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Antifouling Activity of a Dibrominated Cyclopeptide from the Marine Sponge *Geodia barretti*[⊥]

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Many sessile suspension-feeding marine organisms rely on chemical defense to keep their surfaces free from fouling organisms. The brominated cyclopeptides barettin (cyclo[(6-bromo-8-entryptophan)arginine]) (**1**) and 8,9-dihydrobarettin (cyclo[(6-bromotryptophan)arginine]) (**2**) from the cold-water sponge *Geodia barretti* have previously displayed settlement inhibition of barnacle larvae in a dose-dependent manner. In this paper, we describe a novel dibrominated cyclopeptide, bromobenzisoxazolone barettin (cyclo[(6-bromo-8-(6-bromobenzioxazol-3(1*H*)-one)-8-hydroxy)tryptophan)]arginine) (**3**), which we have isolated from *G. barretti* and which displays settlement inhibition of barnacle larvae (*Balanus improvisus*) with an EC_{50} value of 15 nM. The chemical structure was determined using MS and 2D-NMR.

The vast ocean hosts a relatively unexplored and unique biodiversity where many species produce compounds with potential use in drug research and biotechnology, such as the antitumoral agent Ecteinascidin-743 or the painkiller Ziconotide.[1,2] Most marine invertebrates living on the seabed have free-swimming larvae that settle on suitable substrates before growing to their adult stage. After settlement, many marine animals remain permanently attached or have very limited ability to move. These animals have few behavioral or morphological defenses against predators and fouling overgrowth. An alternative strategy is to develop a chemical defense based on the production of toxic, noxious, or repellent compounds.[3] All surfaces in the sea are exposed to fouling, even the surfaces of algae and other sessile marine organisms, and yet some organisms remain free of fouling. Several of these organisms have been studied in antifouling assays; it has been suggested that they leak a broad array of chemical substances into the surrounding water that prevents foulers from attaching.[4,5] Fouling organisms cause extensive technical and economic problems, and hence the great interest in countermeasures against them; for example, the shipping industry avoids fouling by using heavy-metal-based coatings. However, some coatings, such as organotin (TBT), have already been banned, while others face global bans due to their unwanted effects on the environment. There is, therefore, an urgent need for new, eco-friendly substances to replace existing antifouling coatings.[6,7] One possible strategy is to exploit natural chemical defense compounds in protective coatings. Recently, two brominated cyclodipeptides, **1** and **2**, from the marine sponge *Geodia barretti* Bowerbank (Geodiidae, Astrophorida), have been isolated, characterized, and synthesized.[5,8,9] Artificial surfaces painted with coatings including these agents have been shown in field studies to inhibit fouling by the barnacle *Balanus improvisus*and the blue mussel *Mytilus edulis*.[10] When investigated for possible modes of action, **1** was found to interact with 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 receptors transfected in human embryonic kidney cells at concentrations approaching endogenous serotonin, whereas the affinity of **2** was approximately 1 order of magnitude lower.[11] Serotonin receptors are evolutionary conserved and may be present in barnacles.[12] These selective serotonin receptor–ligands may be involved in the chemical defense of *G. barretti* against fouling organisms and grazers.

Table 1. ^{1}H NMR and ^{13}C NMR Data for Bromobenzisoxazolone Barettin (**3**) in DMSO-d_6 (δ in ppm, J in Hz)

position	δ_C[a], mult.	δ_H[b] (J in Hz)	HMBC
NH (1)		11.54 (s)	
2	126.2, CH	7.37 (s)	3, 3a, 22
3	109.2, qC		
3a	125.2, qC		
4	120.9, CH	7.79 (d, 8.6)	3, 7a
5	121.8, CH	7.12 (dd, 8.6;1.4)	
6	114.0, qC		
7	114.0, CH	7.61 (d, 1.4)	3a, 4, 6
7a	136.8, qC		2
8	80.1, qC		
OH (8)		8.41[4]	
9	52.7, CH	4.79 (m)	3, 3a, 8, 14
NH (10)		9.36 (s)	9
11	168.0, qC		
12	53.2, CH	3.04 (m)	
NH (13)		8.27 (s)	8, 11
14	161.9, qC		
15	30.1, CH_2	1.69, 1.58 (m)	
16	23.7, CH_2	1.50, 1.42 (m)	
17	40.1, CH_2	3.02 (m)	15, 16
NH (18)		7.70[4]	
19	156.6, qC		
NH (20/21)		7.20[4]	
22	112.9, qC		2, 24
23	113.5, qC		
24	117.4, CH	6.78 (d, 8.6)	22, 25a, 27a
25	124.1, CH	7.36 (d, 8.6)	22, 26
25a	138.7, qC		
26	170.3, qC		
NH (27)		7.20[4]	
27a	137.7, qC		

[a] Recorded at 151 MHz. [b] Recorded at 600 MHz.

In this study, we present the novel secondary metabolite bromobenzisoxazolone barettin (cyclo[(6-bromo-8-(6-bromobenzioxazol-3(1*H*)-one)-8-hydroxy)tryptophan)]arginine) (**3**), which inhibited settlement of barnacle larvae with an EC_{50} value of 15 nM. We describe the isolation, structure elucidation, and antifouling activity of this new cyclopeptide.

Results and Discussion

Brominated natural products are well distributed in the marine environment, which likely coincides with the extensive presence of bromoperoxidases in marine invertebrates. Brominated amino acid derivatives are present in complex structures, such as cyclic

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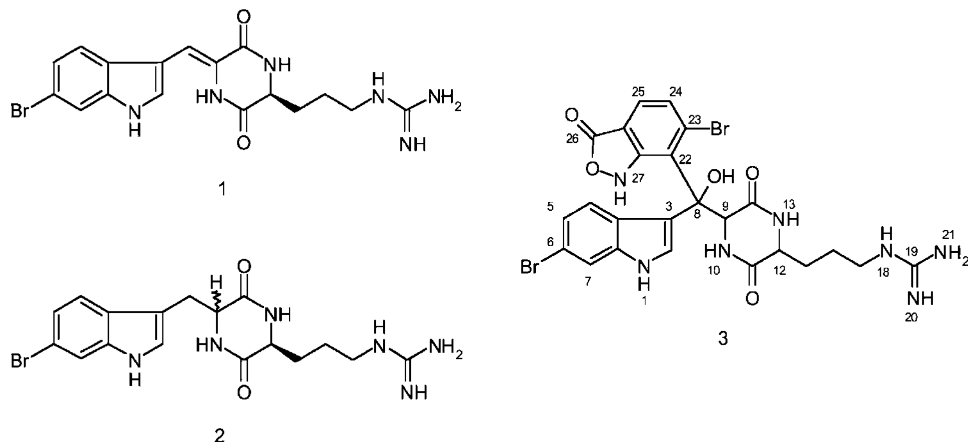

Figure 1. Chemical structures of barettin (**1**), 8,9-dihydrobarettin (**2**), and bromobenzisoxazolone barettin (**3**).

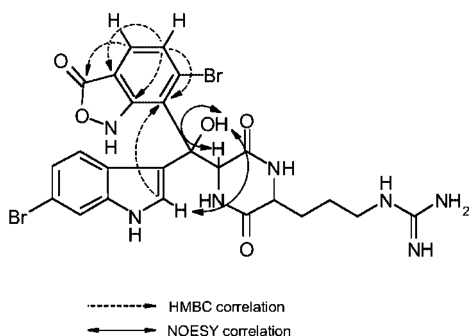

Figure 2. Key long-range NMR correlations of bromobenzisoxazolone barettin (**3**) in DMSO-d_6.

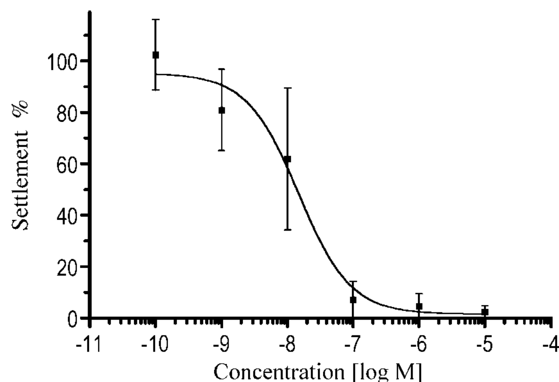

Figure 3. Dose–response inhibition of bromobenzisoxazolone barettin (**3**) on the settlement of cyprid larvae of *B. improvisus*. Data points represent the mean ± SE ($n = 4$).

peptides and indole alkaloids.[13] Several bromotryptophan (BrTrp)-containing compounds have been reported with pharmacological and biological activities. Specifically, the 6-BrTrp unit is present in many bioactive marine peptides, such as the conotoxins and the styelins.[14,15] A tryptophan residue in α-conotoxin has been reported to be modified post-translationally.[16] The barettins discussed in this paper are another group of biologically active marine peptides with 6-BrTrp moieties.

A specimen of the sponge *G. barretti* collected in Koster Fjord, Sweden, was defrosted, extracted repeatedly with a mixture of CH_3CN and H_2O (15:85), and desalted with RP-SPE. The concentrated extract was fractionated on RP-HPLC and screened by ESIMS for traces of brominated compounds. Fraction 36 was found to contain a minor constituent with a 1:2:1 dibrominated isotope cluster and was further purified by analytical RP-HPLC to finally yield metabolite **3**.

NMR and MS analysis showed that compounds **1** and **3** are closely related and, hence, could derive from a similar biosynthetic pathway. Both compounds consist of arginine and tryptophan, which form a diketopiperazine-type cyclic peptide backbone, and **3** is further substituted at position C-8.[5] The ^{1}H NMR spectrum revealed a broad signal at 8.41, which is consistent with a hydroxy group with NOESY correlations to H-2 and H-9. HMBC correlation $^4J_{C\text{-}22,H\text{-}2}$ indicated that position C-8 is further substituted. Two aromatic doublets (δ_H 6.78 and 7.36; $J = 8.6$ Hz) had correlations in HMBC, $^3J_{C\text{-}22,H\text{-}24}$, $^3J_{C\text{-}25a,H\text{-}24}$, $^3J_{C\text{-}26,H\text{-}25}$, $^4J_{C\text{-}27a,H\text{-}22}$, indicating a heterocycle at C-25a/C-27a and a bromine substituent in position C-23. The HRFABMS of **3** (*m/z* 648.0214 [M + H]$^+$, calcd for $C_{24}H_{24}O_5N_7{}^{79}Br_2$, 648.0206) supported the molecular formula $C_{24}H_{24}O_5N_7{}^{79}Br_2$. The ESIMS isotope cluster confirmed the presence of two bromines. N−H bonds were confirmed by ^{1}H NMR at 323 K, where all N−H bonds shifted upfield. NMR data are listed in Table 1. Key HMBC and NOESY correlations are illustrated in Figure 2. The heterocycle side chain skeleton is almost identical to the benzisoxazolones first reported by Wierenga.[17] Benzisoxazolones are modestly cytotoxic against L1210 leukemia cells and show Gram-negative and Gram-positive antibacterial activity.[17]

Many secondary metabolites produced in sponges are likely involved in the chemical defense against foulers and predators. Compounds **1** and **2** have been confirmed to inhibit settlement of cyprids with EC_{50} values of 0.9 and 7.9 μM, respectively,[5] and so metabolite **3** was tested for its effect on settlement of competent barnacle larvae (*B. improvisus*). Compound **3** inhibited cyprids in a dose-dependent manner (Figure 3), and the EC_{50} value was determined as 15 nM, which is approximately 60 times more potent than the inhibition by **1**. This enhanced potency is likely due to the brominated benzisoxazolone side chain. The settlement inhibition was found to be reversible. Cyprids exposed to **3** (10 μM for 48 h) metamorphosed to an extent similar to the control dishes when washed and transferred to fresh seawater (48 h) (Figure 4).

Recently, we investigated the affinity of **1** and **2** against serotonin receptors based on structural resemblance to 5-HT and other potent serotonin receptor ligands.[11] Interestingly, **3** failed to interact with the serotonin receptors 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 (data not shown). This was somewhat unexpected since **1** had affinity to 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 and **2** to 5-HT_4 at concentrations below 10 μM.[11] The brominated benzisoxazolone side chain is a relatively large substituent, and on the basis of our investigations it is plausible that it obstructs the binding site of the serotonin receptor, which prevents the molecule from docking at the receptor.

G. barretti has proven to be a rich source of bioactive secondary metabolites. Our results suggest that **3** is part of the sponge's

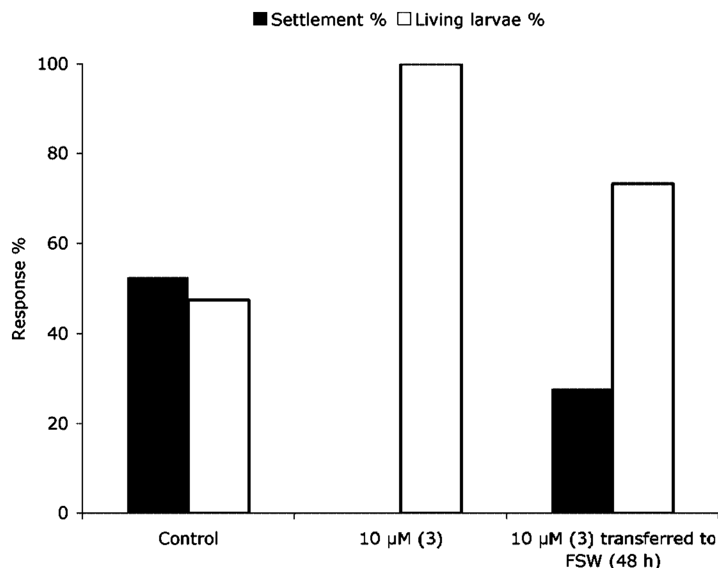

Figure 4. . Reversibility of 10 μM bromobenzisoxazolone barettin (**3**) before and after 48 h when the cyprids were transferred to filtered seawater (FSW).

chemical defense and that it may even have additional targets rather than just the cyprid larvae of *B. improvisus*. The structurally related **1** selectively targets serotonin receptors 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4,[11] whereas **3** does not show any interaction with these serotonin subreceptors. Therefore, we hypothesize that serotonin receptors may be the targets of **1** and **2** but that this is not the case for **3**. Further studies on the functional role and the molecular targets are required to establish the mode of action of these brominated cyclopeptides.

Compounds **1** and **2** have successfully been incorporated in antifouling paints and have shown effects in field tests.[10] There is a need for further studies in order to establish the effect of **3** against common fouling organisms in the natural environment and to determine its potential as a paint additive. It should, however, be noted that **3** is present in *G. barretti* in minute amounts; the yield in the present study was less than 0.1%, implying that the sponge would not be a viable source for scaled-up experiments. The production of **1**, **2**, and **3** is likely performed by a microbial symbiont in *G. barretti*, since sponges lack the shikimic acid pathway and are therefore unable to produce the tryptophan that makes up the cyclic peptide backbone. To provide sufficient quantities of **3**, the next logical step is to synthesize the compound, an approach that has been successful for **1** and **2**.[9] Synthetic studies are currently in progress that will also address the stereochemistry of **3**. The activity of **3** is remarkably potent when compared to **1** and **2**; this high potency, along with the lack of observed toxicity, means that **3** may be an attractive candidate as an active ingredient in marine paints.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter using a 10 cm cell. IR spectra were recorded on a Varian 1000 FT-IR. ^{1}H NMR data were obtained on a Bruker DRX-600 (600 MHz) in DMSO-d_6 and MeOH-d_4. ^{13}C NMR data were obtained on a Bruker DRX-600 in DMSO-d_6 (151 MHz) and MeOH-d_4 (100 MHz). Spectra were referenced to residual solvent signals with resonances at δH/C 2.50/39.5 (DMSO-d_6) and δH/C 3.31/49.0 (MeOH-d_4). Delay time was 120 ms for HMBC and 300 ms for NOESY. ESIMS was obtained using a nanospray ion trap MS on a LCQ (Thermo Finnigan, San Jose, CA), and ESIMS^2 was obtained using nanospray MS (Protana's NanoES source [MDS Protana A/S, Odense, Denmark]) mounted on the same LCQ. HRFABMS was obtained using a JEOL JMS-SX102 spectrometer. A Shimadzu LC-10 system was used for preparative and analytical RP-HPLC, equipped with a SPD-M10Avp photodiode array detector. UV data were collected between 200 and 320 nm. Quantitative amino acid analysis was performed at the Amino Acid Analysis Centre, Department of Biochemistry, Uppsala University.

Extraction and Isolation. A sponge specimen of *Geodia barretti* Bowerbank (1617.6 g wet weight) was collected by dredging at a depth of 50 m in Koster Fjord on the Swedish west coast (58°53′ N, 11°8′ E) in March 2001. It was homogenized and extracted repeatedly with 15% CH_3CN. The extract was then subjected to reversed-phase solid-phase extraction (RP-SPE), and the captured compounds were eluted with CH_3CN (60%) for concentration and desalting (Isolute C18 EC, International Sorbent Technologies, Mid Glamorgan, UK). The extract was diluted to 10% CH_3CN, 0.05% TFA before being loaded onto a ReproSil-Pur C18-AQ column (20 × 250 mm, 5 μm, 300 Å) and subjected to RP-HPLC (Shimadzu SCL-10A, 5 mL/min). A linear gradient elution from 0 to 100% B (A: 10% CH_3CN, 0.05% TFA; B: 90% CH_3CN, 0.045% TFA) over 90 min produced 90 fractions. The separation was monitored at UV 200–320 nm. ESIMS profiling indicated that fraction 36 contained a compound with a dibrominated isotope cluster, and this was selected for further isolation. Purification was performed on RP-HPLC (Shimadzu SCL-10A, 1 mL/min; Vydac RP-C18, 4.6 × 150 mm, 300 Å) and yielded compound **3** (3.2 mg).

Bromobenzisoxazolone barettin: brownish-yellow solid; $[\alpha]^{25}_D$ +0.011 (*c* 0.01, MeOH); mp 150–151 °C; UV (CH_3CN) λ_{max} 198, 229, 265, 348 nm; IR (ATR, diamond) 3347, 2925, 1672, 1435, 1181, 1132, 1062, 841, 802, 723 cm^{-1}; ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 1; HRMS (FAB+) *m/z* 648.0214 $[M + H]^+$ (calcd for $C_{24}H_{24}O_5N_7{}^{79}Br_2$, 648.0206).

Serotonin Receptor Affinity. Membranes were prepared from human embryonic kidney-293 cells (HEK-293) transfected with human serotonin receptors. Serotonin receptor subtypes 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 were prepared from a glycerol stock (Guthrie, Sayre, PA). For experimental procedures, see our previous publication.[11]

Larval Bioassay. Larval rearing and cyprid preparation was conducted as previously described.[5] The larval bioassay was prepared using polystyrene Petri dishes (Ø = 48 mm, Nunc AS Denmark, no. 240045). Bromobenzisoxazolone barettin was dissolved in 10 mL of filtered seawater (0.2 μm) in the following concentrations: 10 μM, 1 μM, 0.1 μM, 10 nM, 1 nM, and 0.1 nM. Competent cyprids (20 ± 2 individuals) were added to each dish ($n = 4$). Petri dishes with filtered seawater served as controls. The dishes were maintained for 5 days at room temperature and then investigated under a stereomicroscope for attached/metamorphosed individuals and nonattached individuals.

Reversible Effect of Bromobenzisoxazolone Barettin. A total of 40 cyprids were exposed to bromobenzisoxazolone barettin at a concentration of 10 μM ($n = 3$). After 48 h, 20 cyprids were washed and transferred to fresh saltwater. The dishes were maintained for 5 days and then investigated for attached/metamorphosed individuals and nonattached individuals.

Statistical Analysis. Antifouling data were analyzed with nonlinear regression analysis using GraphPad Prism 4 (GraphPad Software, San Diego, CA).

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