Potent antifouling compounds produced by marine *Streptomyces*

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**ABSTRACT**

Biofouling causes huge economic loss and a recent global ban on organotin compounds as antifouling agents has increased the need for safe and effective antifouling compounds. Five structurally similar compounds were isolated from the crude extract of a marine *Streptomyces* strain obtained from deep-sea sediments. Antifouling activities of these five compounds and four other structurally-related compounds isolated from a North Sea *Streptomyces* strain against major fouling organisms were compared to probe structure–activity relationships of compounds. The functional moiety responsible for antifouling activity lies in the 2-furanone ring and that the lipophilicity of compounds substantially affects their antifouling activities. Based on these findings, a compound with a straight alkyl side-chain was synthesized and proved itself as a very effective non-toxic, anti-larval settlement agent against three major fouling organisms. The strong antifouling activity, relatively low toxicity, and simple structures of these compounds make them promising candidates for new antifouling additives.

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1. Introduction

Biofouling poses one of the most serious problems to marine industry and aquaculture development. In the marine environment, natural and artificial surfaces immersed in seawater are colonized by biofoulers including micro-foulers such as marine bacteria, algae, and protozoa, and macro-foulers such as barnacles, bryozoans, and tubeworms (Callow and Callow, 2002; Dobretsov et al., 2006; Wahl, 1989). Biofoulers that accumulate on the ship hulls increase drag and surface corrosion, thereby severely diminishing ships’ maneuverability and carrying capacity (Chambers et al., 2006). In addition, biofouling causes huge material and economic costs in maintenance of mariculture, naval vessels, and seawater pipelines (Yebra et al., 2004). It is estimated that governments and industry spend over US $6.5 billion annually to prevent and control marine biofouling (Bhadury and Wright, 2004).

In order to control biofouling, broad-spectrum metal biocides, such as tributyl tin (TBT) and copper, have been added to marine paints as antifouling compounds (Alberte et al., 1992; Thomas et al., 2001). Although very effective, these biocides are often extremely toxic to a wide range of non-target organisms (Alzieu, 2000; Konstantinou and Albanis, 2004). For example, gastropod imposex, mussel larvae mortality, and oyster shell malformation have all been recorded as ecotoxicological effects of TBT even at extremely low concentrations (in the ng l\(^{-1}\) range) (Alzieu, 2000). The increasing concerns about the negative effects of TBT led the International Maritime Organization (IMO) and Marine Environmental Protection Committee (MEPC) to decide to ban the usage of TBT or other substances containing tin as biocides in antifouling paints beginning in January 2008. An effective alternative to TBT is not currently available; therefore, there is an urgent demand for the development of environment- and human-friendly non-toxic antifouling compounds.

Marine natural products are a promising source of novel antifouling agents. Indeed, in the past few decades, many compounds with strong antifouling activity have been isolated from marine sponges, corals, and algae (Clare et al., 1992, 1999; Dworjanyn et al., 2006; Qi et al., 2008; Tsukamoto et al., 1997, see reviews by Armstrong et al. (2000), Fusetani (2004), and Omae (2003)). However, the supply issues of these antifouling compounds isolated from macroorganisms often hamper their commercial application (Dobretsov et al., 2006). Marine microorganisms have proved to be an excellent source of bioactive compounds. For example, some compounds produced by marine bacteria from both shallow water and the deep sea showed pronounced inhibitive effects on the settlement of both micro- and macro-fouling organisms (Burgess et al., 2003; Dobretsov et al., 2007). In particular, many novel compounds with different biological functions have been recently isolated from marine *Streptomyces* (Davidson,
In addition, supply of bioactive products from marine microorganisms may not be limiting because of their cultivability.

In a previous study, we screened 11 deep-sea *Streptomyces* isolates and all of them showed antifouling activity to some extent (Xu et al., 2009). In this study, one of the most active strains, *Streptomyces albido флавus* strain UST040711-291, was cultured in large scale. Five structurally similar compounds were isolated and identified from its crude extract using bioassay-guided fractionation. The antifouling activity of these compounds and four other structurally-related compounds isolated from another marine *Streptomyces* species were compared to generate structure–activity relationship data. Based on structure–activity relationship analysis, we synthesized another compound with a simplified structure but excellent antifouling activities.

### 2. Methods

#### 2.1. Strain isolation

*S. albido флавus* sp. UST040711-291 was isolated from a deep-sea sediment sample collected from West Pacific (N9°10’25”, W145°22'09”) at a depth of 5100 m during the DY11-105 cruise of DAYANG Number 1 in 2001, and was deposited in the culture collection of marine bacteria in the Third Institute of Oceanography, State Oceanic Administration, Xiamen, China. According to its 16S rRNA gene sequence (Accession number: FJ591130), this strain belongs to *S. albido флавus* group.

#### 2.2. Bacterial fermentation

The bacterium was cultured in multiple 3 l flasks containing MGY culture medium (1% of malt extract, 0.4% of glucose, and 0.4% of yeast extract) in seawater at 25°C with agitation (200 rpm) for 4 days until they reach the stationary phase. In total, 60 l of bacterial culture were obtained.

#### 2.3. Extraction and isolation of bioactive compounds

The spent bacterial culture broth was firstly separated from the bacterial cells by centrifugation at 5000g for 15 min, and then exhaustively extracted by ethyl acetate (EtOAc). In total, 60 l of bacterial culture was extracted. The EtOAc phase was dried under vacuum and the residue was collected. The crude extract was subjected to macroporous resin column chromatography using a gradient solvent system from water to acetone. The fractions collected were tested in bioassay and the active one was further purified exhaustively extracted by ethyl acetate (EtOAc). In total, 60 l of bacterial culture were obtained.

#### 2.4. Structure identification of compounds isolated from *S. albido флавus* strain UST040711-291

The 1H and 13C NMR spectra were recorded on a nuclear magnetic resonance (NMR) spectroscopy (JEOL DRX 500) in CDCl3 with TMS as the internal standard. Structures of the compounds 1–5 were determined using NMR spectroscopy and mass spectrometry (MS). HPLC was carried out on a Waters 600 system with a Waters 996 photodiode array (PDA) detector.

#### 2.5. Anti-larval attachment assay

In order to perform bioassay-guided isolation and evaluate each pure compound for antifouling activity, we tested the activity of compounds 1–10 against cyprids of the barnacle *Balanus amphitrite* Darwin, a common crustacean biofouler widely-used in antifouling studies. Adult brood stocks of *B. amphitrite* Darwin were collected from pilings and floating rafts in the Port Shelter, Hong Kong (22°19’N, 114°16’E). Barnacle larvae were obtained and reared according to the method described in Harder et al. (2001) until larva reached the settlement stage, called cyprids. Each compound was dissolved in a small amount of DMSO and then transferred into filtered seawater to make up the test solution with a known concentration. One millilitre of the test solution was allocated to a well of a 24-well plate (Nunc, USA) and about 20 barnacle cyprids were added to the solution. The plates were incubated overnight at 28°C in darkness; at the termination of incubation, the numbers of the settled and swimming larvae were counted directly under a microscope, and where appropriate, possible toxic effect was noted. The number of the settled larvae was expressed as a percentage of the total number of larvae per well. There were five replicates for each fraction or compound. In addition, we also tested the inhibitive activity of compound 10 on two other common biofoulers: the polychaete *Hydrodoid elegans* and the bryozoan *Bugula neritina*. Batch cultures of polychaete larvae were reared for 5 days to competent stage according to Qian and Pechenik (1998). Adults of *B. neritina* were collected from submerged rafts at the fish farms in Yung Shue O, Hong Kong (14°21’E, 22°24’N) and larvae were obtained according to the method described by Dobretsov et al. (2007). For *H. elegans*, settlement requires an inducer (Qian and Pechenik, 1998), 3-isobutyl-1-methylxanthine (IBMX) was used as an artificial settlement-inducer. Wells containing FSW and the untreated larvae served as the negative control while ones containing FSW and the IBMX-treated larvae as the positive control. *B. neritina* larvae will settle in the absence of any artificial inducer, so newly released larvae were used in the bioassays. About 20 of the test larvae (IBMX-induced *H. elegans* or newly-released *B. neritina*) were added to the test solutions that contain different concentrations of compound 10.

#### 2.6. Calculation of EC50 and LC50 values of each compound

For the calculation of EC50 and LC50 of each compound, a serial 2-fold dilution was made from 100 μg ml⁻¹ of the compound to the concentration where no inhibitory effect (for calculation of EC50) or no lethal effect (for calculation of LC50) could be detected in the larval settlement assay. A concentration–response curve was then plotted and a trend line was constructed for each compound. EC50 was calculated as the concentration where 50% of the larval population was inhibited to settle as compared to the control while LC50 was calculated as the concentration where 50% of the larval population was dead. Each compound was tested using three batches of larvae so that the mean and standard deviation of the EC50 and LC50 values could be determined.
2.7. Field test of compound 10

To field-test the antifouling performance of compound 10, it was incorporated into paints following the method described by Skattebol et al. (2006) with modifications. Briefly, 0.4 g of compound 10 was mixed into the following base ingredients: 1.5 g of a copolymer (butyl methacrylate, methacrylic acid, and methyl methacrylate), 2.0 g of rosin, 1.0 g of CaCO3, 2.0 g of titanium dioxide, 0.2 g of bentonite, and 10.0 ml of xylene. The mixture was mixed well and then painted onto the center part of three PVC panels. After the paint dried completely, three treated panels and three control panels were attached to the floating rafts of a fish farm in Yung Shue O, Hong Kong (114°21’E, 22°24’N) where they remained constantly submerged; typically the rafts are heavily fouled. The panels were examined after 3 months and the area covered by the biofoulers were measured. The coverage of biofoulers on the compound-treated panels was measured compared with the control panels with a Student’s t-test.

3. Results and discussion

3.1. Structures and bioactivities of compounds produced by S. albidoflavus strain UST040711-291

In total, 601 of S. albidoflavus strain UST040711-291 were cultured and exhaustive extraction of the bacterial spent culture broth yielded 12.3 g of crude extract. The fractionation and further purification of the crude extract yielded compounds 1–5 (Fig. 1) with net yield of pure compounds ranged from 2.1 to 23 mg. The spectral data of compounds 1–5 identified in this study match well with compounds previously reported from other marine Streptomyces species (Cho et al., 2001; Mukku et al., 2000). Compounds 1 and 4 were also isolated from a marine bacterium Streptoverticillium luteoverticillatum that is actually a Streptomyces species although the authors used a different yet synonymic name (Li et al., 2006).

Compounds 1–5 belong to a class of α,β-unsaturated lactones and they are usually produced by bacteria, fungi, and gorgonians (Mukku et al., 2000). Some of their saturated analogues play important roles in regulation of antibiotics production and morphological differentiation leading to production of secondary metabolites in Streptomyces (Takano, 2006). The most intensively studied example, A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone), can induce streptomycin production and sporulation in Streptomyces griseus (Takano, 2006). Other members from this group have been reported to have inhibitor activities and antifeedant properties (Karlsson et al., 2007).

Comounds 1–5 have been studied previously for their antibacterial, antifungal and anti-tumor, but did not show any antibacterial or antifungal activities (Li et al., 2006). Only Compounds 1 and 4 exhibit some moderate anti-tumor activities (Li et al., 2006). So far, these compounds have only been discovered in Streptomyces. Although these compounds have no known physiological functions, they may perform some important biological functions in the complex life cycle of Streptomyces. Furthermore, their structures indicate they are likely to be synthesized by polyketide synthases.

In this study, we reported the antifouling activities of compounds 1–3 for the first time. As Table 1 shows, all these three compounds could inhibit the larval settlement of the barnacle B. amphitrite, a model fouling species, with low EC50 values. These compounds did not kill larvae at 100 μg ml⁻¹. The therapeutic ratio (LC50/EC50) is a way of expressing the effectiveness of the compound in relation to its toxicity and the desired target ratio should be much greater than 1 for use in an antifouling coating (Clare et al., 1992). Compounds 1–3 all have small therapeutic ratios, suggesting they might have some toxicity as antifouling agents. To search for environmentally-benign antifouling agents, compounds with much larger therapeutic ratios are desired as their toxicity may be low.

3.2. Structure–activity relationships of related compounds

Following the bioassay-guided fractionation of extracts of S. albidoflavus strain UST040711-291, we found that all the compounds 1, 2, and 3 inhibited the larval settlement of barnacle at relatively low concentrations. The common moiety, the 2-furanone, of these three compounds led to the hypothesis that it may be an important functional group responsible for antifouling activities of these three compounds. In comparison to compound 1, compounds 4 and 5, the former has a shorter fatty acid side-chain than compound 1 and the latter possesses one more hydroxyl group on its side-chain, had much higher EC50 values. Based on the differences of their structures and bioactivities, we suspected that the side-chain might have affected the bioactivities by modifying the lipophilicity of the compounds – compounds 4 and 5 are less lipophilic than compounds 1, 2, and 3, they have less potent antifouling activities than the latter (Table 1). This result corresponds very well with the anti-tumor activities of compounds 1, 2, and 4 described by Li et al. (2006) in which they found compound 4 was the least active compound among the three compounds tested. In fact, lipophilicity has already been demonstrated to strongly influence bioactivities in many cases (Gupta, 2001; Siddiqui et al., 1999). One of the explanations is that the compound can enter the cell by passive diffusion more easily when the molecule is more lipophilic, and consequently, can lead to the expression of its

Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Balanus amphitrite larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (μg ml⁻¹)</td>
</tr>
<tr>
<td>1</td>
<td>14.8±5.54</td>
</tr>
<tr>
<td>2</td>
<td>9.65±1.50</td>
</tr>
<tr>
<td>3</td>
<td>8.67±2.73</td>
</tr>
<tr>
<td>4</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

– Not applicable.
that the 2-furanone moiety may be essential for the observed bioactivity at reduced concentration compared to less lipophilic molecules (Siddiqui et al., 1999).

In order to confirm our hypothesis of the structure–antifouling–activity relationships, the antifouling activity of four more compounds with related structures were compared. Compounds 6–9 (Fig. 2) were produced by another marine Streptomyces species isolated from the North (Dickschat et al., 2005). Compound 6 has the same 2-furanone ring, but its fatty acid side-chain does not contain a hydroxyl group compared to compound 4. The bioassay results showed that compound 6 (Table 2) has an EC₅₀ of around 5 µg ml⁻¹, which is at least 20 times more potent than compound 4. This piece of evidence strongly supported our hypothesis that lipophilicity affects the bioactivity because compound 6 is more lipophilic due to the lack of the hydroxyl group. It was also in a good agreement with our hypothesis that the 2-furanone ring is responsible for the bioactivity. Further tests of compounds 7, 8, and 9 (Table 2) showed none of these three compounds were active against barnacle larvae even at a concentration of 100 µg ml⁻¹. Compared to compound 6, compound 7 lacked a double bond on its ring while compound 8 had a double bond at the C-3, their substantial differences in bioactivities from compound 6 indicated that the 2-furanone moiety may be essential for the observed bioactivity.

In a previous work, Clare et al. (1999) examined the antifouling activity of an array of analogues which contain either furan or lactone rings in order to determine whether furan and lactone rings were important functional groups in two natural antifouling compounds which act as defensive molecules in two fouling Octocoral species. Their results also indicated that both the functional group and lipophilicity are essential for the bioactivity. Due to the limitation of the analogues tested, the authors were not able to determine whether 2-furanone ring is an important functional group in one of the antifouling compound, pukalide, which contains both a furan group and 2-furanone group. Our work proved that 2-furanone ring is an important functional group for antifouling activity, adding to the knowledge about the possible reasons why this natural antifouling compound is so potent (EC₅₀ = 0.02–0.2 µg ml⁻¹ against cyprids of B. amphitrite).

The functional moiety found in this study, 2-furanone ring, is also present in the antifouling furanones found in the red alga Delisea pulchra. The defensive roles of these furanones in the alga have already been validated (Dworjanyn et al., 2006). These halogenated furanones are well known as quorum-sensing blockers that are able to inhibit biofilm formation efficiently at very low concentrations. They are also able to inhibit the settlement of invertebrate larvae and algal spores. The 2-furanone ring may also be responsible, or at least to some extent, for the observed pronounced antifouling activity of these halogenated furanones. In a preliminary experiment, we found that compound 1, 2, and 3 could inhibit the fluorescence emission in Vibrio fischeri, however, they could not inhibit or reduce violacein production in Chromobacterium violaceum. Since the results are not conclusive, further study will be carried out to determine whether they are quorum-sensing inhibitors in near future.

### 3.3. Compound optimization for antifouling application

The successful localization of the functional moiety of these compounds helped us optimize the structure–activity relationship. Compound 10 (Fig. 3) was then synthesized, whose branched side-chain was replaced by a straight chain. Its structure was verified by both NMR and MS spectra. As predicted, compound 10 displayed strong inhibitive activity against barnacle larvae with a low EC₅₀ of 0.6 µg ml⁻¹ (Table 3); more importantly, no larvae were killed at a concentration of 50 µg ml⁻¹ after 24 h. Compound 10 has a therapeutic ratio greater than 80 for barnacle larvae, indicating its low toxicity to this species. In order to test whether this compound was effective at inhibiting settlement of larvae of other fouling organisms, we evaluated its activity against larvae of another hard fouler polychaete H. elegans and a soft fouler bryozoan B. neri-tina. Compound 10 strongly inhibited both species of larvae with EC₅₀ values near 0.02 µg ml⁻¹ for H. elegans and 0.2 µg ml⁻¹ for B. nerteina (Table 3). Moreover, the therapeutic ratio of compound 10 was larger than 100 for H. elegans larvae and 250 for B. nerteina larvae. The large therapeutic ratios of compound 10 for all three major fouling organisms suggest that this compound not only has greatly improved antifouling activity than compounds 1–3, but also has substantially reduced toxicity.

Compound 10 was also incorporated into marine paint for field testing. Only 5% (w/w) of compound 10 was mixed into the basal paints and painted onto the center part of the panels. After

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**Table 2**

Anti-larval settlement activity of compounds 6–9 isolated from the North Sea Streptomyces strain GWS-BW-H5 against cyprid larvae of the acorn barnacle Balanus amphitrite. EC₅₀ represents the concentration of a compound where 50% of larval population was inhibited to settle compared to control while LC₅₀ represents the concentration of a compound required to kill 50% of larvae of a tested population.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Balanus amphitrite larvae</th>
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<tbody>
<tr>
<td></td>
<td>EC₅₀ (µg ml⁻¹)</td>
</tr>
<tr>
<td>6</td>
<td>&gt;100</td>
</tr>
<tr>
<td>7</td>
<td>&gt;100</td>
</tr>
<tr>
<td>8</td>
<td>&gt;100</td>
</tr>
<tr>
<td>9</td>
<td>&gt;100</td>
</tr>
</tbody>
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- Not applicable.

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**Fig. 2.** Chemical structures of compounds 6–9, which were identified from a North Sea Streptomyces strain GWS-BW-H5 and synthesized.

**Fig. 3.** Chemical structure of compound 10, which was synthesized based on the analysis of the structure–activity relationship.

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**Table 3**

Anti-larval settlement activity of compound 10 against larvae of three common fouling species. EC₅₀ represents the concentration of a compound where 50% of larval population was inhibited to settle compared to control while LC₅₀ represents the concentration of a compound required to kill 50% of larvae of a tested population.

<table>
<thead>
<tr>
<th>Organism</th>
<th>EC₅₀ (µg ml⁻¹)</th>
<th>LC₅₀ (µg ml⁻¹)</th>
<th>LC₅₀/EC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balanus amphitrite larvae</td>
<td>0.518 ± 0.062</td>
<td>&gt;50</td>
<td>&gt;97</td>
</tr>
<tr>
<td>Hydrides elegans larvae</td>
<td>0.0168 ± 0.0002</td>
<td>&gt;2</td>
<td>&gt;119</td>
</tr>
<tr>
<td>Bugula neritina larvae</td>
<td>0.199 ± 0.018</td>
<td>&gt;50</td>
<td>&gt;250</td>
</tr>
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submergence in seawater for 3 months, these treated areas were much less fouled compared to the outside area of the same panels. In the control group without compound 10, the entire surface of the panels was seriously fouled by all kinds of fouling organisms (Fig. 4). The field experiment suggested compound 10 is a highly potent antifouling agent even in real practice. Moreover, its high lipophilicity would reduce its solubility in seawater and improve its incorporation in a potential antifouling paint.

4. Conclusion

Here we have tried to fill the urgent need for non-toxic, effective antifouling agents by isolating antifouling compounds from marine bacteria. A group of compounds with similar structures were isolated from two marine Streptomyces species and their antifouling activities were reported here for the first time. Their structure–activity relationships were analyzed and a more potent
compound was designed based on the finding. This compound proved to have outstanding antifouling activity even at low concentrations. It meets many criteria to be a good candidate for a low-toxic/non-toxic antifouling additive and has been filed for its application for antifouling purposes (PCT/CN2009/071302).

Acknowledgements

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