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Antifouling potentials of eight deep-sea-derived fungi from the South China Sea

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Abstract Marine-derived microbial secondary metabolites are promising potential sources of nontoxic antifouling agents. The search for environmentally friendly and low-toxic antifouling components guided us to investigate the antifouling potentials of eight novel fungal isolates from deep-sea sediments of the South China Sea. Sixteen crude ethyl acetate extracts of the eight fungal isolates showed distinct antibacterial activity against three marine bacteria (*Loktanella hongkongensis* UST950701–009, *Micrococcus luteus* UST950701–006 and *Pseudoalteromonas piscida* UST010620–005), or significant antilarval activity against larval settlement of bryozoan *Bugula neritina*. Furthermore, the extract of *Aspergillus westerdijkiae* DFFSCS013 displayed strong antifouling activity in a field trial lasting 4 months. By further bioassay-guided isolation, five antifouling alkaloids including brevianamide F, circumdatin F and L, notoamide C, and 5-chlorosclerotiamide were isolated from the extract of *A. westerdijkiae* DFFSCS013. This is the first report about the antifouling potentials of metabolites of the deep-sea-derived fungi from the South China Sea, and the first stage towards the development of non- or low-toxic antifouling agents from deep-sea-derived fungi.

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Keywords Deep-sea-derived fungi · Antibacterial · Antifouling · *Aspergillus westerdijkiae* · Alkaloids

Introduction

In the marine industry, the accumulation of living organisms on artificial surfaces by adhesion, growth, and reproduction is known as marine biofouling [7]. More than 4,000 kinds of marine biofouling species live primarily in the shallower water along the coast and in harbors that provided abundant nutrients [36]. In general, these biofouling species can be divided into two major categories including microfouling (or biofilm organisms) and macrofouling. The first of these are bacteria and diatoms, and the other category includes macrofouling organisms such as algae, ascidians, barnacles, bryozoans, calcarina, mussels, polychaete worms, and seaweeds [7]. Marine biofouling affects a wide range of human activities in the aquatic environment, especially in shipping; it is associated with economic loss due to speed reduction and higher costs both for fuel and for hull [23]. Due to the economic significance of biofouling on technical objects, various antifouling measures are adopted by maritime industries. Current antifouling technology is based on the application of toxic substances, such as copper and tributyltin that can be harmful to the natural environment [16]. However, since 2008, an increasing number of countries have completely banned the application of antifouling coatings based on organotin compounds (e.g., tributyltin and triphenyltin) on ships due to their extreme danger to the environment [30]. There is, therefore, an urgent need for effective but environmentally friendly natural antifoulants, particularly those of marine organisms.

In the marine environment, many organisms including corals, algae, sponges, ascidians, and others are believed

to produce many natural antifouling substances by either marine organisms themselves or their symbionts in order to get rid of undesirable encrusting organisms [16]. These diverse natural antifouling substances are advantageous over conventional toxic biocides and hold a broad-spectrum of antifouling activity; they are also less toxic, effective at low concentrations, biodegradable, and reversible in effects [30].

Continuing to search for antifouling natural products, we have found many diterpenoids [26, 27], steroids [24], and flavonoids [28] from marine corals and seagrasses of the South China Sea showing strong antifouling activity against *Balanus amphitrite* and *Bugula neritina* larval settlement. However, since bioactive compounds are often at extremely low concentration in macroorganisms, supply problem makes it almost impossible for industry to develop those marine bioactive compounds into antifouling agents [35]. Thus, great efforts have also been made to search for antifouling metabolites from marine-derived microorganisms. Recently, we also have obtained some antifouling metabolites from the marine-derived bacterium *Bacillus amyloliquefaciens* SCSIO 00856 [11] and the fungi *Aspergillus* sp. SCSGAF 0076 [3], *Cladosporium* sp. F14 [25], *Penicillium* sp. OUCMDZ-776 [14], *Penicillium* sp. SCSGAF 0023 [2], and *Xylariaceae* sp. SCSGAF 0086 [21].

At present, an increasing number of marine-derived microbes have been reported to produce many antifouling natural products, but most of these microbes are derived from shallow-sea environment [30]. Deep-sea-derived microbes are still poorly understood as antifouling sources. In this study, we investigated the indirect and direct inhibitive effects on larval settlement of 16 ethyl acetate extracts obtained from eight deep-sea-derived fungal cultures by evaluating their antibacterial activity against three marine bacteria and antifouling activity against *B. neritina* larval settlement in laboratory test. Furthermore, in order to confirm the antifouling potential, two strong active extracts were evaluated by their antifouling activity in field tests. Further study was performed to obtain some compounds with antifouling activity against *B. neritina* larval settlement from the strongest active extract using bioassay-guided isolation by silica gel column chromatography and high-performance liquid chromatography.

Materials and methods

Microorganisms

Eight novel deep-sea-derived fungal isolates including *Acremonium implicatum* DFFSCS001 (AI001), *Aspergillus westerdijkiae* DFFSCS013 (AW013), *Alternaria tenuissima* DFFSCS003 (AT003), *Cladosporium cladosporioides* DFFSCS016 (CC016), *C. sphaerospermum* DFFSCS019 (CS019), *Engyodontium album* DFFSCS021 (EA021), *Geomyces vinaceus* DFFSCS022 (GV022), and *Tritirachium* sp. DFFSCS034 (TS034) were selected for this study. These fungal strains were isolated from deep-sea sediments of the South China Sea, and maintained on mPDA (marine PDA, 200 g of potatoes, 20 g of glucose, 20 g of agar, and 1,000 ml seawater) slants, stored at 4 °C, and renewed periodically. Detailed information about these fungal isolates except for *Tritirachium* sp. DFFSCS034 was given by [37], and more information about *Tritirachium* sp. DFFSCS034 was described in GenBank with the accession number JX156380.

Cultivation and extraction

Liquid and solid fermentations were applied for culturing all the eight fungal isolates to obtain crude extracts. Each fungal isolate was inoculated into a 500-ml Erlenmeyer flask containing 150 ml of mPDB medium (marine PDB, 200 g of potatoes, 20 g of glucose, and 1,000 ml of seawater) and then cultivated on a rotary shaker at 200 rpm and 26 °C for 4 days as seed culture.

Then, 10-ml seed culture was inoculated into a 500-ml Erlenmeyer flask containing 150 ml mPDB medium and then cultivated without shaking at 28 °C for 30 days. A total of 15 l of fungal culture broth was filtered through cheesecloth to separate the broth supernatant and fungal mycelia. The broth supernatant was extracted three times with an equal volume of ethyl acetate, and the fungal mycelia were extracted three times with 80 % acetone. The acetone extract was evaporated under reduced pressure to afford an aqueous solution and then extracted three times with ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under reduced pressure at 45 °C to yield 10–20 g of crude extracts. Extracts 1–8 were obtained from the broths of fungal isolates AI001, AW013, AT003, CC016, CS019, EA021, GV022, and TS034, respectively.

For solid fermentation, 50 ml of seed culture was transferred into autoclaved 5,000-ml Erlenmeyer flask containing about 500 g of solid rice medium (400 g of commercially available rice, 2 g of yeast extract, 2 g of glucose, 600 ml of sea water) and incubated at 26 °C as static culture for 30 days. A total of 2,000 g of rice culture was crushed and extracted with 80 % acetone three times. The acetone extract was evaporated under reduced pressure to afford an aqueous solution and then extracted three times with ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness under reduced pressure at 45 °C to yield 20–30 g of crude extracts. Extracts 9–16 were obtained from these rice cultures of fungal isolates AI001, AW013, AT003, CC016, CS019, EA021, GV022, and TS034, respectively.

Determination of antibacterial activity

The antibacterial activities of the extracts were determined against two larval settlement-inducing bacteria *Loktanella hongkongensis* UST950701-009 (LH009), *Micrococcus luteus* UST950701-006 (ML006), and one marine pathogenic bacterium *Pseudoalteromonas piscida* UST010620-005 (PP005) [10, 18, 25], using the standard paper–disk–diffusion technique [1]. Sterile disks (6 mm in diameter) were impregnated with the individual extracts (in ethyl acetate) of interest (50 μg per disk) and dried. A disk loaded with 50 μg of streptomycin was used as a positive control. Detailed information of the determination method was given by Dash et al. [10]. The antibacterial activity was expressed as the diameter of the growth inhibition zone (mm). Each test was performed three times. All the indicator microorganisms were supplied by Prof. Peiyuan Qian (Hong Kong University of Science and Technology/Department Biology) and deposited in the South China Sea Institute of Oceanology, Chinese Academy of Sciences.

Measurement of antifouling settlement activity

The bryozoan *B. neritina* was used to test the antilarval settlement activities of these extracts and compounds. Colonies of *B. neritina* containing mature and developing embryos in their ovicells were collected in March 2013 from Daya Bay (114°32′59 E, 22°40′37 N), Shenzhen, China. Adult colonies were transferred to a small glass aquarium and kept overnight in the dark in non-aerated seawater. To induce the release of larvae, the water in the aquarium was changed and the colonies were exposed for about 30 min to sunlight. The positively phototactic larvae that were attracted to the beam of sunlight were then transferred into a clean beaker by using a wide-mouthed pipette, and immediately used for testing [6, 13].

The antifouling activities of 16 extracts were measured using larvae of the bryozoan *B. neritina*. Briefly, the test extracts were dissolved in a small amount of dimethyl sulfoxide (DMSO) and then diluted with filtered sea water (FSW) to achieve final concentration of 50 μg/ml for their antifouling evaluation. Fifteen to 20 competent larvae were gently transferred into each well with 1 ml of testing solution in three replicates, and the wells containing larvae in FSW with DMSO alone served as the controls. The plates were incubated for 1 h at 23 °C. The extracts' effects on larval settlement were determined by examining the plates under a dissecting microscope to check for (1) settled larvae, (2) non-settled larvae, and (3) any possible toxic effects, such as killed larvae. The settled ratio was the ratio of settled larvae to total larvae [10].

Field antifouling assay

After screening antibacterial and antifouling activities in a laboratory test, the two most active extracts (extracts 10 and 11) were subjected to conventional submerged assay based on Xu et al. [34] with modification to confirm their antifouling potential. Briefly, 0.4 g of each extract was mixed with 10 ml 45 % degradable polyurethane in xylene [20], and the mixed sample was painted on a polyvinyl chloride (PVC) plate (10 × 10 × 0.3 cm). A 10-ml 45 % polyurethane mixture in xylene was used as the control. Three replicates were set up for the treatment and control. After being air dried completely, the treated and control plates were exposed to biofouling at a depth of 1 m at a fish farm in Daya Bay (114°32′59 E, 22°40′37 N), Shen Zhen, where they remained constantly submerged; typically the rafts are heavily fouled. The plates were examined after 2 and 4 months (from January 2013 to May 2013) and the areas covered by the biofoulers were measured. The differences in coverage of biofoulers on the treated plates and control plate were statistically analyzed using one-way ANOVA analysis.

Isolation of antifouling compounds from the active extract 10

Bioassay-guided (antifouling activity against *B. neritina* larval settlement) isolation was carried out to the active extract 10 by silica gel column chromatography (CC) and high-performance liquid chromatography (HPLC) to obtain AF compounds **1–5**. The isolation process was the same as reported [22].

Determination of EC_{50} and LC_{50} values of isolated compound

Because the isolated quantity was not sufficient for field settlement assay, the antifouling activity of compounds 1–5 were evaluated by antilarval bioassay against *B. neritina* larvae settlement in the laboratory. The tested compounds were first dissolved in a small amount of DMSO and diluted to 50, 25, 12.5, 6.25, 3.13, and 1.65 μg/ml with FSW. The antifouling activity measurement was the same as the above mentioned.

The EC_{50} (the concentration that inhibited 50 % of larval settlement compared with the negative control) and LC_{50} (the lethal dose that killed 50 % of the larvae compared with the negative control) values were calculated as described [34]. For the calculation of the EC_{50} and LC_{50} values of the compounds, a concentration−response curve was plotted, and a trend line was then constructed for each compound.

Table 1 Antibacterial growth activity of the extracts of the eight deep-sea-derived fungal isolates from the South China Sea

Extracts	Fungal isolates and fermentation methods	Antibacterial growth activity (mm)		
		LH009	ML006	PP005
1	AI001, liquid fermentation	1.45 ± 0.12	–	–
2	AW013, liquid fermentation	2.33 ± 0.21	1.18 ± 0.08	–
3	AT003, liquid fermentation	–	5.24 ± 0.78	3.12 ± 0.32
4	CC016, liquid fermentation	4.12 ± 0.51	–	–
5	CS019, liquid fermentation	–	–	–
6	EA021, liquid fermentation	–	–	–
7	GV022, liquid fermentation	–	–	2.10 ± 0.32
8	TS034, liquid fermentation	1.70 ± 0.18	–	–
9	AI001, solid fermentation	–	4.70 ± 0.18	–
10	AW013, solid fermentation	5.48 ± 0.46	5.56 ± 0.31	7.13 ± 0.62
11	AT003, solid fermentation	4.10 ± 0.25	3.62 ± 0.54	6.21 ± 0.52
12	CC016, solid fermentation	3.13 ± 0.21	–	–
13	CS019, solid fermentation	–	5.07 ± 0.47	–
14	EA021, solid fermentation	–	–	–
15	GV022, solid fermentation	–	–	–
16	TS034, solid fermentation	–	–	5.23 ± 0.61
Control		8.87 ± 0.75	6.32 ± 0.42	7.91 ± 0.51

Each test was performed three times. The control was a disk embedded with 50 μg of streptomycin. –: no activity; weak activity: <1 mm clear zone; moderate activity: 1–5 mm clear zone; strong activity: >5 mm clear zone. Test bacteria are two larval settlement inducing bacteria *Loktanella hongkongensis* UST950701–009 (LH009) and *Micrococcus luteus* UST950701–006 (ML006), and one marine pathogenic bacterium *Pseudoalteromonas piscida* UST010620–005 (PP005)

Results

Antibacterial activity of the 16 extracts

To indirectly evaluate the antifouling activity of the 16 extracts from eight novel deep-sea-derived fungal isolates, all the extracts were tested against two larval settlement-inducing bacteria and one marine pathogenic bacterium. Among the extracts, 12 showed distinct antibacterial activity (Table 1). Extracts 10 and 11 showed relatively strong and broad antibacterial activity against all the three indicator bacteria, followed by extracts 2 and 3 that inhibited two of the three indicator bacteria. The remaining six active extracts demonstrated distinct activity against one of the three indicator bacteria.

Antilarval settlement activity of 16 extracts in laboratory test

Laboratory testing was performed to measure the antilarval settlement activity of the 16 extracts. Compared with the settled ratio of bryozoans *B. neritina* larvae in the control wells, 12 of the 16 extracts showed significant antilarval settlement activity, and the remaining four extracts (3, 4, 13, and 16) displayed possible toxic effects (Fig. 1). Extracts 10 and 11 demonstrated the strongest antilarval activity with only 3.3 and 10.8 % settle ratio at the concentration of 50 μg/ml, respectively, which were selected for further evaluating their antifouling activity in field test.

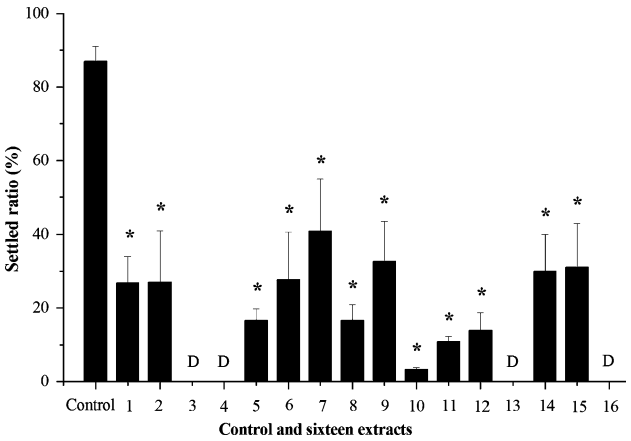


Fig. 1 Antilarval settlement activity of the 16 extracts against larvae of the bryozoans *Bugula neritina* in laboratory test. The concentration of each sample was 50 μg/ml. The settlement rate was the percentage of the settled larvae in each tested solution with three replicates per sample test, the mean and SD of which are shown as *column* and *bar*, respectively. The statistical differences between the control and the samples were calculated using one-way ANOVA analysis and indicated at the significance level of $*p < 0.01$ on the top of each bar. Extracts marked by a letter (D) showed these extracts were possible toxic effect, as high levels of death or paralysis of larvae were observed in these treatments

Antifouling activity of extracts 10 and 11 in field testing

The antifouling potentials of extracts 10 and 11 were further evaluated in field testing. After submersion in seawater for 2 months, a lot of calcarina and bryozoans

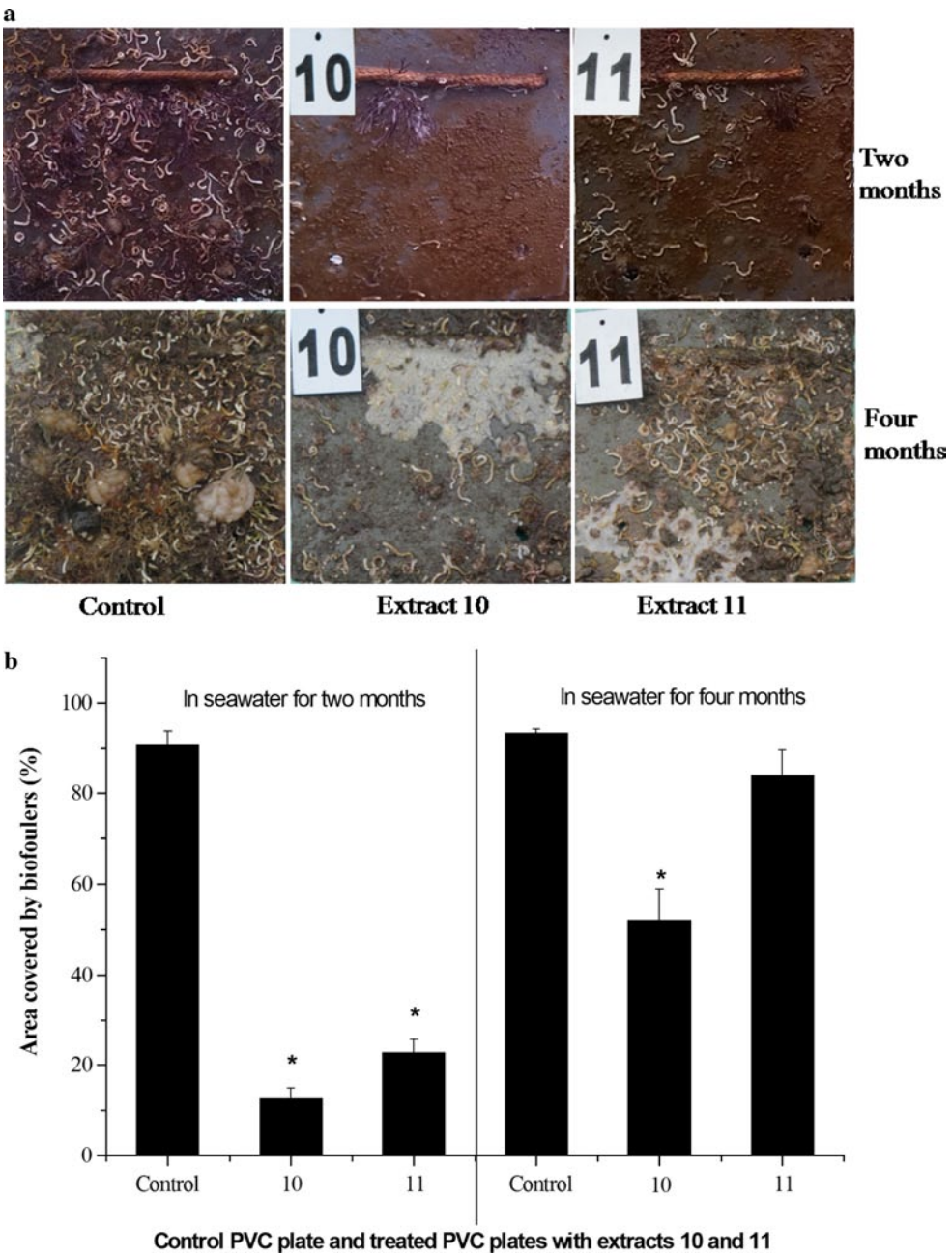
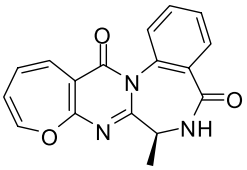


Fig. 2 Field tests of control PVC plates and treated PVC plates with extracts 10 and 11 after submersion in seawater for 2 and 4 months. *Asterisk* indicates data that significantly differ from the control in one-way ANOVA analysis ($p < 0.01$)

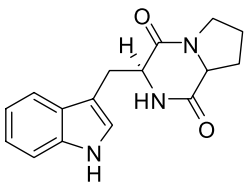
were registered on the surface of the control PVC plates (Fig. 2a). Only a few of calcarina *Hydroides elegans* and bryozoans *B. neritina* were covered on the treated PVC plates with extracts 10 and 11. Analysis of the photos that characterized the macrofouling of tested plates showed that the coverage of adherent biofouler on the control plates' surfaces were several times greater than that of the treated plates (Fig. 2b). After submersion in seawater for 4 months, besides of calcarina and bryozoans, many ascidians were also registered on the surfaces of the control and treated PVC plates (Fig. 2a). The coverage of adherent biofouler on the treated PVC plates with extracts 10 was much less fouled compared to the control PVC plates, while the antifouling effect of extract 11 was not significant as compared to the control (Fig. 2b).

Antifouling potential of compounds isolated from the extract 10

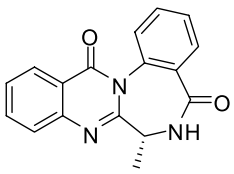
Bioassay-guided isolation generated five compounds from the extract 10. Compounds 1−5 were identified as alkaloids circumdatin L [22], brevianamide F [4], circumdatin F [33], notoamide C [15], and 5-chlorosclerotiamide [22] by comparison with the data reported in these above literatures.



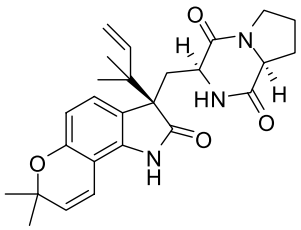
Compound **1:** circumdatin L



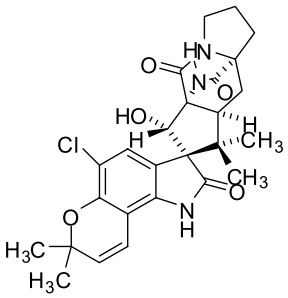
Compound **2:** brevianamide F



Compound **3**: circumdatin F



Compound **4**: otoamide C



Compound **5**: 5-chlorosclerotiamide

Fig. 3 Chemical structures of compounds **1–5** and their antifouling activity against *Bugula neritina* larval settlement

Compounds	Antilarval settlement activity		
	LC_{50} (μg/mL)	EC_{50} (μg/mL)	LC_{50}/ EC_{50}
1	>200	34.91	>5.73
2	>200	6.35	>31.50
3	>200	8.81	>24.69
4	>200	9.85	>20.30
5	>200	13.52	>14.79

Their chemical structures and antilarval settlement activity against *B. neritina* are shown in Fig. 3. Among the five compounds, brevianamide F, circumdatin F, and notoamide C displayed strong antilarval settlement activity with EC_{50} values of 6.35, 8.81, and 9.85 μg/ml, respectively, and low toxicity with LC_{50} values of >200 μg/ml and therapeutic ratios of >20.

Discussion

Deep-sea-derived fungi are known to produce a source of cold-tolerant proteases and diverse antimicrobial substances [9, 37]. However, the potential application of metabolites produced by deep-sea-derived fungi in antifouling has hardly been explored. In this study, 16 extracts of eight novel fungal isolates from deep-sea sediments of the South China Sea were screened for antifouling potential. All of them showed distinct antibacterial activity against two larval settlement inducing bacteria and one marine pathogenic bacterium or significant antifouling activity against larval settlement of bryozoans *B. neritina*. More significantly, the extract 10 exhibited promising antifouling activity in a preliminary field test, and five antifouling alkaloids were isolated from the extract. The present investigation is the first report demonstrating that deep-sea-derived fungi of the South China Sea can be a potential source of antifouling compounds and thus deserve more intensive study in the future.

In this study, five fungal isolates (AI001, AW013, CC016, GV022, and TS034) displayed distinct antibacterial

activity (Table 1), while they showed no antibacterial activity in an "extract-free" assay using the same fungal isolates in previous study [37]. The main reasons for this may be that different cultivation methods (for example, media compositions, culture vessel, and culture time) for these same fungal isolates can produce different diverse secondary metabolites. Bode et al. [5] had investigated the systematic alteration of easily accessible cultivation methods in order to increase the number of secondary metabolites available from a single microbial source, and isolated up to 20 different metabolites covered nearly major natural product families. Our previous studies also found more bioactive compounds could be isolated when the same microbial strain *P. oxalicum* SCSGAF 0023 was cultured by different methods [2, 31]. These results indicated that different cultivation methods could offer a good alternative to high-throughput screening that focuses on the active principle in a distinct bioassay.

Among the five antifouling alkaloids obtained from the extract 10, brevianamide F, circumdatin F, and notoamide C showed significant antifouling activity in this study (Fig. 3). This is the first time to report the antifouling activity of the three compounds. Besides antifouling efficacy, toxicity is a major concern of marine coating industry because effective marine natural compounds are often as toxic as heavy metals [17]. Thus, the toxicity profiles of the three compounds were also determined in this study. The LC_{50} values and therapeutic ratios of the three compounds were >200 μg/ml and >20, respectively (Fig. 3), suggesting that they had low toxicity towards larvae and low effective concentrations. Additionally, compared with the LC_{50} of tributyltin, which is generally less than 0.00001 μg/ml [8], these results indicate that the three compounds are much less toxic than tributyltin.

Because the isolated quantities of brevianamide F, circumdatin F, and notoamide C were not sufficient for the field settlement assay, their antifouling activity could not been confirmed in this study. However, fortunately brevianamide F could be synthesized through a new strategy for side-chain anchoring of tryptophan to dihydropyranyl functionalized polystyrene resins [32], circumdatin F could be synthesized via rearrangement to amidine intermediate, deprotection with 45 % HBr in acetic acid, and cyclization on silica gel [33], and notoamide C could be totally synthesized through an oxidation of a proposed biosynthetic precursor [12], which may do favor to offer sufficient brevianamide F, circumdatin F, and notoamide C for further study.

In summary, although these fungal species could produce many bioactive products [22, 37], and the three active compounds, including brevianamide F, circumdatin F, and notoamide C, could be isolated from many other *Aspergillus* and *Penicillium* ssp. [4, 15, 33], they are reported for the first time to display antifouling activity in this study. Increasing economic and scientific interest has now shifted to the biotechnological potentials of deep-sea-derived fungi that have high biodiversity and have possibly developed unique adaptations to live dark, cold, and high-pressurized environments [19]. Our results suggest that novel metabolic pathways of deep-sea-derived fungi may offer a wealth of opportunities for obtaining antifouling natural products. We anticipate a good potential for deep-sea-derived fungal metabolites as alternative source for novel antifouling compounds.

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