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Two Brominated Cyclic Dipeptides Released by the Coldwater Marine Sponge *Geodia barretti* Act in Synergy As Chemical Defense

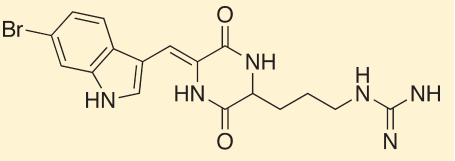
Martin Sjögren,[†] Per R. Jonsson,[‡] Mia Dahlström,[§] Tomas Lundälv,[‡] Robert Burman,[†] Ulf Göransson,[†] and Lars Bohlin*[,†]

[†]Division of Pharmacognosy, Department of Medicinal Chemistry, Biomedical Center, Uppsala University, Box 574, SE-751 23 Uppsala, Sweden

[‡]Department of Marine Ecology, Tjärnö Marine Biological Laboratory, University of Gothenburg, SE- 452 96 Strömstad, Sweden

[§]Swedish Board of Fisheries, Ekelundsgatan 1, Box 423, SE-401 26 Göteborg, Sweden

ABSTRACT: The current work shows that two structurally similar cyclodipeptides, barettin (**1**) and 8,9-dihydrobarettin (**2**), produced by the coldwater marine sponge *Geodia barretti* Bowerbank act in synergy to deter larvae of surface settlers and may also be involved in defense against grazers. Previously, **1** and **2** were demonstrated to bind specifically to serotonergic 5-HT receptors. It may be suggested that chemical defense in *G. barretti* involves a synergistic action where one of the molecular targets is a 5-HT receptor. A mixture of **1** and **2** lowered the EC_{50} of larval settlement as compared to the calculated theoretical additive effect of the two compounds. Moreover, an in situ sampling at 120 m depth using a remotely operated vehicle revealed that the sponge releases these two compounds to the ambient water. Thus, it is suggested that the synergistic action of **1** and **2** may benefit the sponge by reducing the expenditure of continuous production and release of its chemical defense substances. Furthermore, a synergistic action between structurally closely related compounds produced by the same bioenzymatic machinery ought to be the most energy effective for the organism and, thus, is more common than synergy between structurally indistinct compounds.



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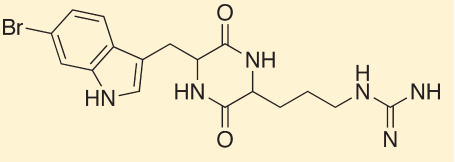

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Sessile organisms have developed different means of defending themselves from predators, grazers, or fouling epibionts to compensate for their lack of motility and an inability to escape. Morphological defense mechanisms in the form of spines, spicules, or barbs are common both in the terrestrial, e.g., ref 1, and in the marine environment, and calcified body armor is typical of many marine invertebrates, e.g., refs 2 and 3. Alternatively, attacks from predators or settling foulers may be avoided by means of a chemical defense, so that the organism produces and releases deterrant or poisonous molecules that target vital functions in the primary metabolism of the attacker.[4,5] There are, however, expenditures associated with the production of an efficient chemical defense since both necessary chemical pathways and the defense compounds require primarily carbon, nitrogen, and phosphorus that otherwise are available for growth and reproduction.[6,7] Therefore, strategies to reduce such biochemical costs are suggested to have evolved, and, for example, many enzymatic pathways responsible for the production of defense metabolites are highly inducible by different threats,[8,9] so that less energy is used than for a continuous release of defensive compounds.[10,11] A proposed but hitherto poorly documented strategy to reduce the cost of a chemical defense is the use of two or more compounds acting in concert to achieve the desired effect at lower concentrations than either substance alone.[11] Such synergistic effects of secondary metabolites recently have been proposed to constitute a major evolutionary driving force in organisms with multiple secondary metabolite production.[12]

In the cold waters of the North Atlantic, large areas of the seabed are covered with sponge reefs, of which some may be several thousand years old.[13] The larger sponges in these fields, mainly *Geodia barretti* Bowerbank, *Geodia macandrewii* Bowerbank, *Isops phlegraei* Sollas, and *Geodia atlantica* Stephens, are long-lived and slow-growing and form a complex three-dimensional deep-sea habitat similar to coral reefs. It is well known that these sponge fields are highly important biodiversity hot-spots,[14] which may serve as refuges as well as hunting grounds for a number of other species including commercially important fish species.[15] Despite their role as key species in deep-sea communities, little is known of the ecology, physiology, or chemistry of these sponges.

Both *G. barretti* and *G. macandrewii* have a remarkably clean and undamaged body surface despite their inability to escape potential predators or fouling organisms.[16] Marine sponges are known to produce an impressive diversity of secondary metabolites,[17] and chemical defense against predators and

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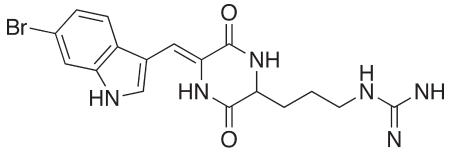
foulers has been proposed as one adaptive function of these compounds.[18] Several studies also show that extracts from sponges may deter predators, e.g., ref 19, and foulers, e.g., ref 20, although the active compounds have rarely been identified. Recently our group isolated two congeneric cyclodipeptides from *G. baretti*,[21] i.e., barettin (**1**) and 8,9-dihydrobarettin (**2**), guided by their ability to inhibit larval settlement of the cosmopolitan barnacle *Balanus improvisus* Darwin.[20] It was found that the barettins inhibit larval settlement in a dose-dependent manner with EC_{50} values of 0.9 μM (**1**) and 8 μM (**2**). We further demonstrated that the barettins bind to mammalian serotonergic receptors,[22] suggesting an action through a specific molecular target in foulers and predators.

In the present study, the hypothesis was tested that *G. barretti* can aquire a more efficient chemical defense by excreting structurally related compounds that together act in synergy to inhibit the larvae of surface foulers. This hypothesis was tested by combining three studies: (1) quantifying a possible synergistic effect of the two compounds **1** and **2** in a larval settlement assay; (2) measuring the relative release of **1** and **2** in the laboratory; and (3) measuring the relative release of barettin (**1**) and 8,9-dihydrobarettin (**2**) in situ from *G. barretti* in its natural habitat at 120 m depth. We also discuss the possibility of **1** and **2** being involved in defense against grazers based on a prey-choice experiment with hermit crabs.



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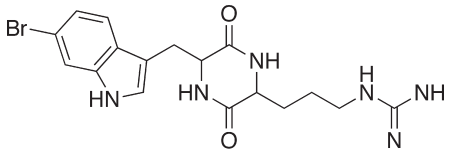

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RESULTS AND DISCUSSION

Test for Synergistic Action of Barettin (1) and 8,9-Dihydrobarettin (2) against Settling Barnacle Larvae. Figure 1A and Table 1 show the effects on larval settlement using different combinations of **1** and **2**. When tested alone, only the highest concentration of **1** showed activity as compared to the control (fresh seawater). Estimates of the reduction in settlement for each compound alone and when combined are given in Table 1. In all the treatments where a mixture of the compounds **1** and **2** was examined, a significant settlement inhibition was detected as compared to the effect of either compound alone, excluding the treatment where the highest concentration of **1** was used. That concentration alone (2.4 μM), as well as in the mixture, resulted in complete settlement inhibition. Furthermore, all the mixture treatments gave rise to a significant settlement reduction as compared to filtered sea water (FSW) controls and to all concentrations of 8,9-dihydrobarettin alone.

To further illustrate the synergistic effects of **1** and **2**, the results are also presented in an isobologram, shown in Figure 1B.

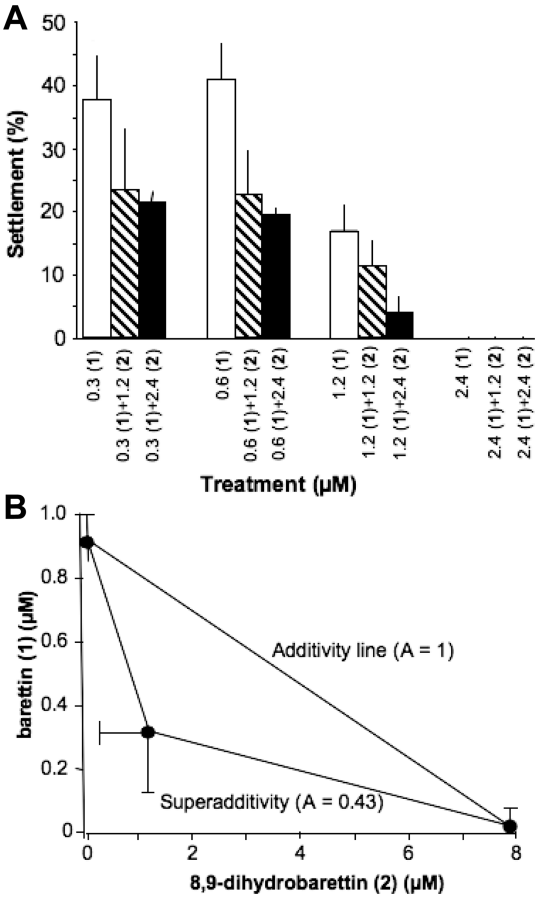

Figure 1. (A) Effects of different ratios of barettin (**1**) and 8,9-dihydrobarettin (**2**) on settling of barnacle larvae. Four different concentrations of **1** (from left to right 0.3, 0.6, 1.2, and 2.4 μM) were combined with two concentrations of **2** (1.2 and 2.4 μM). (B) Isobologram of the 50% larval settlement inhibition for the combination of the compounds barettin (**1**) and 8,9-dihydrobarettin (**2**). The concentration combination between the compounds that gave 50% effect was located under the line of additivity showing a superadditive (synergistic) effect.

In the plot the doses of the individual compounds required to generate 50% effect were plotted as points on the axes of a Cartesian plot. The straight line connecting the two individual EC_{50} values is the locus of points that will produce an additive effect. This line of additivity allows comparison with the actual dose pair that produces this effect level experimentally. It should be noted that dose combinations that deviate from the line are either subadditive (antagonistic) or superadditive (synergistic).[23] Of the mixtures tested, the lowest concentrations yielding 50% effect were 0.3 μM **1** and 1.2 μM **2**; hence they are plotted in the isobologram.

Ratio and Rate of Release of Barettin (1) and 8,9-Dihydrobarettin (2) under Laboratory Conditions and in the Field. To test if the sponge itself exploits the evident synergistic effect between **1** and **2**, the ratio and rates of release of these two compounds were assayed by LC-MS. First, a specimen of *G. barretti* was held at the laboratory in an aquarium: over a 3 h period, it released an average quantity of 2.8 ng of barettin (**1**) and 0.375 ng of 8,9-dihydrobarettin (**2**), giving a ratio of 7:1. Then, the release in situ in the sea close to the sponge in its natural habitat at the sea bottom (at a depth of 120 m) was

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Table 1. Effects of Different Mixtures of Barettin (1) and 8,9-Dihydrobarettin (2) on Cyprid Settlement

mixture of **1** and **2** (μM)	reduction of settlement mixture vs **1** alone	reduction of settlement mixture vs **2** alone[b]	reduction of settlement of **1** vs FSW controls
0.3:1.2	48% ($F_{1,15}$ = 5.6, p = 0.032)	49% ($F_{1,15}$ = 6.0, p = 0.027)	no reduction
0.3:2.4	52% ($F_{1,15}$ = 7.55, p = 0.015)	53% ($F_{1,15}$ = 8.0, p = 0.013)	no reduction
0.6:1.2	57% ($F_{1,15}$ = 12.6, p = 0.003)	50% ($F_{1,15}$ = 7.6, p = 0.015)	no reduction
0.6:2.4	63% ($F_{1,15}$ = 14.1, p = 0.002)	58% ($F_{1,15}$ = 9.0, p = 0.009)	no reduction
1.2:1.2	58% ($F_{1,15}$ = 5.0, p = 0.04)	75% ($F_{1,15}$ = 24.0, p = 0.0002)	43% ($F_{1,15}$ = 8.44, p = 0.01)
1.2:2.4	85% ($F_{1,15}$ = 7.5, p = 0.015)	91% ($F_{1,15}$ = 24.4, p = 0.0002)	43% ($F_{1,15}$ = 5.9, p = 0.02)
2.4:2.4	0%[a]	100% ($F_{1,15}$ = 30.5, p = 0.0001)	100% ($F_{1,15}$ = 33, p = 0.0001)

[a] Both the mixture and **1** alone gave rise to 100% inhibition of larval settlement. [b] None of the concentrations of **2** alone gave rise to any reduction in cyprid settlement as compared to FSW controls.

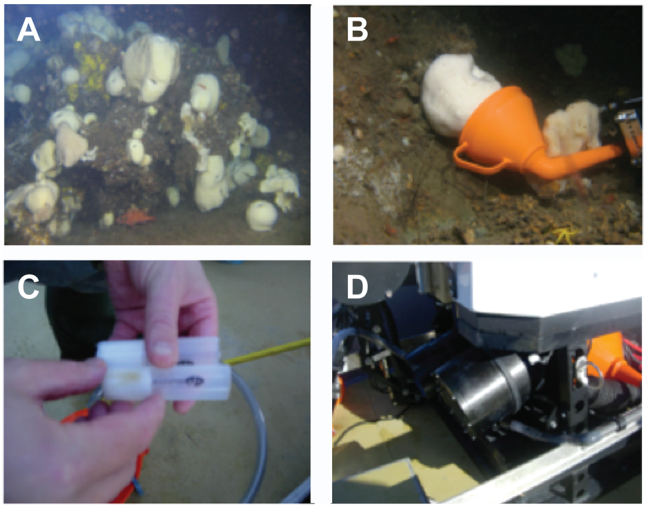

Figure 2. Photographs from the *G. barretti* expedition in the Kosterfjord. (A) Sponge reef of *G. barretti* at 120 m depth. (B) Sampling of compounds released into the surrounding water from a specimen of *G. barretti*. (C) Collected sample compared to an unused SPE column. (D) Position where the SPE column was rigged at the remotely operated vehicle (ROV).

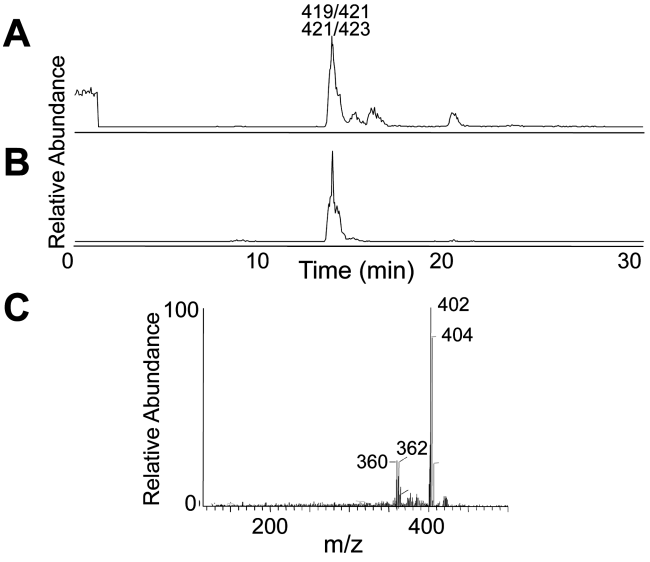

Figure 3. LC-MS and MS/MS analysis of barettins sampled in situ at the sea bottom by the ROV. The base peak ion (BPI) chromatogram (positive mode, m/z 150−1000) is shown in A. The barettins form the major peak, which is assigned by the ions for **1**: m/z 419/421 and (**2**) m/z 421/423. Their identity was confirmed by LC-MS/MS analysis in B, which display the BPI chromatogram of ions between m/z 359 and 363, which are characteristic fragments for the loss of the guanidino group of the Arg residue. (C) Summarized MS/MS spectrum of the barettin (**1**) peak from the LC-MS/MS analysis. At the conditions used, the Z/E isomers of **1**[20] eluted in one peak.

measured using a remotely operated vehicle (ROV). The experimental setup is shown in Figure 2. The water collected in the field close to an individual of *G. barretti* at 120 m depth was analyzed using LC-MS and LC-MS/MS. The results of the analysis revealed that both **1** and **2** were present in the field sample (Figure 3). The amounts found in this sample were calculated from a MS standard curve. It was found that the field sampling collected 0.34 ng of **1** and 0.025 ng of **2**, or a 14:1 ratio.

Test of Deterrence of the Hermit Crab *Pagurus bernhardus*. Out of 12 tested hermit crabs, 10 chose a channel with a bait but without water passing through the head tank containing a single *G. barretti* sponge (one-tailed binomial test, p = 0.019), as shown in Table 2. The two hermit crabs that made for the bait flushed with water from the *G. barretti* head tank moved backward immediately when reaching the bait and made no attempt to feed from it. Analysis with LC-MS showed that the *G. barretti* used in the hermit crab experiment released 0.23 ng of **1** and 0.031 ng of **2** during a 20 min period, giving a release ratio of 7:1. No barettins (**1** and **2**) were detected from the empty head tank.

In the marine environment, sponges are unequaled producers of bioactive compounds.[5] One likely adaptive function of these compounds is as chemical defensive substances against foulers, predators, grazers, pathogens, and possibly some competitors

Table 2. Concentrations Released and Ratio of Barettin (1) and 8,9-Dihydrobarettin (2) in the Different Experiments

experiment	amount released of **1** (ng)	amount released of **2** (ng)	ratio of **1**:**2**
field (ROV)	0.34	0.025	14:1
aquarius (lab)	0.39	0.04	10:1
preference (hermit crab)	0.23	0.031	7:1

(allelopathy). Sponges are sedentary filter-feeders with a simple body structure made up by a limited number of cell types. They lack specialized tissues and, thus, have no possibility of concentrating defensive compounds in glands or tissues. Also, they lack vascular tissue for transportation of compounds from the site of production to the external pinacoderm. However, sponges do possess well-defined water-conducting canals, cavities, and

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choanocyte chambers,[24] which may, to some extent, regulate the concentration of defensive compounds inside the sponge. It is suggested that a constitutive defense by continuous release of defense compounds into the ambient water is costly for the producing organism. One alternative strategy is to rely on cues that can induce release (and/or production) of compounds only when defense is necessary, as in predator attacks or bursts of settling foulers.[9] At present, it is not known if production of defense compounds in sponges can be upregulated in the presence of threats, and, owing to their physiological limitations, these organisms may have to rely on a continuous release of bioactive compounds to ensure a potent and effective chemical defense. Another strategy to reduce the cost of a continuous production and release of bioactive compounds is the use of a multiple chemical defense with compounds that act in synergy to ward off potential attackers.[12,25] In the present study, it was shown that *G. barretti* releases into its natural environment two defensive cyclic dipeptides, barettin (**1**) and 8,9-dihydrobarettin (**2**), and that in laboratory assays these two compounds act in synergy to prevent settlement of fouling larvae albeit at different ratios. Sponges are particularly sensitive to fouling of their pinacoderm where the water enters the sponge. Since settling attempts by larvae probably are numerous and unpredictable over time, it may be necessary to prevent fouling with a continuous release of defense compounds. A defense compound may act when larvae are exploring the surface of the sponge and either repel, anesthetize, or kill the organism. We also show that *G. barretti* deters the approach of hermit crabs that potentially could graze on sponge tissue. Although the present experiments cannot link deterrence specifically and exclusively to compounds **1** and **2**, it may be speculated that these compounds may also be involved in defense against grazers. It is known that very few grazers will attack *G. barretti*, and only some specialist chitons may be able to feed on this sponge.[26]

A synergistic action of two or more defense compounds requires that the compounds interact with different molecular targets. In turn, that implies that two chemically distinct compounds are required. However, similar chemical structures may interact with different molecular targets; for example, dopamine and noradrenaline bind to different G-protein-coupled receptor families, but the only difference in the two is an additional hydroxy group in noradrenaline. A certain degree of cross-reactivity is found, but in essence, the two have different molecular targets and evoke different molecular responses when interacting with these targets. Where barettin (**1**) and 8,9-dihydrobarettin (**2**) are concerned, the only difference in chemical structure is a double bond in the tryptophan residue on **1**. We have also found that **1** specifically interacts with 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 receptors with the corresponding K_i values being 1.93, 0.34, and 1.91 μM, respectively.[22] Barettin (**1**) interacts with these receptors at concentrations close to that of endogenous serotonin, while 8,9-dihydrobarettin (**2**) interacted only with the 5-HT_{2C} receptor, with a K_i value of 4.63 μM. It is therefore suggested that one of the molecular targets in the synergistic action of **1** and **2** is a 5-HT_2 receptor and that **1** is the primary ligand. We further suggest that **2** has an additional molecular target, apart from the cross-reactivitiy displayed with the interaction to the 5-HT_{2C} receptor, which remains to be elucidated and that mediates the synergistic action shown. Previous studies have found serotonin in barnacle cyprids[27] and serotonin-like immunoreactive neuron cell bodies have also been described from the central nervous system in cyprids.[28] In an extensive study it was shown that a number of serotonin antagonists inhibited attachment, preventing the cyprids from settling.[27] The most effective substance, cyproheptadine, is a known 5-HT_2 antagonist and inhibited settlement at concentrations above 0.1 to 1 μM. This corresponds very well to the activity and receptor interactions[22] of the barrettins and suggests that 5-HT receptors in cyprids may indeed be a target for the barrettins produced by *G. barretti*. Furthermore, it has been shown that the intracellular titer of cAMP was positively correlated to the propensity to settle in barnacle larvae.[29] It is known that release of intracellular cAMP is stimulated by 5HT-4 and by 5HT-2 in, for example, rats.[30,31] The suggested blocking of 5HT-2 and 5HT-4 by the barrettins may thus be linked to the cAMP signaling pathway affecting banacle settlement, although the link between 5HT and cAMP release has not, to our knowledge, yet been shown in barnacles.

It may be speculated that this type of chemical defense exploiting a nonlethal mechanism is favored in the evolutionary arms race. The suppression of larval settlement at the sponge epidermis (pinacoderm) allows larvae to settle elsewhere, while the production of a lethal defense compound would cause strong natural selection for tolerance, which could lead to rapid evolution to evade the defense.

The production of structurally similar compounds within the same pathway where "promiscous" enzymes[32] may transform different substrates into a diversity of similar compounds with different bioactivities may be an efficient way to acquire multiple defenses and synergistic action.[25]

EXPERIMENTAL SECTION

General Experimental Procedures. To detect and quantify barettin (**1**) and 8,9-dihydrobarettin (**2**), LC-MS and LC-MS/MS methods were set up using a LCQ ion-trap MS (Thermo Finnigan, San Jose, CA). Samples were analyzed in the positive-ion mode. The spray voltage was set to 4.5 kV and the capillary temperature to 220 °C. For MS/MS the CID was set at 35%. An Äkta Basic HPLC equipped with a 2.1 mm (i.d.) × 150 mm C_{18} column was used for the separation and operated with a 30 min linear gradient from 10% to 60% MeCN in 0.1% HCOOH. The analysis revealed peaks at m/z 419/421 and 421/423, representing the $[M + H]^+$ ions of barettin (**1**) and 8,9-dihydrobarettin (**2**), respectively.

Settlement inhibition assays using cypris larvae *of B. improvisus* were performed as outlined by ref 30. When using live specimens of *G. barretti* in laboratory experiments, the sponges were placed in aquaria after the experiments were finished to check for reattachment to the surface. All sponges showed reattachment and were considered to have been in good condition during the experiments.

Test for Synergistic Action of Barettin (1) and 8,9-Dihydrobarettin (2) against Settling Barnacle Larvae. Compounds **1** and **2** were isolated from *G. baretti* as previously described.[20] The compounds (as trifluoro acetic acid salts) were stored at −20 °C until used. Stock solutions were prepared by dissolving the compounds in dimethylsulfoxide (DMSO) prior to each experiment. The stock solutions were then further diluted in filtered seawater (FSW, Millipore 0.2 μm) to give the desired concentrations of 0.3, 0.6, and 1.2 μM (**1**) and 1.2 and 2.4 μM (**2**). Settlement experiments were conducted including a concentration series of 0.3, 0.6, and 1.2 μM of **1** prepared in polystyrene dishes (0.3 and 0.6 μM have in earlier assays not shown any settlement inhibition activity) to which 1.2 or 2.4 μM of **2** was added. Settlement assays were performed using Petri dishes of untreated polystyrene (Nunc no. 240045, | 48 mm) containing 10 mL of FSW to which 20 ± 2 cyprids were added. Cyprids were used on their first or second day

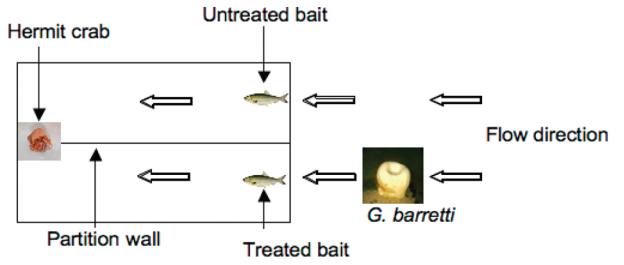

Figure 4. Experimental setup for test of deterrence on the hermit crab *Pagurus bernhardus*. Two head tanks containing either the sponge *G. barretti* or only seawater are connected to the flow-through aquarium. The flow-through aquarium contains two lanes partly separated by a wall. At the upstream end baits (*Sprattus sprattus*) are fixed close to the pipes from the head tanks. Hermit crabs were released at the downstream end and were allowed to choose between the two lanes.

after molting. Each treatment was replicated four times, and dishes with FSW only or FSW containing DMSO (0.1%) served as controls. As a negative control, dishes with one or two in the respective concentration series were used. Dishes were maintained for 3−4 days at room temperature with a prevailing light:dark cycle of 9:15 h. The experiment was concluded by examining the dishes under a stereomicroscope and checking for (1) attached and metamorphosed individuals, (2) non-metamorphosed, alive, nonattached cyprids, and (3) dead cyprids. Each treatment was tested in at least two independent experimental series. The data were further analyzed using an isobologram as outlined by ref 23.

In Situ Measurement of Release of Barettin (1) and 8,9-Dihydrobarettin (2) Using a ROV. To measure the release of **1** and **2** from a *G. barretti* in its natural environment, sponge field sampling was conducted in the Koster Fjord area (59°03′08″ N, 11°08′26″ E). The sampling took place at 120 m depth using a Sperre SubFighter (ROV). An RP-SPE column (Isolute C_{18}, 1 g) was fitted to the ROV to enable the in situ capture of the barettins (**1** and **2**) around a *G. barretti* sponge. Using a videocamera, the sponge was monitored during sampling, and it was ensured that it was not touched or otherwise damaged or disturbed by the ROV. A water sample (1 L) was collected at a distance of 4−5 cm from the sponge body at a flow rate of 0.1 L min^{-1} and led through the RP-SPE column. After the ROV had been lifted up on deck the column was removed and placed on ice. The column was then brought to the laboratory, where it was immediately desalted with three column volumes of MQ-water and three volumes of 10% MeCN. Thereafter, the barettins were eluted with 60% MeCN. The eluate was further analyzed with LC-MS and MS/MS. The amount of released barettin (**1**) and 8,9-dihydrobarettin (**2**) was then calculated using LC-MS/MS: a standard curve was constructed using isolated barettins (**1** and **2**) and the fragment ions of *m/z* 360 (**1**) and *m/z* 362 (**2**).

Test of Deterrence of the Hermit Crab *Pagurus bernhardus*. To broaden the discussion about possible adaptive significance of a synergy between barettin (**1**) and 8,9-dihydrobarettin (**2**), it was also tested if *G. barretti* may deter potential grazers, such as the hermit crab *Pagurus bernhardus* L. Due to experimental constraints, this experiment tested only if the presence of *G. barretti* will deter hermit crabs not the actual compounds responsible for such a response. In the first experiment, hermit crabs were allowed to choose between two channels in a flow-through aquarium where bait (a dead herring) was fixed at the upstream end of each channel (Figure 4). Filtered seawater (30 μm, 32 psu) entered both channels through a plastic tube (Ø = 0.5 cm). Before entering the flow-through aquarium, seawater first passed through two smaller aquaria: one empty and one with a living specimen of *G. barretti* (weight 0.7 kg). Hermit crabs were started at the downstream end of the flow-through aquarium and could choose either the channel fed only seawater or the channel fed seawater from a head tank containing a *G. barretti*. After each choice experiment, the head tank with *G. barretti* was disconnected from the flowing system and seawater without a *G. barretti* was allowed to flush the system for 5 min before the next choice experiment. The channel receiving water from the *G. barretti*-containing head tank was shifted between every test. This choice experiment was repeated with 12 hermit crabs. During the experiment, three replicates of 1 L of seawater from the two head tanks, with and without a *G. barretti* specimen, were flushed trough a SPE column (Isolute C_{18}, 1 g). The columns were then desalted with three column volumes of MQ-water and three volumes of 10% MeCN, and the barettins were eluted with 60% MeCN. The eluted samples were further analyzed with LC-MS and MS/MS, as described above.

Statistical Analysis. Results are reported as means ± SE where not otherwise stated. Effects of the studied treatments were tested in a one-factor analysis of variance (ANOVA) with dose as a fixed factor. Effects of treatments were tested against controls with means comparison contrasts (MCC). Data were checked for homoscedasticity using Cochran's test prior to the ANOVA.[31] In cases where data displayed heterogeneity of variances the data were square-root-transformed. For all tests, a type I error rate (α) of 0.05 was used.

AUTHOR INFORMATION

Corresponding Author

*Tel: +46 18 471 44 92. Fax: +46 18 50 91 01. E-mail: lars.bohlin@fkog.uu.se.

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DEDICATION

Dedicated to Dr. Koji Nakanishi of Columbia University for his pioneering work on bioactive natural products.

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