Antifouling Activity of Brominated Cyclopeptides from the Marine Sponge *Geodia barretti*

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In this work, we show the potent antifouling effects of two compounds, barettin (cyclo[(6-bromo-8-entryptophan)arginine]) (**1**), isolated as a *Z*/*E* mixture (87/13), and 8,9-dihydrobarettin (cyclo[(6-bromotryptophan)arginine]) (**2**), isolated from the marine sponge *Geodia barretti*. The compounds were isolated guided by their ability to inhibit the settlement of cyprid larvae of the barnacle *Balanus improvisus*, and their structures were determined by means of mass spectrometry, NMR, and quantitative amino acid analysis. The activities of these brominated diketopiperazine-like cyclic dipeptides are in the range of antifouling agents in use today, as shown by their EC_{50} values of 0.9 and 7.9 μM, respectively. However, contrary to today's antifouling agents, the effects of barettin and 8,9-dihydrobarettin are nontoxic and reversible. A small set of synthetic analogues, including L-arginine, L-tryptophan, 5-bromo-D,L-tryptophan, 6-bromo-D,L-tryptophan, and 6-fluoro-D,L-tryptophan, were tested for possible structure−activity relationships. None of these compounds showed any effect at a concentration of 10 μM. We hypothesize that the isolated compounds are part of the sponge's chemical defense to deter fouling organisms. This theory is supported by the fact that barettin is found in water exposed to living specimens of *G. barretti* in concentrations that completely inhibit barnacles from settling.

The presence of antifouling strategies for sessile organisms is well documented,[1−3] and several compounds have been isolated that prevent settlement and/or attachment of invertebrate larvae.[4] This chemical defense plays a crucial role for filter-feeding marine sponges, as a body surface free from fouling organisms is necessary for their nutrition. As the sessile life and the nonmuscular tissue of marine sponges prevent any escape from predators, chemical defense may also have a predator deterrent role.[5,6]

Secondary metabolites involved in this defense attract a great interest as a source for biologically active compounds.[6] To date, a large number of such compounds have been isolated from different marine sponges that display a diverse array of activities, e.g., antimicrobial, antiviral, and antitumor.[7−9] In addition, recent reports have described compounds from marine sponges, including terpenes, steroids, kalihines, and ceratinamides,[10−13] that display inhibitory effects on the settlement of barnacle larvae. This is an interesting finding, as these compounds most likely have a crucial role for the survival of the sponge itself, but also because they may provide us with a solution to the severe problem of fouling marine organisms.

Biofouling on ship hulls and on static constructions such as offshore oil rigs and pipelines causes substantial economic losses worldwide. Today, heavy metal-based coatings are used to fight this problem. However, these coatings are facing bans due to their environmental hazard,[14] and thus the search for new antifouling methods is intense. The idea to search for antifouling substances among secondary metabolites in the marine environment was first introduced in 1982 by Targett et al.,[15] and several studies have since reported the isolation of molecules from marine organisms with antifouling activity.[16−18] Such a compound would have an additional advantage when used in an antifouling coating over traditional synthetic biocides, as pathways for degradation are very likely to already exist. This is of utmost importance, as the risk for bioaccumulation thus may be avoided.

In the present work we have examined the marine sponge *Geodia barretti* Bowerbank (family Geodiidae, class Demospongiae, order Astrophorida) for the presence of substances with antifouling activities. This sponge lives on the Atlantic continental shelf (at a depth of 10−500 m), and it shows a fouling-free body surface, which strongly suggests that some compound(s) produced by the sponge may be efficient against larval settlement and fouling growth. In addition, this sponge is known to produce a wide spectrum of secondary metabolites such as histamine, inosine, amino acids such as taurine and several sterols,[19] and a diketopiperazine-type cyclic dipeptide named barettin.[20] The latter has been a substance of controversy since its structure was first reported by our group as cyclo[(6-bromo-8-entryptophan)proline] in the mid 1980s.[20] This structure was disproved by total synthesis of cyclo[(6-bromo-8-entryptophan)proline] by Lieberknecht et al.[21] In addition Sölter et al.[22] recently reported the isolation and structural characterization of a compound from *G. barretti* with the structure cyclo[(6-bromo-8-entryptophan)arginine] which showed NMR data similar to those originally reported for barettin. Consequently it was suggested that this diketopiperazine represented the correct structure for barettin.

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In this work the bioassay-guided isolation of an aqueous ethanol extract of *G. barretti* led to the isolation of two substances. Their structures were determined by MS, NMR, and quantitative amino acid analysis as cyclo[(6-bromo-8-entryptophan)arginine] (**1**) and cyclo[(6-bromo-tryptophan)arginine] (**2**). Compound **1** is identical to the structure reported by Sölter et al.,[22] and as shown by comparison with original data for barettin and the original compound (isolated in 1980s),[20] it similarly represents the correct structure for this compound. (The current isolation and structure elucidation of **1** was done independently and in parallel to the work by Sölter et al.[22]) Hereafter, the name barettin refers to compound **1**. In addition, we show that *G. barretti* produces another, structurally related brominated cyclopeptide with a potent inhibitory effect on the settlement of barnacle larvae. This substance **2** is hereafter referred to as 8,9-dihydrobarettin.

1

2
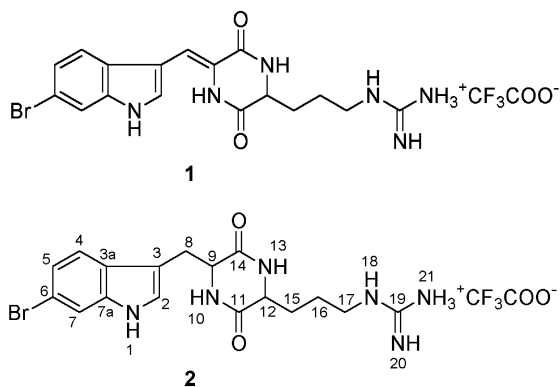
Since the isolated compounds consist of the amino acids tryptophan and arginine with the tryptophan residue bearing the bromine atom, we also evaluated the effect of the synthetic amino acids L-arginine and L-tryptophan and the halogenated amino acids 5-bromo-D,L-tryptophan, 6-bromo-D,L-tryptophan, and 6-fluoro-D,L-tryptophan to address the question if the complete molecular structure is causing the inhibition or if a specific part of the molecule in itself could be responsible for the effect. We also address the question whether the mechanism of **1** and **2** is toxic or reversible. Furthermore, an initial observation regarding the release into the water of these compounds from living *G. barretti* is reported.

Results and Discussion

In this work, the fractionation and isolation of an aqueous ethanol extract from the marine sponge *G. barretti* (guided by the inhibitory effect on the settlement of *B. improvisus*) led to the isolation of two cyclic dipeptides, barettin (**1**) and 8,9-dihydrobarettin (**2**), as their TFA salts. Their chemical structures, determined by a combination of NMR and MS, showed them both to consist of an arginine and a tryptophan residue, joined N-terminal to C-terminal, forming a diketopiperazine-type cyclic peptide backbone.

The spectroscopic properties of **1**, including MS, ^{1}H NMR, HMBC, and ROESY, were identical to the data reported by Sölter et al.[22] The presence of an arginine residue was also shown by quantitative amino acid analysis. The spectroscopic properties of compound **2** (with the molecular weight of m/z 421, 423 [M + H]$^+$) were very similar to those of **1**. The combined NMR analysis, including 2D experiments, also revealed a cyclic dipeptide (consisting of tryptophan and arginine) brominated in the 6-position of the indole nucleus, as determined for **1**. The differences in

Table 1. ^{1}H NMR and ^{13}C NMR Data for Compound **2** in DMSO-d_6

position	δ_C	δ_H (m, J in Hz)	COSY	HMBC
N-1		11.07 (s)	2, 7	3, 3a, 7a
C-2	125.7	7.09 (s)	1, 8	3, 3a, 7a
C-3	108.7			
C-3a	126.6			
C-4	120.7	7.54 (d, 8.3)	5	3, 6, 7a
C-5	121.3	7.10−7.06 (dd, 8.5; 1.7)	4, 7	3a, 7
C-6	113.6			
C-7	113.7	7.51 (s)	1, 5	3a, 5
C-7a	136.7			
C-8	28.7	3.28−2.97	2, 9	2, 3, 3a, 9
C-9	55.3	4.12−4.05 (m)	8, 10	3, 11
N-10		8.15 (s)	9	12, 14
C-11	167.3			
C-12	52.9	3.03−2.97 (m)	13, 15	14, 16
N-13		7.92 (s)	12	9, 11
C-14	167.9			
C-15	29.2	1.55−1.48 (m)	12, 16	
C-16	23.4	1.47−1.24 (m)	15, 17	
C-17	40.2	3.03−2.97 (m)	18	
N-18		7.45 (t, J = 5.4)	17	19, 15
C-19	156.6			
N-20/21		7.30−6.75 (br)		

molecular weight (i.e., the molecular weight of **1** + 2 amu) indicated that the structure of **2** was cyclo[(6-bromotryptophan)arginine], that is, 8,9-dihydrobarettin. This was further substantiated by [1]H−^{1}H-COSY sequence 8-H, 9-H, 10-H and by HMBC correlations $^3J_{C−3,H−9}$, $^3J_{C−11,H−9}$. The ^{13}C NMR data featured four signals from CH$_2$ groups and two signals from CH groups. All proton signals were assigned to their respective carbon by HMQC. ^{1}H and ^{13}C NMR data as well as correlations from [1]H−^{1}H-COSY and HMBC experiments are listed in Table 1. (Absolute stereochemistry at C9 and C12 was not determined.)

The chemical structures of **1** and **2** are notable in their possession of a brominated tryptophan residue. Tryptophan-containing substances have been found in organisms such as bryozoans[23] and gastropods and in the venomous cone snail *Conus imperialis*, which produces a heptapeptide possessing a 6-bromotryptophan unit.[24] In marine sponges, the predominating aromatic amino acids in secondary metabolites are tyrosine or phenylalanine,[25] which makes the presence of the tryptophan residue itself conspicuous.

In addition to the structural determination of **1** and **2** we have tested these metabolites for their effect on the settlement of competent barnacle larvae of *B. improvisus*. The results and statistical data (1-factor-ANOVA) of the different settlement experiments using fractions and purified substances are summarized in Table 2. When cyprids were exposed to barettin, settlement was inhibited in a dose-dependent manner between 0.12 and 19.0 μM (Figure 1a). The EC$_{50}$ value (i.e., the concentration where the settlement was 50% of that in the control dishes) of barettin was determined to 0.9 μM, and at 1.9 μM the inhibition was complete. The potency of 8,9-dihydrobarettin was approximately 1 order of magnitude lower (Figure 1b); the corresponding EC$_{50}$ was determined to be 7.9 μM. We also tested for the settlement-inhibiting properties of synthetic barettin and 8,9-dihydrobarettin. These substances caused an identical dose−response inhibition of barnacle larvae settlement (Table 2).

The settlement inhibition evoked by **1** and **2** was reversible: we found that cyprids that had been exposed to these compounds (at concentrations of 1.9 and 19 μM, respectively, for 48 h) and which then were washed and transferred to fresh seawater metamorphosed to an extent

Table 2. Effect of the Extract, Fractions, and Isolated Substances from *G. barretti* and Some Synthetic Derivatives of Tryptophan and Arginine on Cyprid Settlement.
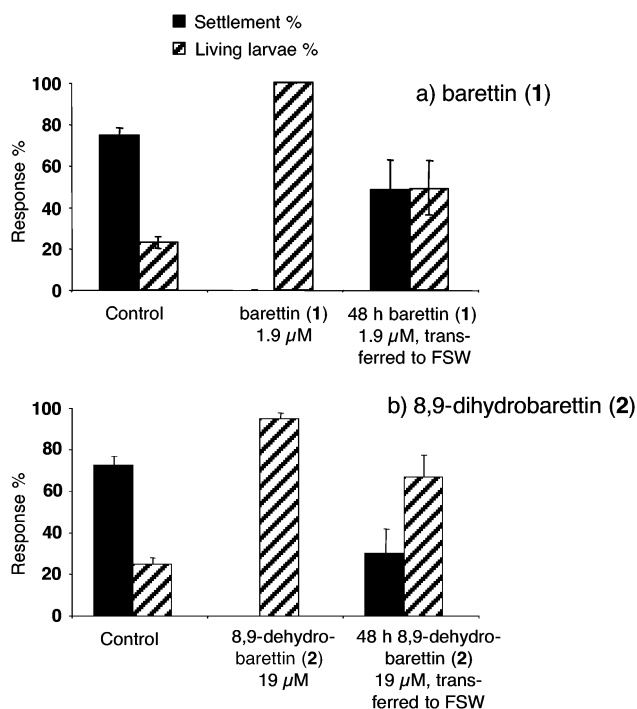
treatment	effect on settlement	conc μM (EC_{50})	*F*-value
ethanol extract	$+^a$	55.0 (μg mL^{-1})	7.2
fraction 6	++	10.0 (μg mL^{-1})	13.9
incubation water	+++	2.4	41.9
barettin	+++	0.9	22.7
8,9-dihydrobarettin	++	7.9	14.3
barettin (synthetic)	+++	0.8	5.2
8,9-dihydrobarettin (synthetic)	++	7.0	27.8
L-tryptophan	$-^b$	−	3.3
L-arginine	−	−	1.4
5-bromo-D,L-tryptophan	−	−	3.3
6-bromo-D,L-tryptophan	−	−	3.7
6-fluoro-D,L-tryptophan	−	−	3.3

^{a} (+++), (++), and (+) denote the relative efficiency of the individual treatments in the settlement inhibition assay and also denote that these treatments significantly differ from the controls ($p < 0.05$). ^{b} (−) denotes treatments with no settlement-inhibiting activity as compared to controls ($p > 0.05$). *F*-value is defined as F = mean square (factor "treatment")/mean square (residual).
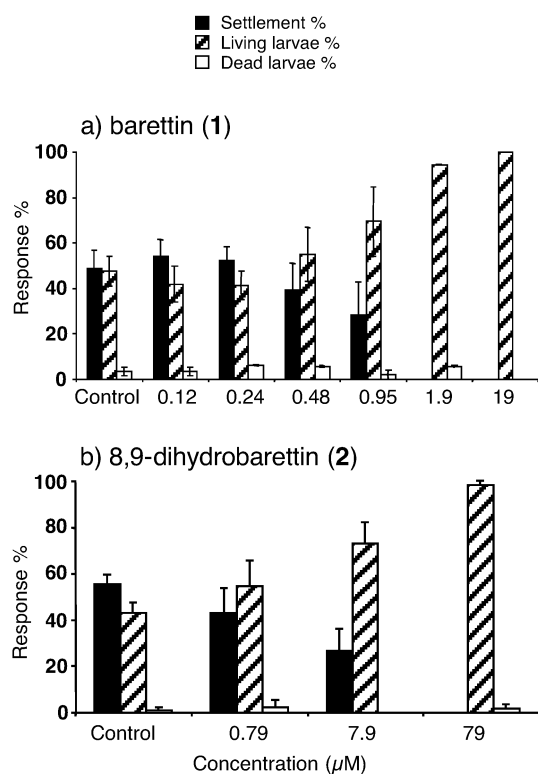
Figure 1. Effect of (a) barettin (**1**) and (b) 8,9-dihydrobarettin (**2**) on the settlement of cyprid larvae of *B. improvisus* as reflected by percentages for settled, living (but not settled), and dead cyprids and given as means ± SE ($n = 4$).

Figure 2. Reversibility of (a) barettin (**1**) and (b) 8,9-dihydrobarettin (**2**) after the cyprids were transferred to fresh seawater (FSW) as reflected by percentages for settled cyprids and given as means ± SE ($n = 4$).
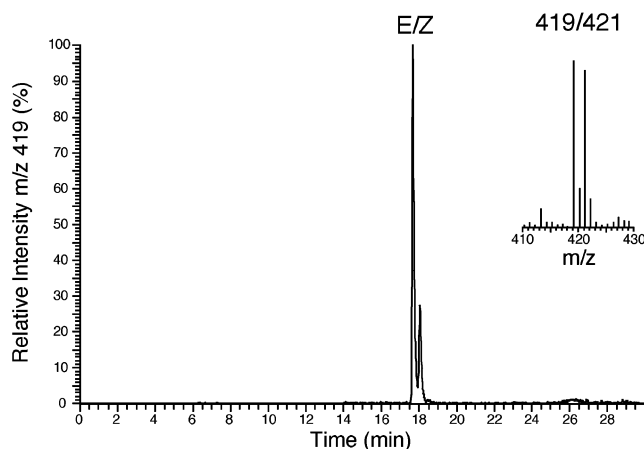
Figure 3. Analysis of the release of barettin from live *G. barretti*. RP-HPLC-MS analysis monitored at *m/z* 419 after injection of water surrounding an incubated specimen. The presence of both isomers of barettin (*Z/E*) is shown. The mass spectrum of the main peak is inserted, clearly showing the typical isotopic distribution pattern of bromine. When injecting the isolated substances as reference, equivalent chromatograms were obtained.

similar to the cyprids in the control dishes. In the dishes where the cyprids remained in **1** and **2** at concentrations of 1.9 and 19 μM, respectively, the settlement was completely inhibited (Figure 2a,b).

To assess possible structure−activity relationships, a set of synthetic amino acids were tested in the assay, including L-arginine and L-tryptophan, and the halogenated tryptophan derivatives, 5-bromo-D,L-tryptophan, 6-bromo-D,L-tryptophan, and 6-fluoro-D,L-tryptophan. None of these compounds showed any effect in concentrations ranging between 0.1 and 10 μM (Table 2). This suggests that the complete chemical structures of **1** or **2** are necessary for the inhibiting effect observed in the cyprid settlement

studies. In addition, the single structural difference between **1** and **2**, the presence/absence of the double bond at position 8, seems to be crucial for the activity, as its presence increases the activity 10-fold.

Seawater incubated with a living and undamaged species of *G. barretti* completely inhibited the settlement of *B. improvisus* cyprids, and indeed this water was shown to contain barettin (*Z/E*, 87/13) by means of LC-MS (Figure 3) and by MS fragmentation studies using nanospray infusion (data not shown). The concentration of released barettin in the tested seawater was determined to 20.5 μM by LC-UV, and this inhibited 90% of the settlement even after a 10-fold dilution (Figure 4). These results were all in good agreement with the effect mediated by the pure compound. The ability to release the compound in the
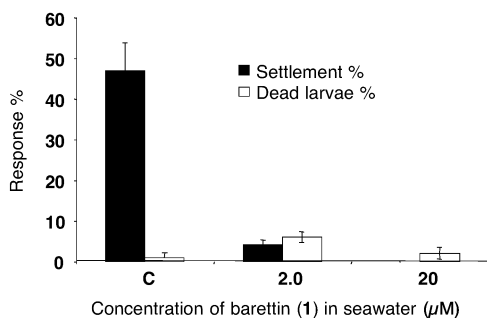
Figure 4. Effect of seawater exposed to live *G. barretti* on the settlement of cyprids of *B. improvisus* as reflected by percentages for settled and dead cyprids and given as means ± SE (*n* = 4). The concentration of barettin in intact seawater was established as 20 μM (see Experimental Section). Data are shown for intact seawater and water diluted 10 times.

surrounding water is most likely a function to deter attempts of attachment in an early phase. As many settling larvae and algal spores have a sticky body surface or secrete a permanent glue preceding an often speedy metamorphosis,[26] this would be highly advantageous in the sponge's defense.

Both **1** and **2** are highly efficient in preventing the settlement of cyprid larvae of *B. improvisus*. These two compounds also display a dose-dependent inhibition of settlement, which suggests a specific physiological mechanism. Furthermore, both **1** and **2** have strongly hydrophobic elements due to their tryptophan residue, a feature that probably enables good tissue and cell wall penetration. However, for both compounds the effect was reversed when cyprids were washed and transferred to seawater, indicating that the substances do not exert their action through an irreversible toxic mechanism.

Due to the low toxicity of **1** and **2**, both compounds are attractive candidates as active ingredients in marine antifouling paints. TBTO (tributyltin-oxide) and other biocides, such as Sea-Nine, which are widely used as antifouling substances, are toxic to cyprid larvae, causing death within 24 h. The LC_{50} values for TBTO and Sea-Nine are 0.09 and 0.3 μg mL^{-1}, respectively,[27] while the corresponding EC_{50} values for **1** and **2** are 0.4 (0.9 μM) and 4 μg mL^{-1} (7.9 μM), respectively. A comparison of these values shows that **1** and **2** are effective in the same range as these biocides. In addition when cyprids were exposed to a 20-fold increase in the effective concentration of barettin (19 μM), there was no significant increase in mortality, as shown in Figure 2a, which further supports the idea that barettin does not act through a toxic mechanism. The hydrophobic portion of **1** and **2** also makes them suitable for antifouling purposes since a hydrophobic molecule has the possibility of interacting with important components in the paint formulation, making the active ingredients less prone to leak out into the water. In addition, **1** and **2** possess the attractive feature of being biosynthesized in the marine environment, where pathways of degradation are likely to already exist.

Our initial observations regarding the release of barettin into ambient water show that the concentration in water surrounding live and undamaged specimens of *G. barretti* placed in aquaria is sufficient to completely inhibit settlement of cyprid larvae of *B. improvisus*. This suggests that barettin is released in effective concentrations and reaches settling larvae as a part of the chemical defense of the sponge *G. barretti*. While we in this study have concentrated on one possible target, i.e., barnacles, there may be targets other than fouling by invertebrate larvae, including

selective targets, such as microbial fouling and predation. Further studies are in progress on the functional role of these brominated cyclopeptides in the marine sponge *G. barretti*.

Experimental Section

General Experimental Procedures. Optical rotation was determined on a Perkin-Elmer 241 polarimeter, using a 1 mL cell. The UV spectrum was recorded on a Shimadzu-160A spectrophotometer. The IR spectrum was recorded using a Perkin-Elmer FT-1600 instrument. NMR spectra were recorded on a Bruker DPX 300 (300 MHz). For MS and MS^2 a nanospray-ion trap MS [Protana's NanoES source (MDS Protana A/S, Odense, Denmark) mounted on a LCQ (Thermo Finnigan, San Jose, CA)] was used. Samples were analyzed in the positive ion mode, directly after fractionation, or were lyophilized and dissolved in 60% MeOH with 1% HOAc. The spray voltage was set to 0.5 kV and the capillary temperature to 150 °C. For MS^2, the CID was set at 35%. The high-resolution mass spectrum was measured on a JEOL JMS-SX/SX 102A spectrometer. Quantitative amino acid analysis was performed at the Amino Acid Analysis Centre, Department of Biochemistry, Uppsala University.

Isolation of Barettin (1) and 8,9-Dihydrobarettin (2) from *Geodia barretti*. *G. barretti*, Bowerbank, was collected at 50−60 m depth in the Kosterfjord on the Swedish west coast in March 2001. A 1.1 kg specimen was homogenized and freeze-dried. The freeze-dried material was defatted with dichloromethane five times for removal of lipophilic compounds, and the residue was extracted five times with 50% aqueous ethanol, as outlined in a fractionation protocol described by Claeson et al. (1998).[28] The extract was then concentrated and desalted by reversed-phase solid-phase extraction (RP-SPE) [Isolute C18 (EC), International Sorbent Technologies, Mid Glamorgan, U.K.]. After applying the sample, the RP-SPE column was washed with 0.1% trifluoroacetic acid (TFA) and then 60% acetonitrile (AcN) with 0.1% TFA to elute captured substances. AcN was removed from the latter fraction in vacuo, before the extract was subjected to RP-HPLC using an Äkta Basic equipped with a 50 mL Superloop (Amersham Pharmacia Biotech, Uppsala, Sweden), enabling repeated injections of 4 mL of extract on a Rainin Dynamax RP18 column (10 × 250 mm, 5 μm, 300 Å). A linear gradient elution from 10 to 100% B [A: 0.1% TFA (v/v); B: 60% AcN, 0.1% TFA] over 25 min was used at a flow rate of 4 mL min^{-1}. The separation was monitored by UV at 215, 254, and 280 nm, and the five collected fractions were tested for inhibition of barnacle settlement. Fractions 4 and 5 were found to be most active and were subjected to rechromatography on the same system using more shallow gradients. In this manner barettin was isolated from fraction 5, and 8,9-dihydrobarettin, from fraction 4. The purity of the isolated compounds was >95% as determined by analytical HPLC on a GROM ODS-4HE column (2 × 100 mm, 3 μm, 200 Å). For barettin, the ratio of isomers was approximately 87/13 (*Z/E*) according to peak integration at 280 nm. 8,9-Dihydrobarettin and a mixture of the two isomers from barettin were used for testing in the bioassay described below.

8,9-Dihydrobarettin (2): brownish solid; [α]$^{21}_D$ −24° (*c* 0.096, MeOH); UV (MeOH) λ_{max} (log ϵ) 225 (1.27), 286 (0.16) nm; IR (CHCl$_3$) ν_{max} 3352, 3210, 1670, 1456, 1330, 1198, 802, 760 cm^{-1}; NMR data, see Table 1; HRMS (FAB+) *m/z* 421.0996 (calcd for $C_{17}H_{22}N_6O_2{}^{79}Br$ 421.0988).

Larval Bioassay. The brood stock of adult barnacles (*B. improvisus*, Darwin 1854) was allowed to settle on Plexiglass panels in the sea on a raft outside Tjärnö Marine Biological Laboratory (latitude 58°53′ N; longitude 11°8′ E). Cleaned from epiphytes, they were brought to the laboratory and immediately placed in trays with running seawater (salinity 32 ± 1‰). When regularly fed, with nauplii of *Artemia salina*, *B. improvisus* will spawn throughout the year. For larval rearing we used the method described by Berntsson et al. (1999).[29] The experiment for evaluating the effect of **1** and **2** on settlement

and mortality was run using polystyrene Petri dishes (diameter 48 mm) (Nunc AS Denmark, no. 240045) to which 10 mL of *G. barretti* fractions dissolved to different concentrations in filtered seawater (0.2 μm) was added. Competent cyprids (20 ± 2 individuals) were added to each dish in four replicates, and dishes containing filtered seawater served as controls. Dishes were maintained for 3–4 days at room temperature, after which they were examined under a stereomicroscope for attached and metamorphosed individuals, and also for dead cyprids.

Experiments for Determining the Effect of Synthetic Barettin and 8,9-Dihydrobarettin, L-Arginine, and Derivates of Tryptophan. Synthetic barettin (cyclo[(6-bromo-8-entryptophan)-L-arginine]) (*Z*-configuration) and 8,9-dihydrobarettin (cyclo[(6-bromo-D,L-tryptophan)-L-arginine]) were obtained in collaboration with Bergman and co-workers, who recently synthesized these molecules.[30] The synthetic amino acids L-arginine, L-tryptophan, 5-bromo-D,L-tryptophan, 6-bromo-D,L-tryptophan (Biosynth), and 6-fluoro-D,L-tryptophan were purchased from Sigma-Aldrich (St. Louis, MO) and Biosynth (Switzerland). Synthetic barettin and 8,9-dihydrobarettin were dissolved in milliQ water and added to FSW (filtered seawater) (10 mL) to give the desired concentration series (0.019, 0.19, and 1.9 μM for barettin; 0.19, 1.9, and 19 μM for 8,9-dihydrobarettin). L-Arginine was dissolved in milliQ water and used in the concentrations 0.1, 1.0 and 10 μM. The tryptophan derivatives were dissolved in dimethyl sulfoxide (DMSO) and prepared as above. Petri dishes of polystyrene (Nunc no. 240045) were filled with the different substances in FSW, and 20–25 cyprids were added to each dish. Dishes with FSW or FSW plus DMSO 0.1% served as controls. The experiment, with each treatment replicated four times, was maintained for 4 days before the dishes were viewed under a stereomicroscope to check for attached and metamorphosed individuals, nonattached larvae, and dead cyprids.

Reversible Effect of Barettin (1) and 8,9-Dihydrobarettin (2). A series of experiments was carried out to determine whether the effect of **1** and **2** on the settlement of cyprid larvae was reversible. Cyprids were exposed to **1** and **2** in the effective concentration as revealed by the dose–response experiment described above. After 24 h, the cyprids were washed and then transferred to fresh saltwater. The experiment, with the treatment replicated four times, was maintained for 4 days. The dishes were then checked in a stereomicroscope for attached and metamorphosed individuals, nonattached larvae, and dead cyprids.

Release of Barettin (1) by Living *G. barretti*. A living and undamaged specimen of *G. barretti* (0.85 kg) was placed for 24 h in an aquarium containing 5 L of filtered seawater. Samples of 10 mL were then taken from four different places in the aquarium and were used in the cyprid settlement assay. These samples were then diluted 10 times and examined for properties inhibiting cyprid larval settlement.

LC-MS analysis was done on the LCQ electrospray ion trap (MS operated in the positive ion mode) connected to the Äkta Basic HPLC. A 50 μL sample of incubated water was injected [Grom-Sil ODS-4 HE (2 × 100 mm, 3 μm, 200 Å)] and eluted at a flow rate of 0.3 mL min⁻¹ with a linear gradient from 10 to 100% B over 15 min [A: 0.1% HCOOH (v/v) in H₂O; B: 60% AcN, 0.1% HCOOH]. The spray voltage was set at 4.5 kV and the capillary temperature at 230 °C. For determination of the concentration of released barettin, a calibration curve was created [same HPLC setup as above, eluents (A: 0.1% TFA; B: 60% AcN, 0.1% TFA), and detection changed (UV 215 nm)] after establishing the molar UV absorbance at 337.5 nm (log ε = 3.12) in MeOH.

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