Original Article

MARINE BIOTECHNOLOGY

Antifouling Activity of Bromotyrosine-Derived Sponge Metabolites and Synthetic Analogues

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Abstract

Eighteen brominated sponge-derived metabolites and synthetic analogues were analyzed for antilarval settlement of *Balanus improvisus*. Only compounds exhibiting oxime substituents including bastadin-3 (**4**), −4 (**1**), −9 (**2**), and −16 (**3**), hemibastadin-1 (**6**), aplysamine-2 (**5**), and psammaplin A (**10**) turned out to inhibit larval settling at 1 to 10 μM. Analogues of hemibastadin-1 (**6**) were synthesized and tested for structure activity studies. Debromohemibastadin-1 (**8**) inhibited settling of *B. improvisus*, albeit at lower concentrations than hemibastadin-1 (**6**). Both **6** and **8** also induced cyprid mortality. 5,5′-dibromohemibastadin-1 (**7**) proved to be nontoxic, but settlement inhibition was observed at 10 μM. Tyrosinyltyramine (**9**), lacking the oxime function, was not antifouling active and was non-toxic at 100 μM. Hemibastadin-1 (**6**) and the synthetic products showed no general toxicity when tested against brine shrimp larvae. In contrast to the lipophilic psammaplin A (**10**), the hydrophilic sulfated psammaplin A derivative (**11**) showed no antifouling activity even though it contains an oxime group. We therefore hypothesize that the compound needs to cross membranes (probably by diffusion) and that the target for psammaplin A lies intracellularly.

Keywords: antifouling — *Balanus improvisus* — bastadins — bastadin analogues — bromotyrosine derivatives — natural products

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Introduction

In 1952, Woods Hole Oceanographic Institution referred to biofouling as growth of animals and plants on artificial submerged surfaces. Biofouling includes microfouling (i.e., bacteria and diatoms) and macrofouling (i.e., macroalgae and invertebrates) (Clare, 1996a) and causes significant economical and environmental losses worldwide by reducing boat speeds and increasing fuel consumption (Wahl, 1989). Until today organotin (tri-*n*-butyl tin oxide, TBTO), copper oxide, and herbicide coatings have been commercially used for preventing on-growth. These coatings are, however, toxic to the aquatic environment (Alzieu et al., 1986, 1989) and were recently banned by the International Maritime Organisation (IMO) in a resolution that called for a stepwise reduction of the use of organotin compounds by 2003 and complete prohibition by 2008 (IMO, 2001). New antifouling strategies, involving chemical, physical, and mechanical mechanisms, are being investigated to replace these toxic agents (Bers and Wahl, 2004).

Barnacles are an example of severe macrofouling organisms with economical importance, because they settle and grow on ships' hulls, cooling systems, and similar man-made substrates. Attempts are now being made to understand the physiology of barnacle larvae, their settlement behavior, and their ability to metamorphose (Fusetani, 2004; Sjögren et al., 2004a; Dahlström et al., 2005). This is important not only for understanding ecological processes, such as competition and successions of sessile marine invertebrates, but also for finding environmentally

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sound non-toxic antifouling agents. In the settling process the barnacle larvae, or cyprids, search for a surface where they settle in a gregarious manner and metamorphose into juvenile barnacles. The organisms generally used for cyprid settling assays are either *Balanus amphitrite* Darwin 1854 or *Balanus improvisus* Darwin 1854.

Sponges (phylum Porifera) are soft bodied and sessile animals, which lack spine and shell (Armstrong and Quigley, 1999). For their defense against fouling organisms, predators, and neighbours competing for space, sponges rely on bioactive natural products instead (Proksch et al., 2002). As sponges are filter feeders, overgrowth by fouling organisms would be devastating for their survival as inhaling pores would likely be clogged. Among the thousands of natural products isolated from sponges so far (Blunt et al., 2006) halogenated compounds are frequently encountered. In the past, numerous brominated sponge-derived compounds were already shown to possess antifouling activity against the barnacle *B. amphitrite*. Among these active compounds are, for example, heterocyclic substances such as pseudoceratidine (Tsukamoto et al., 1996c), bromotyrosine derivatives including ceratinamines (Tsukamoto et al., 1996b) and ceratinamides (Tsukamoto et al., 1996a), as well as brominated peptides against *B. improvisus* (Sjögren et al., 2004b). Most of the current research is focusing on compounds originating from tropical water organisms, but recently brominated secondary metabolites with antifouling activity have also been reported from cold water invertebrates (Sjögren et al., 2004b). Even though halogenation is a frequently observed phenomenon in antifouling substances, a recent review also lists nonhalogenated antifouling compounds such as the sesquiterpene axinyssimide, the steroid halistanol sulfate, the triterpene glycoside famoside, and the phakellin-derived alkaloid styoluanidine (Fusetani, 2004). Not only marine macroorganisms, such as sponges, provide antifouling compounds; several interesting metabolites with antifouling potential have also been isolated from marine microbes (Dobretsov et al., 2006).

In this study we investigated 15 brominated natural products isolated from several marine sponges such as *Ianthella basta*, *Pseudoceratina purpurea,* and others and three synthetic analogues for their inhibition of cyprid settlement. The compounds analyzed included bromopyrrole derivatives such as sceptrin (**14**), debromosceptrin (**15**), or hymenidin (**17**) and bromotyrosine derivatives such as bastadin derivatives (**1–4**, **6, 7**), psammaplins (**10**, **11**) and others (Figure 1).

Bastadins are tyrosine-derived, brominated, oxime-bearing peptides, which can be either cyclic or linear. In earlier studies, they have been reported to be cytotoxic (Miao et al., 1990; Pordesimo and Schmitz, 1990; Carney et al., 1993; Jaspars et al., 1994; Pettit et al., 1996), to inhibit inosine 5′-phosphate dehydrogenase (Jaspars et al., 1994), and to modulate ryanodine-sensitive sarcoplasmatic retriculum calcium channels (Mack et al., 1994). So far, however, bastadins have not been studied for antifouling activity.

We found several bastadin derivatives including the bastadins 3 (**4**), 4 (**1**), 9 (**2**), and 16 (**3**) which were all isolated from *Ianthella basta*, aplysamine-2 (**5**) from *Pseudoceratina purpurea*, and psammaplin A (**10**) obtained from *Aplysinella rhax* to be active in the barnacle bioassay at low micromolar concentrations. All other analyzed sponge metabolites irrespective of their halogen substituents, however, proved to be inactive (Figure 1). The naturally occurring bastadin derivative hemibastadin-1 (**6**), which is chemically less complex compared to the other bastadins, as well as several hemibastadin analogues were synthesized (**7–9**) and tested for antifouling activity in the barnacle bioassay. In these experiments it was unequivocally proven that the oxime function is the decisive structural element for the antifouling activity of hemibastadin-1 (**6**) and its synthetic analogues, even though the presence of bromine atoms modulates the activity of the respective derivatives. The toxicity of the synthesized derivatives was also tested in a brine shrimp assay, showing no increase of the mortality of the brine shrimp nauplii compared to the controls.

Materials and Methods

Origin of Natural Products Analyzed in this Study. All sponge-derived natural products analyzed in this study (**1–5** and **10–18**) were from the collection of P.P. and P.S. and had been structurally charac- terized by mass spectrometry as well as by one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy (^{1}H, ^{13}C, Correlation SpectroscopY (COSY), Heteronuclear Multiple Bond Coherence (HMBC)). The NMR data of the known compounds was compared to the literature; bastadin 3 (**4**) (Kazlauskas et al., 1980), bastadin 4 (**1**) (Fattorusso), bastadin 9 (**2**) (Pordesimo and Schmitz, 1990), bastadin 16 (**3**) (Park et al., 1994), hemibastadin-1 (**6**) (Butler et al., 1991), aplysamine 2 (**5**) (Xynas and Capon, 1989), sceptrin (**14**) (Walker et al., 1981), debromosceptrin (**15**) (Shen et al., 1998), ageliferin (**13**) (Kobayashi et al.,

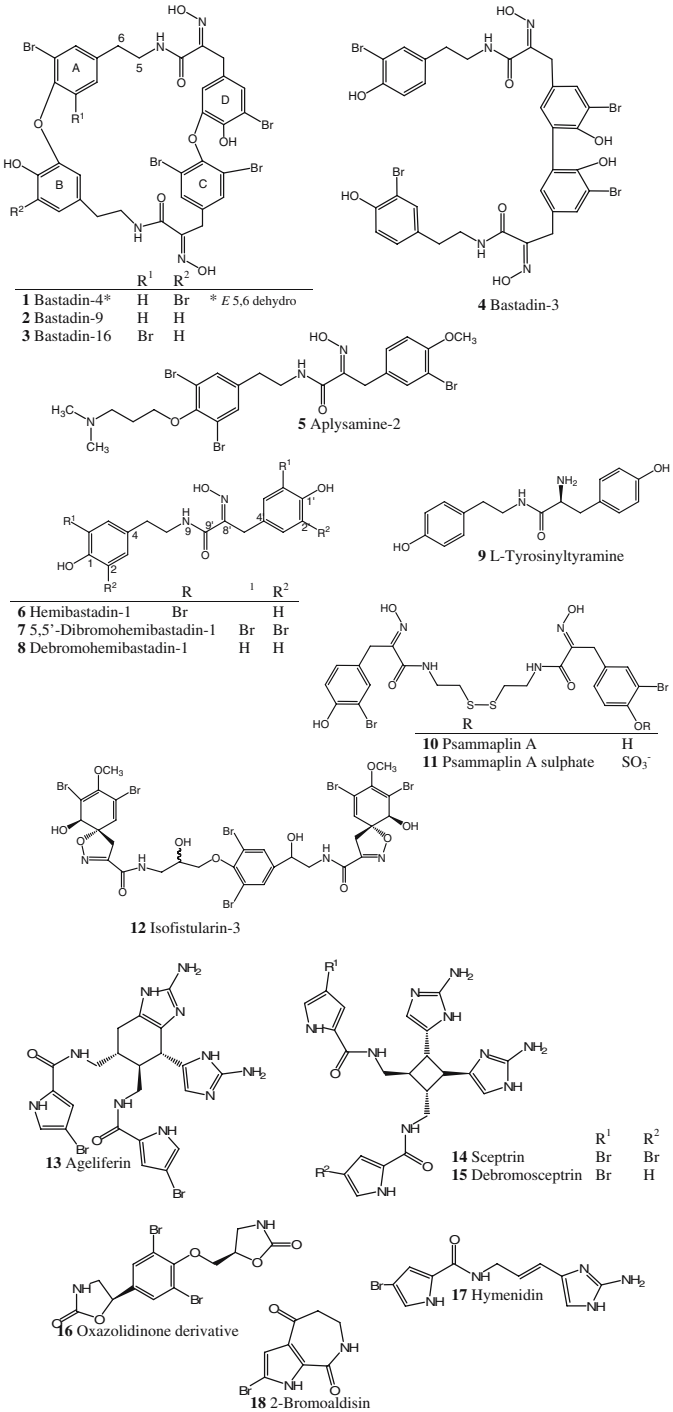


Figure 1. Structures of the tested compounds.

1990), hymenidin (**17**) (Kotoku et al., 2005), oxazolidinone derivative (**16**) (Borders et al., 1974), isofistularin-3 (**12**) (Cimino et al., 1983), 2-bromoaldisin (**18**) (Xu et al., 2001), psammaplin A (**10**) (Arabshahi and Schmitz, 1987), and psammaplin A sulfate (**11**) (Pham et al., 2000).

The bastadins (**1–4**) were isolated from *Ianthella basta* and aplysamine-2 (**5**) as well as the oxazolidinone derivative (**16**) originated from *Pseudoceratina purpurea*. Isofistularin-3 (**12**) was from *Aplysina aerophoba*, the psammaplins (**10**, **11**) from *Aplysinella rhax*, ageliferin (**13**) from *Agelas conifera*, and sceptrin (**14**) as well as debromosceptrin (**15**) were isolated from *Agelas nakamurai*. Hymenidin (**17**) came from *Stylissa carteri*, and 2-bromoaldisin (**18**) was isolated from *Axinella damicornis*.

Chemical Synthesis of Analogues of Hemibastadin-1 and L-Tyrosinyltyramine. Debromohemibastadin-1 (**8**) was prepared in a three-step synthesis starting from the phenyl pyruvic acid (**I**), which was converted to the oxime (**II**) and transformed to the ester (**III**). After trituration of **III** together with a threefold excess of tyramine the mixed powder was fused in an open flask under stirring in an oil bath at 130°C for 30 min without any solvent. After usual work up and chromatography on silica gel with toluene-ethyl acetate (1:1) the product (**8**) was obtained as colorless crystals, mp 179°C.

Successive reaction of (**8**) with various amounts of bromine (two and five equivalents respectively) in ether/methylene chloride at room temperature resulted in a mixture of brominated products (**6**, **7**), which could be separated by column chromatography (silica gel, methylene chloride / ether 100+5). (See Scheme 1.)

l-tyrosinyltyramine (**9**) was synthesized starting from **l**-tyrosine-methylester, which was carefully triturated and melted with a double excess of tyramine in an open flask at 150°C without any solvent for 3 h. After cooling the residue was dissolved in dilute HCl, adjusted to pH 8 to 9 with sodium carbonate, and extracted with ethyl acetate. After concentration and cooling, colorless crystals of **9** (87%) were separated and dried *in vacuo,* mp 161°C.

Details on the synthesis of compounds **6–9** will be published elsewhere.

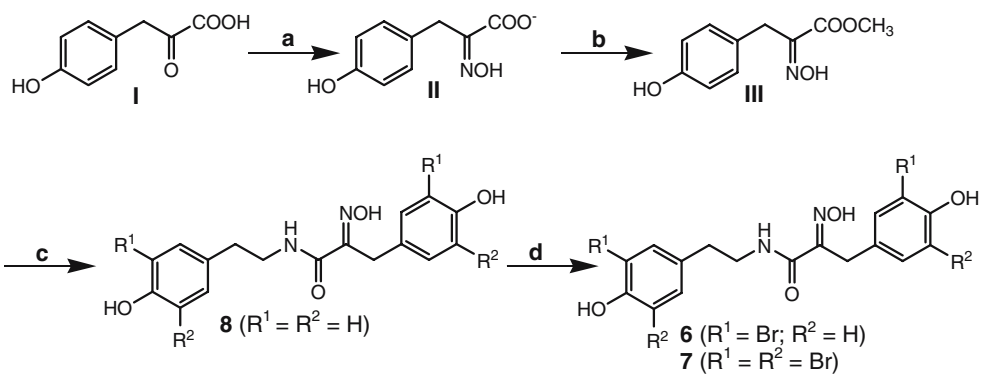
Compounds **6–9** were fully characterized by mass spectrometry (MS), infrared (IR), and ^{1}H-nuclear magnetic resonance (^{1}H-NMR).

5,5′-Dibromohemibastadin-1 (**7**): ^{1}H-NMR (500 MHz, DMSO-d_6) 2.66 (2 H, t, *J*=6.9 Hz, H-7), 3.31 (2 H, m, H-8), 3.68 (2 H, s, H-7′), 7.33 (2 H, s, H-6, H-6′) 7.32 (2 H, s, H-2, H-2′), 8.00 (1 H, t, *J*=5.8 Hz, NH-9), 9.73 (1 H, s, OH) 9.65 (1 H, s, OH), 11.90 (1 H, s, NOH). ESIMS *m/z* (%): $[M+H]^+$ 630 (100), 627 (18), 629 (75), 632 (80), 635 (30), $[2M+H]^+$ 1259 (100), 1252 (2), 1253 (9), 1255 (34), 1257 (83), 1261 (78), 1263 (43), 1265 (12), 1266 (2).

Debromohemibastadin-1 (**8**): ^{1}H-NMR (500 MHz, DMSO-d_6) 2.61 (2 H, t, *J*=6.9 Hz, H-7), 3.28 (2 H, m, H-8), 3.67 (2 H, s, H-7′), 6.59–6.69 (4 H, m, H-2, H-6, H-2′, H-6′), 6.91–7.01 (4 H, m, H-3, H-3′, H-5, H-5′), 7.82 (1 H, t, *J*=5.6 Hz, NH-9), 9.17 (2 H, s, OH) 11.67 (1 H, s, NOH). MS 70 eV, *m/z* (%): M^+ 314 (2), 163 (16), 134 (10), 133 (40), 132 (17), 107 (100), 78 (31), 77 (38), 51 (25).

L-Tyrosinyltyramine (**9**): ^{1}H-NMR (200 MHz, pyridine-d_5) 2.2 (2 H, s, NH_2-8′), 2.85 (2 H, t, *J*=7.0, H-7), 3.00 (1 H, dd *J*=8.2, 13.5 Hz, H-7′A), 3.39 (1 H, dd, *J*=4.7, 13.5 Hz, H-7′B), 3.70 (2 H, m, H-8), 3.84 (2 H, dd, *J*=4.7, 8.2 Hz, H-8′), 7.2 (8 H, m, H-Ar), 8.4 (1 H, t, *J*=5.6 Hz, NH-9), 11.5 (2 H, s, OH). MS 70 eV, *m/z* (%): [M-2H] 298 (2), [M-H_2O] 283 (5), 194 (18), [M-C_7H_7O] 193 (36), 164 (13), [M-$C_8H_{10}NO$] 136 (100), 121 (33), 120 (23), [M- $C_{10}H_{13}N_2O_2$] 107 (41), 91 (20), 77 (19), 51 (19).

Bioassays. Antifouling assay. Cyprid larvae of *Barnacle improvisus* Darwin were reared in a laboratory culture as previously described (Berntsson et al., 2000). Experiments for evaluating the effects of the respective substances on larval settlement and mortality of *B. improvisus* were run using polystyrene Petri dishes (48 mm diameter, Nunc AS, Denmark, no 2440045). To each Petri dish 10 ml of filtered seawater (filter pore size 0.2 μm) was added. Seawater for controls contained 0.1% dimethyl sulfoxide (DMSO) whereas treated samples contained 0.1% DMSO in which a defined amount of a given natural product or synthetic analogue had been dissolved. Competent cyprids (*n*=16) were added to each dish and each



Scheme 1. Synthesis of derivatives of hemibastadin-1. (**a**) NH_2OH / NaOH/60°C/3 h. (**b**) CH_3I/DBU/DMF/0°C/3 h. (**c**) tyramine/130°C/30 min. (**d**) Br_2/ether/CH_2Cl_2/25°C/24 h.

experiment was run in four replicates (n=4). Dishes were maintained for 3 to 4 days at room temperature (approximately 20°C), under normal light conditions (day/night), followed by examination of the cyprids under a stereomicroscope for attached and metamorphosed, swimming, or dead individuals. All substances were tested at concentrations of 0.1, 1, and 10 μM and in some cases even at 100 μM.

Brine shrimp assay. The assay was carried out using nauplii of *Artemia salina* Leach as previously described (Meyer et al., 1982). The compounds were dissolved in 40 μl of DMSO and added to 10-ml vials. After addition of artificial seawater to the vials, the final concentrations of test samples amounted to 1, 10, or 100 μM whereas the DMSO concentration was 0.8%. Brine shrimp eggs (Dohse Aquaristik KG) were hatched in a small tank filled with artificial seawater, which was prepared with sea salt (Wiegandt GmbH) 33 g/L in tap water. After 2 days, 20 nauplii were taken by pipette and transferred to each sample vial. The vials were maintained under illumination, and after 24 h surviving brine shrimp larvae were counted. Solvent controls, with 0.8% DMSO in seawater, were run parallel to the samples.

Statistical Analysis of Data. The means are reported ± standard error (SE). Effects of the various substances on cyprid settlement were tested using one-factor analysis of variance (ANOVA) with the dose as a fixed factor. The minimum dose leading to a significant difference compared to the control was determined using the Student–Newman–Keuls (SNK) test. All data were also subjected to a Cochran's homoscedasticity test. In cases where data displayed significant heterogeneity, log-transformations were successfully performed. The level of significance was set at P<0.05.

Results

A total of 16 different halogenated compounds and two nonhalogenated derivatives (**8**, **9**) that either occur in different marine sponges (**1–6** and **10–18**) or represent synthetic products (**7**) were investigated for antifouling activity using larvae of *B. improvisus*. The effects of the substances on larval settlement and mortality are summarized in Tables 1 and 2, including F-values, P-values, and degrees of freedom. Minimum inhibitory concentrations of cyprid settlement and toxicity are presented in the tables.

Four of the natural products analyzed in this study including bastadins 3 (**4**), 4 (**1**), 9 (**2**), and aplysamine-2 (**5**) significantly inhibited larval settlement at concentrations of 1 or 10 μM, without increasing larval mortality (Figure 2). Bastadin-16 (**3**), hemibastadin-1 (**6**), and psammaplin A (**10**) likewise inhibited larval settlement at a dose of 10 μM but in addition caused significant mortality of the cyprids (Figures 2 and 3). All other analyzed natural products turned out to be inactive in this bioassay with exception of isofistularin-3 (**16**), which was toxic to the cyprids at only 1 μM but did not inhibit settlement of surviving *B. improvisus* larvae (data not shown). Interestingly, psammaplin A sulfate (**11**) did not inhibit settling, but proved to be mildly toxic to the cyprids at 10 μM.

Hemibastadin-1 (6), because of its simplified structure (when compared to the bastadins 3, 4, 9 or 16, [1–4]), was selected for the preparation of synthetic analogues and for evaluation of structure–activity relationships. Three synthetic congeners including debromohemibastadin-1 (8), 5,5′-dibromohemibastadin-1 (7), and l-tyrosinyltyramine (9) were prepared and studied for their effects on larval settlement and mortality (Figure 3). Hemibastadin-1 (6) and its 5,5′-dibromo-derivative (7) were essentially comparable with regard to the suppression of larval settlement (Figure 3).

However, only the natural product hemibastadin-1 (6) caused significant larval mortality at this concentration whereas no toxicity was observed for its synthetic analogue (7) (Figure 3). In comparison to compounds 6 and 7 the second synthetic analogue debromohemibastadin-1 (8) was considerably less active causing inhibition of larval settlement only when tested at a dose of 100 μM (Figure 3). At this concentration the debromo-derivative (8) proved to be toxic to the cyprids thus resembling the activity profile of the parent compound hemibastadin-1 (6), albeit at higher concentrations. l-tyrosinyltyramine (9), which lacks bromine atoms and an oxime function but features an amino substituent instead, was completely inactive even when analyzed at a dose of 100 μM, showing no adverse effect on larval settlement or survival (Figure 3).

None of the synthesized compounds caused mortality of the brine shrimp nauplii when compared to controls.

Discussion

In this study we investigated the ability of selected sponge metabolites to inhibit the settlement of *Balanus improvisus* larvae under laboratory condi-

Table 1. Inhibition of larval settlement of *B. improvisus* by brominated sponge-derived secondary metabolites

	Settlement inhibition		*Toxicity*	
Compound	Minimum significant dose to inhibit settlement (μM)	Result of statistical test of effect on settlement (ANOVA)	Minimum significant dose to affect larval mortality (μM)	Result of statistical test of effect on larval mortality (ANOVA)
Active and toxic compounds				
Bastadin-16 (***3***)	10	$F_{1, 18}$=6.9, P<0.05	10	$F_{1, 18}$=13.1, P=0.002
Psammaplin A (***10***)	10	$F_{5, 18}$=10.9, P<0.05	10	$F_{4, 15}$=3.2, P=0.04[1]
Active compounds without toxicity				
Bastadin-4 (***1***)	10	$F_{4, 15}$=5.2, P<0.05	n.e.	$F_{5, 18}$=0.7, P>0.05
Bastadin-9 (***2***)	1.0	$F_{1, 18}$=6.4, P<0.05	n.e.	$F_{5, 18}$=1.0, P>0.05
Bastadin-3 (***4***)	10	$F_{5, 18}$=7.9, P<0.05	n.e.	Non-analyzable (zero percent dead in all treatments)
Aplysamine-2 (***5***)	10	$F_{5, 18}$=19.7, P<0.05	n.e.	$F_{4, 15}$=1.0, P>0.05
Inactive compounds				
Psammaplin A sulfate (***11***)	n.e.	$F_{3, 12}$=3.2, P>0.05	10	Significant at 10 μM
Isofistularin-3 (***12***)	n.e.	$F_{5, 18}$=1.3, P>0.05	1.0	Significant at 1 μM but not at any other concentration
Ageliferin (***13***)	n.e.	$F_{5, 18}$=0.75, P>0.05	n.e.	$F_{5, 18}$=2.76, P>0.051
Sceptrin (***14***)	n.e.	$F_{3, 12}$=2.9, P>0.05	n.e.	Non-analyzable (zero percent dead in all treatments)
Debromosceptrin (***15***)	n.e.	$F_{4, 15}$=2.3, P>0.05	n.e.	Non-analyzable (zero percent dead in all treatments)
Oxazolidinone derivative (***16***)	n.e.	$F_{4, 15}$=2.8, P>0.05	n.e.	Non-analyzable (zero percent dead in all treatments)
Hymenidine (***17***)	n.e.	$F_{5, 18}$=0.7, P>0.05	n.e.	$F_{5, 18}$=0.4, P>0.05
2-Bromoaldisin (***18***)	n.e.	$F_{3, 12}$=0.9, P>0.05	n.e.	$F_{5,18}$=0.6, P>0.05

n.e. = no effect, 1 data log-transformed.

tions. All compounds (except compounds **8** and **9**) selected for this bioassay are brominated (Figure 1). Several of the selected sponge-derived natural products display pronounced activity in other bioassays. For example, ageliferin (**13**), isofistularin-3 (**12**), and sceptrin (**14**) are known to be deterrent against fishes (Assmann et al., 2000; Thoms et al., 2004). Sceptrin (**14**) and hymenidin (**17**) are serotonin inhibitors (Kobayashi et al., 1990). Serotonin appears to be involved also in the settling behaviour of cyprids (Yamamoto et al., 1998). Oxazolidinone derivatives are known for their antibacterial activity (Renslo et al., 2006). Hence, their previously documented bioactivity made these compounds interesting candidates for an assessment of their antifouling activity.

Table 2. Inhibition of larval settlement of *B. improvisus* by synthetically obtained hemibastadin-1 analogues

Settlement inhibition			*Toxicity*	
Compound	Minimum significant dose to inhibit settlement (μM)	Result of statistical test of effect on settlement (ANOVA)	Minimum significant dose to affect larval mortality (μM)	Result of statistical test of effect on larval mortality (ANOVA)
Active and toxic compounds				
Hemibastadin-1 (***6***)	10	$F_{5, 18}$=8.6, P<0.05	1	$F_{5, 18}$=1225, P<0.05
Debromo-hemibastadin-1 (***8***)	100	$F_{5, 18}$=15.6, P<0.05	100	$F_{5, 18}$=13.5, P<0.05
Active compounds without toxicity				
5,5′-Dibromohemibastadin-1 (***7***)	10	$F_{5, 18}$=8.2, P<0.05	n.e	$F_{5, 18}$=1.6, P=2.8
Inactive compounds				
L-Tyrosinyltyramine (***9***)	n.e	$F_{5, 18}$=3.9, P=0.04	n.e	$F_{5, 18}$=0.8, P=0.5

n.e. = no effect.

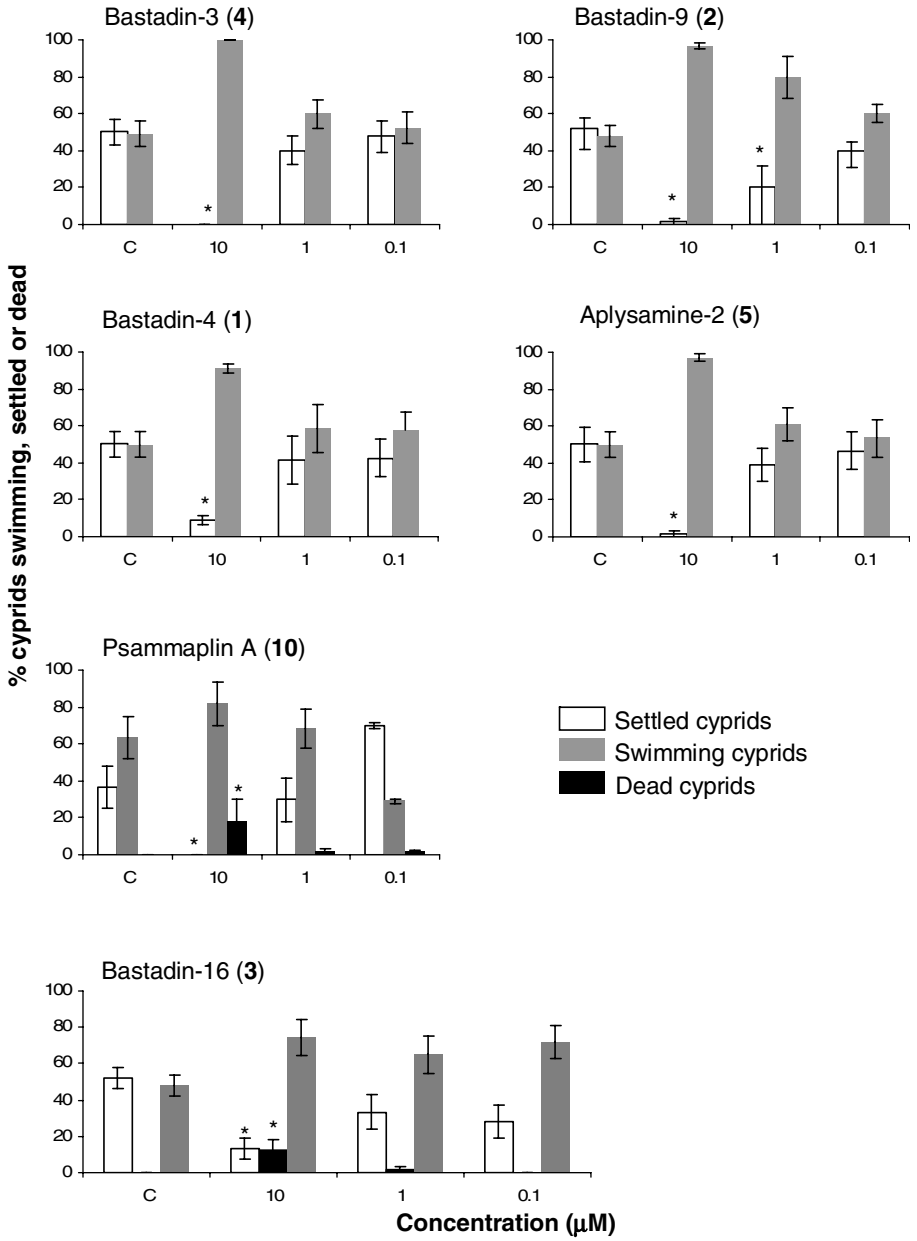


Figure 2. Effects of the active natural products on *B. improvisus* cyprid settlement. Results are expressed as percentage settlement (± SE) and percentage living swimming cyprids (± SE). C = control, n=4. *Results significantly different from the solvent controls.

From all tested naturally occurring sponge metabolites, however, only bastadins 3 (**4**), 4 (**1**), 9 (**2**), 16 (**3**), hemibastadin-1 (**6**), aplysamine-2 (**5**), and psammaplin A (**10**) inhibited settlement of barnacle larvae in a dose-dependent manner in a range of 0.1 to 10 µM (Figures 2 and 3). In addition hemibastadin-1 (**6**), psammaplin A (**11**) and, to a lesser degree, bastadin-16 (**3**), were also toxic to *B. improvisus* when tested at a dose of 10 µM (Figure 2). Especially the toxicity of bastadin-16 (**3**) when compared to bastadin-4 (**1**) is difficult to explain, as the former differs from the latter merely by the presence of two instead of one symmetrically substituted tyrosine rings. Whereas the number of bromine atoms is the same for both compounds, bastadin-4 (**1**) differs from bastadin-16 (**3**) by an additional double bond in the side chain of ring A (Figure 1)

For the remaining natural products (**11**–**18**) with no obvious effect on larval settlement, only psammaplin A sulfate (**11**) and isofistularin-3 (**12**), both representing brominated tyrosine derivatives,

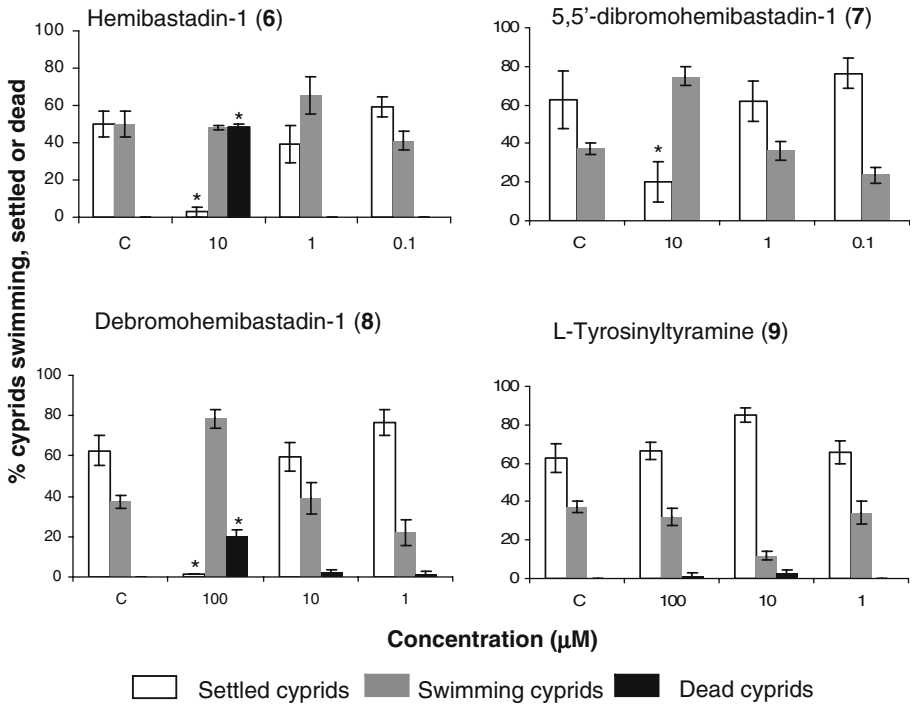


Figure 3. Effects of the synthesized hemibastadin-1 analogues on *B. improvisus* cyprid settlement. Results are expressed as percentage settlement (± SE) and percentage living swimming cyprids (± SE). C = control, n=4. *Results significantly different from the solvent controls.

showed toxicity against *B. improvisus* larvae at 10 and 1 μM, respectively but had no adverse effects on settling of the cyprids. Other isoxazoline alkaloids such as aerothionin and homoaerothionin which are structurally closely related to isofistularin-3 (**12**) are known to possess antifouling activity against other fouling organisms (e.g., ectoprocta, polychaeta, and gastropoda) (Thompson, 1985; Thompson et al., 1985). In the barnacle assay, however, only bromotyrosine compounds with an oxime moiety as a uniting structural feature inhibited settling of barnacle larvae in a dose-dependent manner (Figures 2 and 3), giving rise to the hypothesis that the oxime substituent is important for the biological activity. This assumption was proven by synthesizing debromohemibastadin-1 (**8**) and the corresponding L-tyrosinyltyramine (**9**) which differs from the former merely by the lack of the oxime function. Both compounds furthermore lack bromine atoms, in contrast to the natural product hemibastadin-1 (**6**), which showed a pronounced inhibition of cyprid settling and was also toxic to the barnacle larvae (Figure 3). Whereas L-tyrosinyltyramine (**9**) turned out to be completely inactive even at a concentration of 100 μM, the oxime-bearing debromohemibastadin-1 (**8**) inhibited barnacle settling at the same concentration almost completely (Figure 3), thus corroborating the importance of the oxime function. On the other hand, debromohemibastadin-1 (**8**) was clearly less active than the brominated natural product hemibastadin-1 (**6**), which demonstrates the importance of bromine atoms in the molecule with regard to antifouling activity. The toxic side effects that were rather severe for hemibastadin-1 (**6**) and less so for debromohemibastadin-1 (**8**) were not observed when assaying the synthetic product 5,5′-dibromohemibastadin-1 (**7**), at least up to a concentration of 10 μM (Figure 3). These results suggest that the degree of bromination is important for the toxicity for this bastadin analogues.

However, compound **7** was comparable with regard to antifouling activity to hemibastadin-1 (**6**). It is interesting to note though that none of the synthesized hemibastadin-1 analogues, including hemibastadin-1 itself, showed any toxicity against brine shrimps, *Artemia salina*, which are an established model for the assessment of cytotoxicity (Carballo et al., 2002).

The importance of the oxime group for the antifouling activity of bastadin derivatives was unequivocally proven in this study. In this context, it is of interest to note that ianthelline, which is a further oxime-bearing bromotyrosine

derivative isolated from the Caribbean sponge *Ailochroia crassa*, was recently shown to inhibit bacterial attachment (Kelly et al., 2005). Up until now a growing number of marine natural products with antifouling activity against *Balanus* larvae has been reported in the literature. In addition to bromotyrosines, these compounds include, for example, diketopiperazines, sesquiterpenoids, steroids, or saponins (Fusetani, 2004). Given the large structural diversity of these natural products it is obvious that more than one target responsible for the inhibition of settling exists in the cyprids. In fact it has been shown that dopamine and serotonin receptor agonists as well as modulators of intracellular calcium levels influence settling of *Balanus* larvae (Rittschof et al., 1986; Clare, 1996b; Yamamoto et al., 1999).

The mode of action of the antifouling bastadin derivatives described in this study remains unknown so far. One possible explanation involves an alteration of the intracellular Ca^{2+} levels of cyprids. It has previously been demonstrated that Ca^{2+} ions are important for barnacle settling (Rittschof et al., 1986) and a reduction in the intracellular Ca^{2+} generally inhibits cyprid settlement (Clare, 1996b). It is interesting to note that bastadin derivatives affect intracellular Ca^{2+} levels through interaction with the ryanodine-sensitive sarcoplasmatic reticulum Ca^{2+} channel (Mack et al., 1994). Further studies are necessary to test this hypothesis. However, the differences in the observed activity for the structurally closely related oxime bearing compounds psammaplin A (**10**) and psammaplin A sulfate (**11**) suggest, that the target of these bioactive compounds is localized intracellularly. Only psammaplin A (**10**) inhibits settling of the cyprids (Figure 2), whereas no settling inhibition was observed when assaying psammaplin A sulfate (**11**). As both compounds differ merely by the presence of the highly polar sulfate substituent in compound **11** membrane transport of the more lipophilic analogue **10** appears to be necessary for settling inhibition.

The relatively easy accessibility of hemibastadin-1 (**6**) and of several of its analogues (**7**, **8**) through organic synthesis and lack of general toxicity, at least for **7** and **8**, makes these compounds interesting candidates in the search for new antifouling compounds from nature.

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