diluted to an optical density (OD600) of about 0.01 with fresh LB medium, and then 150 μl dilutions were incubated with different concentrations of each compound in 96-well plates without shaking at 37°C. Cells in LB medium with DMSO were used as the control, and LB medium with DMSO was used as the blank control. After 24 h, the contents were discarded, and the plates were washed, dried, and stained with 170 μl of 0.1% crystal violet (Shanghai Chemical Reagents Co. Ltd, China) for 30 min. Subsequently, 200 μl of 30% acetic acid was added to each well after the plates were washed and dried at room temperature. The absorbance at 570 nm was determined using a Multiskan GO Reader (Thermo Scientific, USA). Biofilm inhibition was calculated...
using the following formula: Percentage biofilm inhibition = \([\frac{(\text{Control OD}_{570} - \text{Treated OD}_{570})}{(\text{Control OD}_{570} - \text{Blank OD}_{570})}] \times 100\). At the same time, the optional density at 600 nm of the planktonic cells was also measured to evaluate bacterial growth of planktonic cells. Growth inhibition was calculated using the following formula: Percentage growth inhibition = \([\frac{(\text{Control OD}_{600} - \text{Treated OD}_{600})}{(\text{Control OD}_{600} - \text{Blank OD}_{600})}] \times 100\). All experiments were performed in eight replicates, and the cut-off

### Table 1. The inhibitory percentage (IP: %) of biofilm formation caused by the 18 bioactive compounds against *S. aureus*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Strains</th>
<th>100 μg/ml IP at OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>100μg/ml IP at OD&lt;sub&gt;570&lt;/sub&gt;</th>
<th>25 μg/ml IP at OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>25μg/ml IP at OD&lt;sub&gt;570&lt;/sub&gt;</th>
<th>12.5 μg/ml IP at OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>12.5μg/ml IP at OD&lt;sub&gt;570&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,5-Dihydroxy-toluene</td>
<td><em>Aspergillus</em> sp. SCSGAF 0076 (CCTCC M 2012397)</td>
<td>88.47 ± 0.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88.59 ± 0.91</td>
<td>24.94 ± 2.22</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Citrinin</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>73.38 ± 1.66</td>
<td>59.67 ± 8.05</td>
<td>39.56 ± 11.53</td>
<td>73.75 ± 11.64</td>
<td>-</td>
<td>51.39 ± 2.72</td>
</tr>
<tr>
<td>Sclerotinin A</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>24.69 ± 1.97</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ACDT&lt;sup&gt;b&lt;/sup&gt;</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>-</td>
<td>52.82 ± 2.62</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stoloniferol B</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>-</td>
<td>-39.62 ± 5.48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Engyodontiumone D</td>
<td><em>Engyodontium album</em> DFFSCS021 (CCTCC M 2014521)</td>
<td>-</td>
<td>41.10 ± 2.46</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Conioxanthone A</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>-</td>
<td>-31.07 ± 2.35</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Secalonic acid D</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>66.58 ± 0.86</td>
<td>98.07 ± 1.12</td>
<td>-</td>
<td>101.72 ± 1.43</td>
<td>-</td>
<td>99.80 ± 1.32</td>
</tr>
<tr>
<td>Secalonic acid B</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>69.79 ± 0.50</td>
<td>96.27 ± 0.96</td>
<td>-</td>
<td>100 ± 1.95</td>
<td>-</td>
<td>92.14 ± 3.25</td>
</tr>
<tr>
<td>Penicillixanthone A</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>31.72 ± 0.96</td>
<td>91.25 ± 1.43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Emodin</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>91.57 ± 1.33</td>
<td>91.00 ± 6.12</td>
<td>78.35 ± 1.97</td>
<td>78.25 ± 0.79</td>
<td>61.01 ± 7.19</td>
<td>50.87 ± 1.34</td>
</tr>
<tr>
<td>Citreorosein</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>25.86 ± 1.39</td>
<td>71.95 ± 1.45</td>
<td>24.38 ± 6.18</td>
<td>36.50 ± 5.70</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Penicillic acid</td>
<td><em>Aspergillus</em> sp. SCSGAF 0076 (CCTCC M 2012397)</td>
<td>100 ± 0.96</td>
<td>68.23 ± 4.16</td>
<td>33.25 ± 3.64</td>
<td>43.82 ± 9.51</td>
<td>22.02 ± 2.33</td>
<td>-</td>
</tr>
<tr>
<td>Astetoxin B</td>
<td><em>Aspergillus</em> sp. SCSGAF 0076 (CCTCC M 2012397)</td>
<td>-27.49 ± 4.82</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7α,14-Dihydroxy-6β-β-nitrobenzoylconfertifolin</td>
<td><em>Aspergillus</em> sp. SCSGAF 0076 (CCTCC M 2012397)</td>
<td>68.51 ± 0.66</td>
<td>87.49 ± 2.90</td>
<td>31.75 ± 6.29</td>
<td>31.72 ± 10.34</td>
<td>-</td>
<td>27.99 ± 11.66</td>
</tr>
<tr>
<td>Oxaline</td>
<td><em>Penicillium</em> sp. SCSGAF 0023 (CCTCC M 2012507)</td>
<td>28.96 ± 3.40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ochratoxin A methyl ester</td>
<td><em>Aspergillus</em> sp. SCSGAF 0076 (CCTCC M 2012397)</td>
<td>82.08 ± 0.75</td>
<td>92.81 ± 2.46</td>
<td>82.41 ± 0.68</td>
<td>93.35 ± 4.49</td>
<td>80.18 ± 0.79</td>
<td>-</td>
</tr>
<tr>
<td>Notoamide B</td>
<td><em>Aspergillus</em> westerdijkiae DFFSCS013 (CCTCC M 2013078)</td>
<td>40.11 ± 2.59</td>
<td>81.20 ± 2.60</td>
<td>39.23 ± 8.46</td>
<td>-</td>
<td>31.37 ± 5.72</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Error bar: SD.

<sup>b</sup>ACDT: 1-acetonyl-7-carboxyl-6,8-dihydroxy-3,4,5-trimethylisochroman.

"..." shows the inhibitory rates from −20% to 20%, which means no obvious differences between the treatments with and without compounds.
value was defined as –20% to 20%.

To study the dynamic changes of *S. aureus* biofilm formation caused by potential compounds, the biofilm formation of *S. aureus* with different concentrations of drugs (1.56, 3.12, 6.25, and 12.5 µg/ml) and at different intervals (6, 12, and 24 h) was detected. To further study the effects of potential compounds on already developed biofilms, the biofilms grown in 96-well plates for 24 h as described above were treated with different concentrations of the compounds (3.12, 6.25, and 12.5 µg/ml) for 24 h. After incubation, the plates were stained with crystal violet.

**Cell Growth Curve**

*S. aureus* cells grown overnight were diluted with fresh LB medium to an OD<sub>600</sub> of about 0.01 and then incubated with or without different concentrations of compounds (1.56, 3.12, 6.25, and 12.5 µg/ml) in a Bioscreen C microtiter plate (Labsystems) for 24 h at 37°C with shaking. The OD<sub>600</sub> was monitored and recorded in an automatic spectrophotometer (Bioscreen, Finland) at every 3 h. All experiments were performed in eight replicates.

**Confocal Laser Scanning Microscopy (CLSM)**

Pre-sterilized glass microscope slides were used to observe biofilms by CLSM as described in the previous study [29, 30]. Briefly, cells were grown in LB medium overnight and diluted with fresh LB medium to an OD<sub>600</sub> of about 0.01. Then, 2 ml dilutions were incubated with or without different concentrations of compounds (1.5, 3, and 6 µg/ml) in 12-well plates with a glass microscope slide in each well under static conditions. After 24 h, the glass slides were gently moved out and rinsed with deionized water to remove loosely attached cells. The biofilms on one side were stained with 5 µM SYTO9 dye (Sigma) in the dark, and those on the other side were wiped off. After 15 min, the slides were washed, and observed by CLSM (LSM 710 Zeiss, Germany) with a 60× objective lens to visualize the biofilms. The 488 nm excitation and 520 nm emission filter settings were used for detection of SYTO 9. Furthermore, quantification of biofilm parameters was processed with the COMSAT software using the CLSM images [31].

**Cell Aggregation**

Cell aggregation was assayed as reported previously [32]. Briefly, cells were grown in LB medium overnight and diluted with fresh LB medium to an OD<sub>600</sub> of about 0.01. Aliquots of 5 ml dilution, with or without different concentrations of compounds, were incubated statically in glass tubes. The OD<sub>600</sub> of upper 1 ml of the culture was recorded as OD<sub>600</sub> before. The residual culture was vortexed vigorously and its OD<sub>600</sub> was determined as OD<sub>600</sub> after. Percentage aggregation was calculated using the formula [(OD<sub>600</sub> before – OD<sub>600</sub> after)/OD<sub>600</sub> before] × 100.

**RNA Isolation and qRT-PCR**

*S. aureus* was grown in LB medium overnight and diluted to an OD<sub>600</sub> of about 0.01 and then was incubated with or without potential compounds in 12-well plates for 24 h under static conditions. Planktonic bacteria and formed biofilms in each well were harvested, and the total RNAs were extracted using RNAiso Plus Reagent (Takara, China) and transcribed reversely into cDNA with PrimeScript RT reagent kit (Takara) according to the manufacturer’s directions. Ten-fold dilutions of each cDNA were used as templates to assess the transcriptional levels of 17 genes via qRT-PCR with paired primers and 16S rRNA as an internal standard, using SYBR Premix Ex Taq (Takara) (Table S1). The relative transcription level of each gene was defined as the ratio of its transcript of biofilms grown in the indicated concentration of compounds over that in LB medium with DMSO, using the 2<sup>−ΔΔCt</sup> method [33]. The threshold cutoff was set to 1.5 (relative transcriptional levels > 1.5 or < 0.66), which means that it would be regarded to influence biofilm formation to a degree, when (compared with those in control cells) transcripts in cells treated with SAD either increased by >50% or decreased by >0.34%.

**Combination Testing of SAD with Three Antibiotics on *S. aureus* Growth and Biofilm Formation**

Penicillin and chloramphenicol are known to be important bactericides to *S. aureus*, and vancomycin is a preferred antibiotic to treat the infection caused by gram-positive bacteria in clinics. Therefore, in order to observe the synergistic effects of SAD, three kinds of antibiotics (ampicillin sodium salt, chloramphenicol, and vancomycin HCl) at their sub-MIC concentrations were chosen to combine with 1 µg/ml SAD, which could inhibit the biofilm formation of *S. aureus* by <20%. The minimum inhibitory concentrations (MIC) of the three antibiotics were assayed in 96-well plates. Wells containing 100 µl *S. aureus* dilutions (OD<sub>600</sub> = 0.01) were supplemented with different concentrations of ampicillin sodium salt (0 to 0.2 µg/ml), vancomycin HCl (0 to 2 µg/ml), and chloramphenicol (0 to 26 µg/ml) separately, and incubated at 37°C for 24 h. After incubation, the OD<sub>600</sub> of the wells was measured. For synergist effects of SAD, 150 µl of *S. aureus* dilution (OD<sub>600</sub> = 0.01) with 1 µg/ml SAD and different sub-MIC concentrations of ampicillin sodium salt (MIC: 0.05 µg/ml), vancomycin HCl (MIC: 1.2 µg/ml), or chloramphenicol (MIC: 6.5 µg/ml) were added into 96-well microtiter plates. For cell growth, the microtiter plates were incubated for 24 h at 37°C with shaking. Biofilm formation was initiated in the microtiter plates and incubated at 37°C for 24 h as described above. The absorbance at 570 nm or 600 nm was determined using a Multiskan GO Reader (Thermo Scientific). All experiments were performed in six replicates.

**Data Analysis**

Results from eight replicates were expressed as the mean ± standard deviation (SD) and statistical analysis was subjected to one-factor analysis of variance performed with the Data Processing System software [34]. Significant differences were considered at a *p* value of <0.05 in all experiments.
Results

Anti-Biofilm Effects of 106 Compounds

To find new anti-biofilm drugs, 106 compounds (Fig. S1) isolated from marine-derived fungi were tested in 96-well plates against the biofilm formation of *S. aureus*. The results showed that 18 compounds (Fig. 1) had certain effects on *S. aureus* growth and biofilm formation at the concentration of 100 µg/ml, where the inhibitory percentage is over the cutoff value, and especially 12 compounds exhibited notable anti-biofilm activity with an inhibiting rate of >50% (Table 1), whereas the other 88 compounds had no obvious activity compared with the negative treatment, with inhibitory rates from ~20% to 20%. To further verify the anti-biofilm activity of the 12 bioactive compounds, lower concentrations of these compounds were diluted and added into the culture of *S. aureus*. Four compounds (emodin, citrinin, and secalonic acids B and D) still showed strong anti-biofilm activity at the concentration of 12.5 µg/ml (Table 1). Specifically, secalonic acids B and D inhibited *S. aureus* biofilm formation by >90% without reducing cell growth at lower concentrations (Table 1), whereas biofilm formation by *Pseudomonas aeruginosa* PAO1 was not affected by secalonic acids B and D at concentrations up to 100 µg/ml, where the inhibitory rate was about 10%.

The 106 tested compounds (Fig. S1) included 9 simple

![Image](image-url)
Effects of Natural Products on *S. aureus* Biofilm

**Background**

Potential natural products were evaluated for their effects on *S. aureus* biofilm formation. Phenol derivatives, citrinin derivatives, chromones, anthraquinones, alkaloids, esters, cyclic peptides, pyrroles, and polyols were investigated. Comparison of the structure-bioactivity of these compounds suggested that specific structural features might be important for anti-biofilm activity.

**Secalonic Acid D Inhibited Biofilm Formation and Accelerated Biofilm Detachment**

The inhibitory effects of secalonic acid D (SAD) on *S. aureus* biofilm formation were evaluated. SAD inhibited biofilm formation in a dose-dependent manner, with an inhibitory rate of up to 97% at a concentration of 6.25 μg/ml. Secalonic acid B, an epimer of SAD, also showed dose-dependent inhibition.

Furthermore, SAD affected biofilm development, decreasing the rate of biofilm formation during 12–24 h. After treatment with SAD, the OD₅₇₀ of biofilms decreased by 34.2–43.7%, indicating that SAD accelerated biofilm eradication.

**Conclusion**

SAD was a potent inhibitor of *S. aureus* biofilm formation and maturation. Its effects were characterized by dose-dependent inhibition and acceleration of biofilm eradication.

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Fig. 2. Effects of secalonic acid D (SAD) on *S. aureus* biofilm formation.

(A, B) The relative biofilm formation after treatment with SAD and its analog was quantified relative to the treatment without any SAD after 24 h in 96-well plates, respectively. (C) The biofilm formation at OD₅₇₀ was detected at different intervals. (D) Eradication of the pre-developed biofilms was assayed in the presence of SAD. Error bar: SD. *p < 0.05.
**S. aureus** biofilms (Fig. 2D). These results showed that SAD inhibited biofilm development dose/time-dependently and slightly accelerated biofilm eradication in **S. aureus**.

**SAD Influenced the Biofilm Architecture**

CLSM studies were applied to analyze the effect of SAD on the **S. aureus** architecture, using glass slides after cells were stained with SYTO9. The CLSM images showed a typical biofilm on the surface of the glass slides in LB medium, whereas a sparse biofilm existed on the glass slides in LB medium supplemented with SAD, which varied in response to different concentrations (Figs. 3A and S2). COMSTAT analysis of the CLSM images showed that the total biomass and average thickness of the **S. aureus** biofilms were decreased dose-dependently by 30.9–53.8% and 24.9–44.8%, respectively, but the roughness coefficient and surface-to-biovolume ratio increased dose-dependently by 3,600–6,900% and 19.8–67.8%, respectively, in response to SAD (Figs. 3B–3E). However, there was no significant difference in maximum thickness of **S. aureus** biofilms when **S. aureus** was co-incubated with SAD (Fig. 3C). These results demonstrated that SAD dose-dependently influenced

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**Fig. 3.** Effects of secalonic acid D (SAD) on **S. aureus** biofilm architecture.

(A) Respective confocal scanning laser microscopy images of **S. aureus** in the presence of 0, 1.5, 3, and 6 μg/ml SAD. (B–E) Biofilm parameters of **S. aureus** biofilms were calculated by COMSTAT. Error bar: SD. *p < 0.05.
the architecture of the \textit{S. aureus} biofilm.

**Effect of SAD on Cell Growth and Cell Aggregation**

To monitor the growth of \textit{S. aureus} at different intervals, cells treated with SAD at concentrations ranging from 1.56 to 12.5 µg/ml were incubated in Bioscreen C microtiter plates (Labsystems). In the first 6 h, the growth of cells with SAD was severely delayed, but as the incubation time increased, the gap closed and the growth was similar to those without any SAD after 18 h (Fig. 4A). Moreover, when SAD was added to the media, the percentage aggregation was increased by 53.4%, 89.2%, and 106%, respectively, at the concentrations of 3.12, 6.25, and 12.5 µg/ml, compared with the medium without any SAD (Fig. 4B). The above results indicated that SAD had ultimately little effect on \textit{S. aureus} growth but slowed down the rate of growth in the early phase and promoted cell aggregation.

**SAD Modulated the Transcriptional Expressions of Biofilm-Related Genes**

In order to investigate the underlying molecular mechanism of anti-biofilm activity of SAD in \textit{S. aureus}, the transcriptional expressions of 17 biofilm-related genes in floating cells and biofilms were detected by real-time qRT-PCR, and the transcriptional confidence threshold cutoff was set to 1.5. In planktonic bacteria, SAD obviously up-regulated the transcriptional expression of the quorum-sensing gene (\textit{agr}), biofilm regulator genes (\textit{isaA} and \textit{icaD}), protease genes (\textit{aur}, \textit{sspA}, and \textit{clp}), nuclease gene (\textit{nuc2}), \textit{α}-hemolysin gene (\textit{hla}), phenol-soluble modulin genes (\textit{PSMa}, \textit{PSMβ}, and \textit{hld}), and one staphopain protease gene (\textit{sspB}) by 73.7–2,103%, and down-regulated the transcriptional expression of another staphopain protease gene (\textit{scpA}) by 60.5% (Fig. 5A). In formed biofilms, gene \textit{hla} was also up-regulated by 120%, but the expression of \textit{agrA}, \textit{isaA}, \textit{icaD}, \textit{aur}, \textit{clp}, \textit{nuc2}, \textit{PSMa}, \textit{scpA}, and another staphopain protease gene (\textit{sspC}) was repressed by 35.8–86.9% after 3.12 µg/ml SAD was added into the media (Fig. 5B). In addition, the expression levels of other biofilm regulators (\textit{icaA} and \textit{sigB}) were also down-regulated by 44.4–78.4% in response to SAD (Fig. 5B). These results implicated that SAD inhibited \textit{S. aureus} biofilm formation through the transcriptional regulation of biofilm regulators, proteases, nucleases, PSMs, and staphopains in planktonic cells and biofilm cells in different ways, which may be another example proving that it is a complex process that governs the formation of \textit{S. aureus} biofilms.

**SAD Synergistically Interacts with Three Antibiotics on \textit{S. aureus} Growth and Biofilm Formation**

Interaction of SAD with three antibiotics (ampicillin sodium salt, vancomycin HCl, or chloramphenicol), which are potent for \textit{S. aureus}, on \textit{S. aureus} growth and biofilm formation was tested by the microplate method. Compared with the control and treatments with a single compound, SAD synergistically interacted with the three antibiotics on \textit{S. aureus} growth and biofilm formation in varying degrees (Figs. 6A–6C). At 1 µg/ml, SAD reduced the MIC of ampicillin sodium salt (MIC: 0.05 µg/ml) to 0.02 µg/ml, of vancomycin HCl (MIC: 1.2 µg/ml) to 0.8 µg/ml and of chloramphenicol (MIC: 6.5 µg/ml), to 2.5 µg/ml. For ampicillin sodium salt, 1 µg/ml SAD obviously increased its antibacterial activity at both 0.005 and 0.01 µg/ml concentrations by 35.5%, but did not inhibit the increased

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**Fig. 4.** Effects of secalonic acid D (SAD) on \textit{S. aureus} growth and cell aggregation.

(A) Cell growth was assayed in the presence of different concentrations of SAD at different intervals. (B) The percentages of aggregation were detected in the presence of different concentrations of SAD. Error bar: SD. * \( p < 0.05 \).
biofilm formation caused by ampicillin sodium salt. For vancomycin HCl, SAD improved its antibacterial activity and anti-biofilm activity at concentrations of 0.125 and 0.25 μg/ml by about 42.7–48.3%. For chloramphenicol, SAD enhanced its activities at 0.5 μg/ml (but not 0.2 μg/ml) by 14.9% for antibacterial activity and 39.1% for anti-biofilm activity.

Discussion

Marine fungi have been reported to produce various secondary metabolites with various bioactivities. In the present study, the anti-biofilm activities of 106 compounds isolated from marine-derived fungi against S. aureus were detected using the crystal violet staining method in 96-well plates. The results demonstrated that four compounds (emodin, citrinin, and secalonic acids B and D) showed strong anti-biofilm activity with reduction by ≥50% at the concentration of 12.5 μg/ml (Table 1). Emodin, an anthraquinone, has been reported to reduce the biofilm formation and cell growth of S. aureus MSSA 6538 by ≥70% at 10 μg/ml and Streptococcus mutans by >80% at 5 μg/ml [3, 35], which was in line with our results. However, there is no report about the anti-biofilm activities of secalonic acids B and D and citrinin against S. aureus in previous studies. SAD could inhibit the pellicle formation of Shewanella oneidensis MR-1 at the concentration of 3.125 μg/ml during 12 h of incubation, but the pellicle formation grew as well as those without SAD when the incubation time was increased to 24 h [23]. It was interesting to find that secalonic acids B and D inhibited S. aureus biofilm formation up to 90% without reducing cell growth at the concentration of 6.25 μg/ml, and up to 60% at the concentration of 3.12 μg/ml after 24-h incubation (Figs. 2A and 2B), and 1 μg/ml (about 1.57 μM) SAD could synergistically interact with three antibiotics (ampicillin sodium salt, vancomycin HCl, and chloramphenicol) on S. aureus growth and biofilm formation in varying degrees (Figs. 6A–6C). Furthermore, the results obtained from different incubation hours demonstrated that SAD mainly inhibited biofilm development and slightly detached preformed biofilm, but showed no significant differences at the early phase of S. aureus growth (Figs. 2C and 2D). Meanwhile, SAD influenced the architecture of S. aureus.

Fig. 5. Quantitation of the relative transcriptional levels of selected biofilm-related genes in planktonic bacteria (A) and formed biofilms (B) via qRT-PCR after S. aureus cells were treated with 3.12 μg/ml secalonic acid D. The line represents the transcriptional levels of genes in the control experiments, which was defined as 100%. Error bar: SD. *p < 0.05.

biofilm by forming sparse biofilm, dose-dependently decreasing the biomass and average thickness, and dose-dependently increasing the roughness coefficient and surface-to-biovolume ratio (Fig. 3). Among the various parameters, the increased surface-to-biovolume ratio and roughness coefficient signified that a high fraction of cells in the *S. aureus* biofilms were exposed to the bulk liquid, and biofilm heterogeneity increased in response to SAD [31].

It was reported that two hydroxyl units at the C-1 and C-2 positions of anthraquinones were verified to be important for antibiofilm and anti-hemolytic activities in *S. aureus* [3], and dimeric aaptamine alkaloids, suberitines B and D, showed more potent cytotoxicities than their monomers against the selected P388 cells [36]. Thus, another objective in the present study was to discover the structural motif important for anti-biofilm activities against *S. aureus*. The results suggested that the double bond between C-1 and C-10 of citrinin derivatives and the C-C connection position of two chromone monomers may significantly affect the anti-biofilm activity against *S. aureus* (Fig. 1, Table 1, and Fig. S1).

The mechanism governing the formation of *S. aureus* biofilm is complicated and involves environmental factors, quorum sensing, proteases, DNase, and other global regulators [17]. In the present study, qRT-PCR data showed that SAD regulated in varying degrees the expressions of 17 biofilm-related genes in planktonic *S. aureus* cells and biofilms (Fig. 5). In planktonic cells, the transcriptional levels of negative biofilm regulators (*agr* and *isaA*) and three PSMs controlled by *agr* were increased by SAD (Fig. 5A), which supported its anti-biofilm activity on *S. aureus*, because induction of *agr* and/or *isaA* resulted in the inhibition of *S. aureus* biofilm formation and three PSMs played a negative role in biofilm formation [37–40]. On the other hand, *aur* overexpression inhibited biofilm formation in *S. aureus* sigB mutants and *aur/sspB* deletion increased biofilm formation in *S. aureus* sigB deletions [41, 42]. Meanwhile, when *S. aureus* was incubated with purified Nuc2, the presence of Nuc2 could prevent biofilm formation in a dose-dependent manner [43]. Thus, the increased transcriptional expression of *agr*, *isaA*, PSMs, *aur*, *sspB*, and *nuc2* in planktonic cells might be one explanation.

![Fig. 6. Interaction of 1 µg/ml secalonic acid D (SAD) with different concentrations of ampicillin sodium salt, vancomycin HCl, and chloramphenicol on the MIC of the antibiotics (A), *S. aureus* growth (B), and biofilm formation (C). Error bar: SD. * p < 0.05.](Image)
for the inhibition of \textit{S. aureus} biofilm formation caused by SAD. In formed biofilms, the expression levels of \textit{icaA} and \textit{icaD} were down-regulated (Fig. 5B), which was in accordance with previous studies where repression of \textit{ica} expression is the best understood mechanism in the inhibition of biofilm formation \cite{44-45}. Furthermore, \textit{sigB} disruption led to the inhibition of \textit{S. aureus} biofilm formation \cite{46}, which suggested that the down-regulated expression of \textit{sigB} may be another reason for the inhibition of biofilm formation caused by SAD. Thus, these findings demonstrated that SAD inhibits biofilm formation by regulating the transcriptional levels of biofilm-associated genes in different ways under different conditions in \textit{S. aureus}, which indicated that the biofilm formation of \textit{S. aureus} is a complicated process. Further genetic studies are required to obtain more details of the molecular mechanisms responsible for the effects of SAD on \textit{S. aureus} biofilms.

It is urgently needed to find novel strategies to control pathogenic \textit{S. aureus}. This study demonstrates that many natural products isolated from marine-derived fungi have significant antibacterial and anti-biofilm activities, especially SAD with its strong ability of inhibiting the formation of \textit{S. aureus} biofilm, and which with the increased antibacterial and anti-biofilm activities of the antibiotics ampicillin sodium salt, vancomycin HCl or chloramphenicol may be regarded as a potential biofilm inhibitor against \textit{S. aureus}. The findings strongly support that marine fungi are important resources of biofilm inhibitors.

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