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Antifouling activities of hymenialdisine and debromohymenialdisine from the sponge *Axinella* sp.

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ABSTRACT

Being physically unprotected sessile organisms, marine sponges are thought to protect themselves from surface fouling through the use of antifouling secondary metabolites. In this study, the sponge *Axinella* sp. was extracted with methanol and then partitioned between organic solvents and water. Two main compounds, hymenialdisine (HD) and debromohymenialdisine (DBH), were isolated from the n-BuOH layer using Sephadex LH-20 and C-18 column chromatography. The antifouling activity of HD and DBH were evaluated using the test of byssus thread production with the green mussel *Perna viridis*, and the settlement assays with the bryozoan *Bugula neritina* larvae and the green alga *Ulva prolifera* spores. Both HD and DBH were found to exhibit significant antifouling activities against *P. viridis* (EC_{50} values of 31.77 and 138.18 μg ml^{-1}, respectively), *B. neritina* (EC_{50} values of 3.43 and 8.17 μg ml^{-1}, respectively) and *U. prolifera* (EC_{50} values of 8.31 and 0.67 μg ml^{-1}, respectively). Our results suggested that HD and DBH may play a role in chemical defense against fouling in *Axinella* sp.

1. Introduction

There have been numerous studies reporting the presence of biologically active secondary metabolites in marine organisms (e.g. Faulkner, 1998, 2000, 2001, 2002; Tziveleka et al., 2003; Blunt et al., 2009). Sponges (Porifera) in particular, are the most prolific source of marine natural products, and have long been in focus of natural product chemists (Faulkner, 1998, 2000, 2001, 2002; Sipkema et al., 2005). The chemical diversity of compounds isolated from sponges is remarkable. Compounds include sterols, terpenoids, alkaloids, fatty acids, macrolides, saponins, nucleosides, peroxides, and amino acid derivatives (Faulkner, 1998; Sipkema et al., 2005). These compounds, proven to be antitumor, antiviral, antiinflammatory, antimalarial, immunosuppressive, or antibiotic, exhibited significant potential in drug development (Munro et al., 1999; Sipkema et al., 2005; Mayer et al., 2009). Although a great deal of research has been done on chemical analyses and pharmacological activities of natural products from sponges, the ecological functions of most of these bioactive compounds in sponges remain to be determined.

Being sessile and soft-bodied organisms, marine sponges are physically vulnerable to predation, competition for space, attacks from potential pathogens, and surface fouling. It is commonly accepted that sponges protect themselves using powerful chemical defenses through the production of bioactive metabolites (Proksch, 1994; Engel and Pawlik, 2000; Laport et al., 2009). For example, presence of natural products with antifouling activity have been reported in many sponges and suggested to be involved in the defense of sponges against fouling organisms (Willemsen, 1994; Tsoukatou et al., 2002; Hellio et al., 2005; Limna Mol et al., 2009).

The sponge *Axinella* sp. was observed to be free of epibiosis in the field, suggesting that this species may possess certain antifouling mechanisms. Our preliminary study found that the crude extract from *Axinella* sp. exhibited antifouling activity. Two main alkaloids, hymenialdisine (HD) and debromohymenialdisine (DBH), were isolated from the *Axinella* sp. crude extract using a bioassay-guided approach. It is noteworthy that HD and DBH have been reported to exhibit inhibitory effects against a number of protein kinases in pharmacological studies and have shown their

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promising potential in treating osteoarthritis, Alzheimer's disease and tumors (Chipman and Faulkner, 1996; Meijer et al., 2000; Curman et al., 2001; Roy and Sausville, 2001; Tasdemir et al., 2002; Sipkema et al., 2005). However, little is known about the ecological function of these two compounds in sponges. The aim of the present study was to investigate the antifouling activity of HD and DBH. Here bioassays were performed with two species of animal foulers, the green mussel *Perna viridis* and the bryozoan *Bugula neritina*, and one species of plant fouler, the seaweed *Ulva prolifera*. The evidence from the bioassays supports the hypothesis that HD and DBH may be involved in chemical antifouling defense in the sponge.

2. Materials and methods

2.1. Sponge collection

Specimens of *Axinella* sp. (Class Demospongiae, Order Halichondrida, Family Axinellidae, Fig. 1) were collected by diving from reefs at the depths of 1.5–3.0 m off Yongxing Island (112°20′ E, 16°50′ N, seawater salinity of 32–34) in September 2008. Yongxing Island is one of the major islands of Xisha Island chain in the South China Sea. The samples were transported in a cooler to the laboratory and were stored at −20 °C prior to extraction. A voucher specimen was deposited at our laboratory.

2.2. Extraction and isolation

The sponge *Axinella* sp. (2 kg, wet wt) was extracted with MeOH twice and the combined extracts were partitioned between EtOAc and H_2O. The aqueous layer was subsequently extracted with n-BuOH. The n-BuOH-soluble materials were then subjected to Sephadex LH-20 column chromatography eluting with MeOH to give an alkaloid-containing fraction. This fraction was re-separated by a C-18 column chromatography washing with the mixed solvent MeOH/H_2O/CF_3COOH (35:65:0.1) to give the two main compounds.

2.3. Structural elucidation of compounds

UV spectra were recorded on a Shimadzu UV-1600PC spectrophotometer. NMR spectra were obtained in TFA-*d* on a Bruker Advance-500 FT spectrometer operating at 500 and 125 MHz for ^{1}H and ^{13}C respectively. Chemical shifts are reported in parts per million (ppm, δ), and coupling constants (J) are expressed in Hz. ESIMS spectral data were measured in the positive ion mode on an ABI 3200 Q-Trap mass spectrometer.

2.4. Assay for antifouling activity against the green mussel P. viridis

Specimens of *P. viridis* (shell length, 13–18 mm), were collected from submerged rafts at a fish farm in Zhangzhou, Fujian Province, P.R. China. In the laboratory, they were kept in an aquarium with aerated seawater and acclimatized for 7 days prior to use.

The test of byssus thread production by green mussels was adopted for assessing the antifouling activity of compounds, performed as previously described in Van Winkle (1970) and Rajagopal et al. (2003). The green mussels were gently removed from the aquarium, with particular care taken to cut the byssal mass (stem and old threads) off each mussel with sharp scissors. The purified compounds from the sponge *Axinella* sp. were dissolved in dimethylsulfoxide (DMSO). One mussel was placed in each well (twenty-four-well plates) containing 20 μl of each compound solution and 2 ml filtered (0.22 μm) seawater (FSW). Ten replicates were set up for each of the treatment groups and the control (20 μl DMSO added to 2 ml FSW). After 24 h of incubation, the number of byssus threads produced by each mussel was counted and expressed in threads mussel^{-1} day^{-1} (Van Winkle, 1970; Rajagopal et al., 2003).

2.5. Assay for antifouling activity against larvae of the bryozoan B. neritina

Mature colonies of *B. neritina* were collected from submerged rafts at a fish farm in Zhangzhou and transported to the laboratory

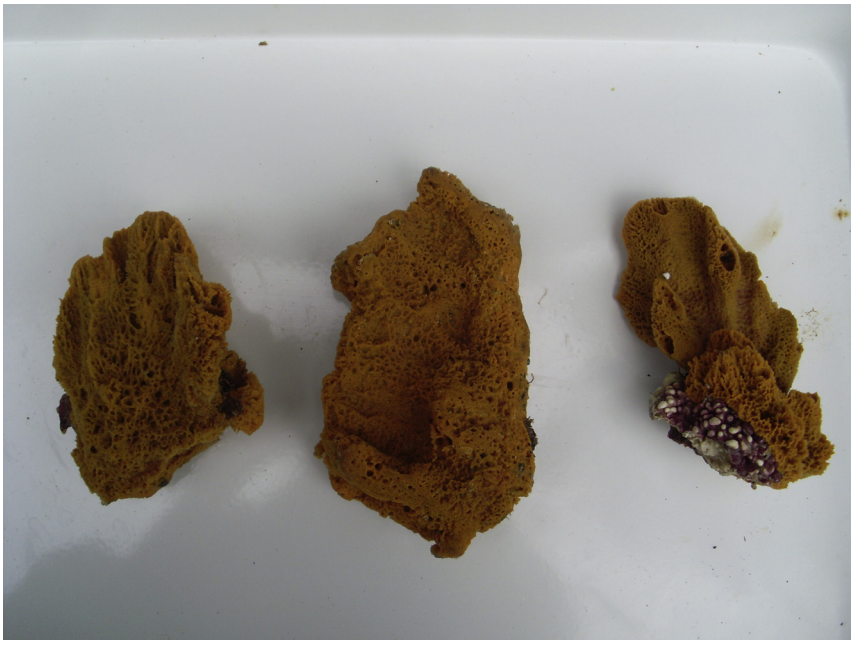

Fig. 1. Samples of the sponge *Axinella* sp.

in plastic buckets with ambient seawater. Upon arriving at the laboratory, they were transferred to glass tanks filled with FSW. Specimens were maintained overnight in total darkness. The next morning, larvae production was induced in mature colonies through exposure to strong artificial light for approximately 30 min. The positively phototactic bryozoan larvae attracted to the rim of the glass tanks were gently collected with a pipette and immediately used for assay.

The settlement assay with bryozoan larvae was carried out using the method of Martín and Uriz (1993). The purified compounds from the sponge *Axinella* sp. were dissolved in ethyl acetate. Aliquots of the solution were applied to glass Petri dishes (6 cm diameter) and spread on the inner surface of the dishes. After complete evaporation of the solvent at room temperature, 10 ml of FSW and approximately 30 *B. neritina* larvae were added to each Petri dish. Three replicates were set up for the FSW control and each of the treatment groups. The Petri dishes were then incubated at a temperature of 25 °C in darkness for 24 h. After this time, the number of settled larvae was counted with the aid of a stereomicroscope. Larvae that firmly attached on the dishes and could not be removed by a gentle jet of water from a pipette were counted as settled (Marshall and Keough, 2003; Yu et al., 2007). The number of the settled larvae was expressed as a percentage of the total number of larvae per dish.

2.6. Assay for antifouling activity against spores of the green alga U. prolifera

Fertile specimens of *U. prolifera* were collected from rock surfaces in Zhangzhou a few days before spring tides. They were rinsed with FSW to remove debris and dried for about 10 h at room temperature. Spores were obtained by placing the algae into a glass tank containing FSW. Artificial light was used to concentrate spores at the edge of the glass tank. Spores were collected with a pipette and kept in a beaker for immediate use.

The settlement assay with macroalgal spores was performed by following of Callow et al. (2002) with some modifications. The concentration of spore suspension was adjusted to 2.5×10^5 spores ml^{-1} by diluting with FSW. The purified compounds from the sponge *Axinella* sp. were dissolved in DMSO. A volume of 50 μl of each compound solution was added to each triplicate well (six-well plates) containing 5 ml spore suspension and a glass coverslip (24 × 24 mm). A 1% (v/v) solution of DMSO in spore suspension was used as control. The plates were incubated in the dark at 25 °C for 3 h. Unsettled spores were removed from coverslips by passing coverslips back and forth 10 times in a beaker of FSW. With the aid of an inverted light microscope (Leica), the number of settled spores was counted in 10 random fields of view on each of three replicate coverslips using a ×20 objective. Spore settlement is presented as a relative rate: (S/C) ×100%, where S is the number of settled spores in treatment and C the number of settled spores in control (Cho et al., 2005).

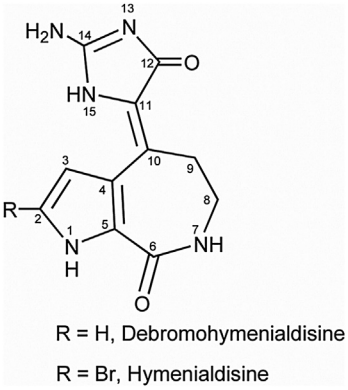

Fig. 2. Chemical structures of hymenialdisine and debromohymenialdisine.

2.7. Statistical analysis

Differences in the byssus thread production of green mussels, the bryozoan larval settlement or the algal spore settlement between the treatments and control were analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett post hoc test. The significance level was set at $P < 0.05$. The concentration of the compounds that inhibited settlement by 50% relative to the control (EC_{50}) was calculated using the Spearman–Karber method (Hamilton et al., 1977, 1978; Reichelt-Brushett and Michalek-Wagner, 2005).

3. Results

3.1. Isolation and identification of compounds

Two pyrrole alkaloids were isolated from marine sponge *Axinella* sp.. Hymenialdisine (Fig. 2) was obtained as a pale red powder with ESIMS *m/z* 324.1 and 326.0 $[M + H]^+$ (1: 1). UV (MeOH) λ_{max} 347, 268, 231, 207 nm; ^1H NMR (500 MHz, TFA) δ_H: 6.63 (1H, s, H-3), 3.69 (2H, brd, H-8), 3.60 (2H, brd, H-9); ^{13}C NMR (125 MHz, TFA) δ_C: 163.1 (C-6), 162.0 (C-12), 152.9 (C-14), 133.9 (C-10), 126.0 (C-2), 122.9 (C-5), 120.3 (C-11), 112.5 (C-4), 110.9 (C-3), 40.5 (C-8), 31.0 (C-9).

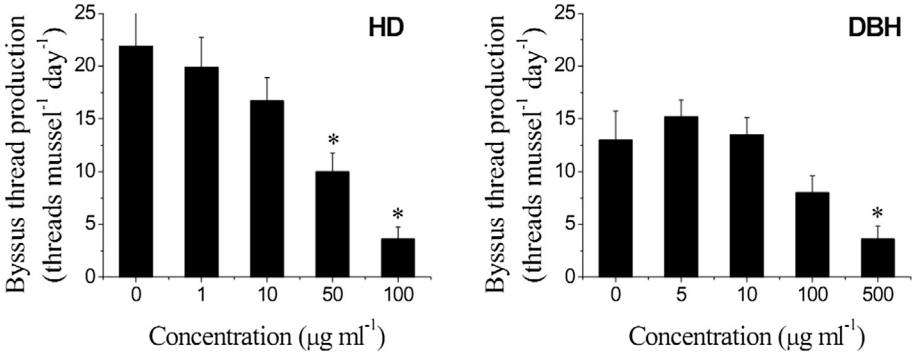

Fig. 3. Effects of hymenialdisine (HD) and debromohymenialdisine (DBH) on byssus thread production of *Perna viridis*. Data shown are the means of ten replicates + standard error. Data that are significantly different from the control according to a Dunnet test (ANOVA: $P < 0.05$) are indicated by an asterisk above the bars.

Table 1
Antifouling activity of hymenialdisine and debromohymenialdisine against the mussel *Perna viridis*, the bryozoan *Bugula neritina* larvae and the alga *Ulva prolifera* spores.

EC_{50} (μg ml^{-1})	Compound	
	Hymenialdisine	Debromohymenialdisine
Mussel bioassay	31.77 (30.90–32.65)	138.18 (131.61–145.08)
Bryozoan bioassay	3.43 (3.33–3.54)	8.17 (7.86–8.48)
Algal bioassay	8.31 (7.90–8.74)	0.67 (0.65–0.70)

The data are expressed as EC_{50} and 95% confidence limits.

Debromohymenialdisine (Fig. 2) was obtained as a yellow powder with ESIMS *m/z* 246.2 $[M + H]^+$ and 268.2 $[M + Na]^+$. UV (MeOH) λ_{max} 346, 266, 230, 206 nm; ^1H NMR (500 MHz, TFA) δ_H: 7.34 (1H, br, H-2), 6.74 (1H, br, H-3), 3.75 (2H, brd, H-8), 3.67 (2H, brd, H-9); ^{13}C NMR (125 MHz, TFA) δ_C: 165.3 (C-6), 163.3 (C-12), 152.9 (C-14), 135.3 (C-10), 127.1 (C-2), 125.5 (C-5), 121.1 (C-11), 119.9 (C-4), 110.8 (C-3), 40.9 (C-8), 30.7 (C-9).

3.2. Antifouling activity

The byssus thread production of *P. viridis* decreased significantly as the Hymenialdisine (HD) concentration increased ($P < 0.05$, Fig. 3). Debromohymenialdisine (DBH) also significantly inhibited the byssus thread production. As shown in Table 1, the antifouling activity of HD against *P. viridis* (EC_{50} 31.77 μg ml^{-1}) was higher than that of DBH (EC_{50} 138.18 μg ml^{-1}).

HD inhibited settlement of *Bugula neritina* larvae in a concentration-dependent manner (Fig. 4). The EC_{50} value of HD against *B. neritina* larvae was 3.43 μg ml^{-1} (Table 1). DBH also significantly inhibited larval settlement of *B. neritina* in a concentration-dependent manner ($P < 0.05$, Fig. 4). This compound showed slightly lower antifouling activity (EC_{50} 8.17 μg ml^{-1}) against *B. neritina* than HD.

Both HD and DBH also significantly inhibited settlement of *U. prolifera* spores compared to the control ($P < 0.05$, Fig. 5). As shown in Table 1, the antifouling activity of DBH against spores of *U. prolifera* (EC_{50} 0.67 μg ml^{-1}) was much higher than that of HD (EC_{50} 8.31 μg ml^{-1}).

4. Discussion

In this study, two pyrrole alkaloids HD and DBH were isolated from marine sponge *Axinella* sp. These two natural products have been isolated from different sponges: HD from sponges of the genera *Hymeniacidon*, *Acanthella*, *Axinella*, *Stylissa*, and *Pseudaxinyssa*, and DBH from the same genera in addition to *Phakellia* (Sharma et al., 1980; Cimino et al., 1982; Kitagawa et al., 1983; De Nanteuil et al., 1985; Tasdemir et al., 2002). Recently, HD and DBH have attracted much attention mainly due to their potent pharmacological activities for treating osteoarthritis, Alzheimer's disease and tumor, showing promising potential as marine drug (Chipman and Faulkner, 1996; Meijer et al., 2000; Curman et al., 2001; Roy and Sausville, 2001; Tasdemir et al., 2002; Sipkema et al., 2005). A total synthesis of HD and DBH has been reported (Annoura and Tatsuoka, 1995). However, no experimental research has investigated their ecological function in sponges.

We observed that both compounds significantly inhibited byssus thread production of the green mussel *P. viridis*, and settlement of the bryozoan *B. neritina* and the alga *U. prolifera*. Some useful information about the structure–activity relationship could be obtained by considering the similar structures of HD and DBH (Fig. 2). The Br atom substituted at position 2 in HD, is absent in DBH. It does not influence anti-settlement activity against *B. neritina* significantly since there is only slight difference in this activity of these two compounds. On the other hand, the Br atom at position 2 enhances the antifouling activity against *P. viridis*, as revealed by comparison of the EC_{50} values of HD and DBH against *P. viridis*. While comparing the EC_{50} values of HD and DBH obtained in the bioassay with *U. prolifera*, it is found that the presence of Br atom at position 2 in HD obviously reduces antifouling activity against *U. prolifera*.

It is suggested that in the marine environment, marine organisms have evolved antifouling defenses due to the ubiquity of fouling organisms and the negative consequences of surface fouling (Wahl, 1989; Kelly et al., 2003). For physically unprotected sessile organisms like sponges, antifouling secondary metabolites are crucial for them to maintain a clean surface. In the present study, HD and DBH were isolated from the sponge *Axinella* sp. and found to be significantly antifouling active. In a recent study Song et al. (2011), it has been shown that HD and DBH are localized in sponge cells rather than in endosymbiotic or associated bacteria in *Axinella* sp. Furthermore, Song et al. (2011) suggested that HD and DBH are abundant metabolites in *Axinella* sp. (up to 1.46% and 2.56% of the sponge dry weight, respectively). That the high concentrations of HD and DBH in the sponge, in combination with the finding of their significant antifouling activities, indicates the possibility of their utility to the *Axinella* sp. sponge as defensive compounds against fouling.

On the other hand, it is noteworthy that stevensine, very similar to HD and DBH in chemical structure, has been proven to function as a chemical defense against fish predators in marine sponge *Axinella corrugate* (Wilson et al., 1999). Further work needs to be done to determine whether HD and DBH also have the ecological function to protect sponges from predation.

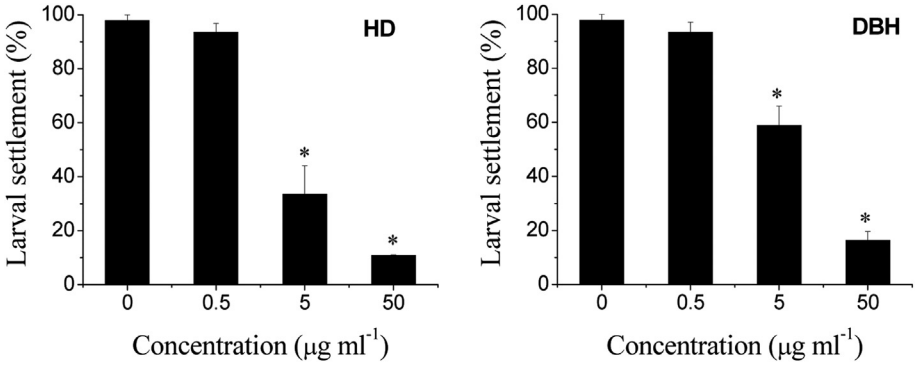

Fig. 4. Effects of hymenialdisine (HD) and debromohymenialdisine (DBH) on settlement of *Bugula neritina* larvae. Data shown are the means of three replicates + standard error. Data that are significantly different from the control according to a Dunnet test (ANOVA: $P < 0.05$) are indicated by an asterisk above the bars.

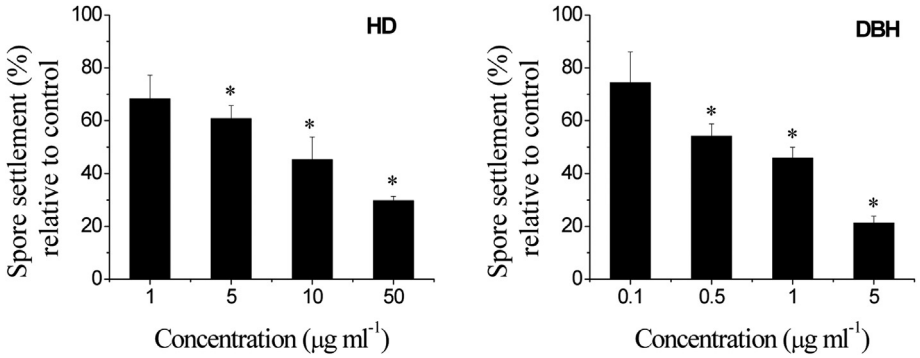

Fig. 5. Effects of hymenialdisine (HD) and debromohymenialdisine (DBH) on settlement of *Ulva prolifera* spores. Data are expressed as settlement (%) relative to the control. Error bars are standard errors. Data that are significantly different from the control according to a Dunnet test (ANOVA: $P < 0.05$) are indicated by an asterisk above the bars.

In this study we provided first laboratory evidence of the antifouling activity of HD and DBH against mussels, bryozoan larvae and macroalgal spores, indicating that they may be involved in chemical defense against fouling in marine sponges.

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