

## MULTIPLE DEFENSIVE ROLES FOR BROMOPYRROLE ALKALOIDS FROM CARIBBEAN *AGELAS* SPONGES

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### ABSTRACT

Previous studies have indicated that Caribbean reef sponges of the common genus *Agelas* are chemically defended from fish predators by brominated pyrrole alkaloids: *Agelas clathrodes* and *A. wiedenmayeri* by 4,5-dibromopyrrole-2-carboxylic acid (**1**) and oroidin (**2**), *A. conifera* by sceptrin (**3**). In this study, we expand our understanding of chemical defense in this sponge genus to include an extensive HPLC quantification analysis of the defensive metabolites in tissues of *A. cerebrum*, *A. cervicornis*, *A. dilatata*, *A. dispar* and *A. sceptrum*. *A. cervicornis* and *A. dispar* contained the same two major metabolites as *A. clathrodes* and *A. wiedenmayeri*, while *A. cerebrum*, *A. dilatata* and *A. sceptrum* contained a mixture of dimeric bromopyrrole alkaloids dominated by sceptrin, similar to *A. conifera*. At natural volumetric concentrations, both crude extracts and purified compounds from each species were unpalatable to a common generalist reef fish, *Thalassoma bifasciatum*, in aquarium assays, and inhibited attachment of the marine bacterium *Vibrio harveyi* in surface fouling assays. Brominated pyrrole alkaloids may play multiple ecological roles in protecting sponges of the genus *Agelas*.

### KEY WORDS

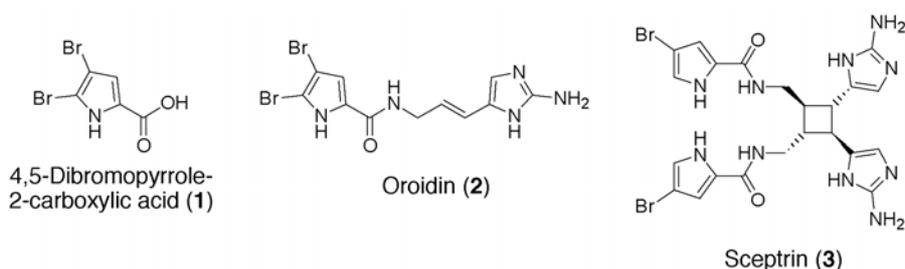
Chemical defense, *Agelas*, bromopyrrole alkaloids, predation, antifouling, antimicrobial.

### INTRODUCTION

Despite decades of natural products isolation and structure elucidation, little is known about the ecological functions of secondary metabolites from marine sponges (PAWLIK, 1993). Sponges are soft-bodied, sessile, and appear to be physically vulnerable to predation in an environment noted for intense grazing activity by fishes. Nevertheless, very few fish species are known to feed on Caribbean sponges (RANDALL & HARTMAN, 1968). Only recently have ecologically relevant methods been used to test whether sponge secondary metabolites deter consumption by predatory fishes (e.g. DUFFY & PAUL, 1992; PENNINGNS *et al.*, 1994; CHANAS *et al.*, 1996; HAY, 1996; WILSON *et al.*, 1999; ASSMANN *et al.*, 2000; KUBANEK *et al.*, 2002).

In a survey of the chemical antipredatory defenses of 73 species of Caribbean sponges (PAWLIK *et al.*, 1995), it has been discovered that all 6 species within the genus *Agelas* yielded crude organic extracts that strongly deterred the feeding of a predatory reef fish, *Thalassoma bifasciatum*, in aquarium assays. Subsequently, 4,5-dibromopyrrole-2-carboxylic acid (**1**) and oroidin (**2**) were identified as the principal fish antifeedant metabolites of *Agelas clathrodes*, present at concentrations of 1-5 mg/ml within the sponge tissue (CHANAS *et al.*, 1996). Marine sponges of the genus

*Agelas* (family Agelasidae) from around the world are well known to produce a variety of bromopyrrole alkaloids (reviewed in BRAEKMAN *et al.*, 1992; LINDEL *et al.*, 2000). Recently, we quantified the major and minor metabolites in the sponges *Agelas wiedenmayeri* and *A. confjera* (ASSMANN *et al.*, 2000), and determined that the major metabolites in *A. wiedenmayeri*, like *A. clathrodes*, are **1** and **2**, while *A. confjera* contains primarily sceptrin (**3**). Sceptrin also deters feeding by the common predatory reef fish *T. bifasciatum* in aquarium assays (ASSMANN *et al.*, 2000).



The conservation of brominated pyrrole alkaloids as metabolites in the tissues of *Agelas* sponges suggests that these compounds have been evolutionarily elaborated and retained as chemical defenses. The purpose of the study reported herein was to determine whether 5 additional species of *Agelas* common to the Caribbean (*A. cerebrum*, *A. cervicornis*, *A. dilatata*, *A. dispar*, and *A. sceptrum*) share the same or similar metabolites, and at what concentrations. To evaluate whether bromopyrrole alkaloids, which are responsible for feeding deterrence of predatory reef fish, have other ecological functions, as *e.g.* protection from fouling organisms, antimicrobial settlement assays were performed employing marine fouling bacteria.

## MATERIAL AND METHODS

This study was conducted over the course of 5 research expeditions: one at the National Undersea Research Center in Key Largo, Florida, USA, in May 1998, one on-board the RV 'Edwin Link' in September and October 1998, and three on-board the RV 'Seward Johnson', in July and August 1999 and 2000, and March 2001, all in the Bahamas, respectively. Collection and treatment of the sponge material prior extraction was performed as previously described (ASSMANN *et al.*, 2000). Sponges were identified on the basis of spicule and tissue preparations at the Zoölogisch Museum, Universiteit van Amsterdam, The Netherlands (ASSMANN *et al.*, 2001).

Isolation of brominated pyrrole alkaloids and preparation of crude organic extracts for quantification was achieved using previously described procedures (ASSMANN *et al.*, 1999, 2000). Analytical photo-diode-array RP-HPLC was used to quantify the major secondary metabolites (**1-3**) according to a previous established method (ASSMANN *et al.*, 2000). Each quantitative HPLC analysis based on peak area calibration was achieved using purified 4,5-dibromopyrrole-2-carboxylic acid (**1**) and oroidin (**2**) for HPLC profile type A sponges (*Agelas cervicornis*, *Agelas clathrodes*, *Agelas dispar*, *Agelas wiedenmayeri*) or sceptrin (**3**) for HPLC profile type B sponges (*Agelas cerebrum*, *Agelas confjera*, *Agelas dilatata*, *Agelas sceptrum*) as an external standard.

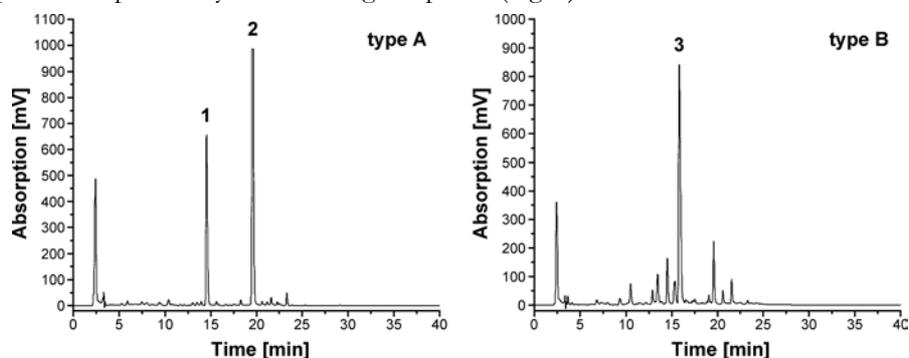
Antimicrobial settlement assays of purified bromopyrrole alkaloids **1**, **2** and **3** were performed on-board the RV 'Seward Johnson' in July/August 2000. An explanation of the

method for running and scoring the assay and statistical analysis, is well described in the literature (KUBANEK *et al.*, 2002; KELLY *et al.*, 2003).

Sponge overgrowth assays with crude extracts of *A. conifera* were conducted in Florida Bay, Key Largo, Florida, USA, in July 2000 using previously described methods regarding deployment, retrieval and statistical analyses (ENGEL & PAWLIK, 2000).

## RESULTS

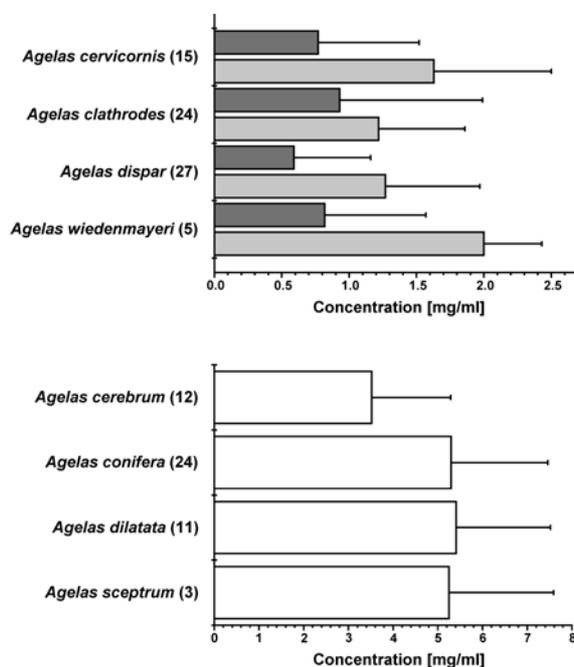
The fact that crude extracts of *Agelas cerebrum*, *A. cervicornis*, *A. clathrodes*, *A. dilatata*, *A. dispar* and *A. sceptrum* are strongly unpalatable in the feeding deterrence assays against *Thalassoma bifasciatum* is not surprisingly when comparing their HPLC profiles to previously described *Agelas* species (Fig. 1).



**Fig. 1.** HPLC profiles of the dichloromethane/methanol crude extracts of *Agelas clathrodes* (left, type A) and *Agelas conifera* (right, type B). The retention times for the major metabolites are as follows: oroidin (**1**) 14.52 min, 4,5-dibromopyrrole-2-carboxylic acid (**2**) 19.57 min in profile type A, sceptrin (**3**) 15.81 min in profile type B. HPLC conditions, column: Kromasil RP18, 4.6 x 250 mm, 5  $\mu$ m; gradient: 20-60 % acetonitrile/water + 0.1 % TFA in 40 min; flowrate: 1 ml/min, UV detection at 280 nm.

According to the HPLC analyses *A. cervicornis* and *A. dispar* can be arranged to the same HPLC profile type as *A. clathrodes* and *A. wiedenmayeri* (type A), whereas *A. cerebrum*, *A. dilatata* and *A. sceptrum* can be assigned to HPLC profile type B (as *A. conifera*). The chemistry within the species of HPLC profile type A (*A. cervicornis*, *A. clathrodes*, *A. dispar* and *A. wiedenmayeri*) is identical from a qualitative point of view as well as for the species within type B (*A. cerebrum*, *A. conifera*, *A. dilatata* and *A. sceptrum*).

Therefore, for all sponges several individuals (from 5 to 27 for type A and from 3 to 24 for type B, Fig. 2) were investigated and the content of the secondary metabolites was quantified for three replicate samples. The average concentrations of the major metabolites in type A specimens are similar for all four species: 1.2 to 2.0 mg/ml of **1** and 0.6 to 0.9 mg/ml of **2**. In type B specimens, the concentration of **3** is in average about 5 mg/ml, only in *A. cerebrum* it is 3.5 mg/ml (Fig. 2).

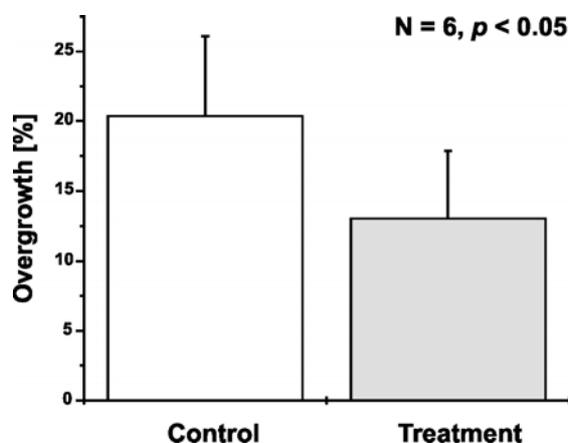


**Fig. 2.** Concentration of 4,5-dibromopyrrole-2-carboxylic acid (**1**) (light grey bar) and oroidin (**2**) (dark grey bar) top plot and of sceptrin (**3**) (white bar) below-mentioned plot, in dichloromethane/methanol crude extracts of *Agelas* spp. quantified by HPLC (Mean + 1 SD indicated). For each species the number of investigated individuals is shown in parentheses and the concentration per ml sponge tissue is given.

These results show no significant variation in the concentration of the secondary metabolites for the averaged values between different species of the investigated *Agelas* specimens. Whereas, the variation within the species is relatively large: for **1** from 0.5 to 3.7 mg/ml, for **2** from 0.1 to 3.6 mg/ml and for **3** from 1.6 to 8.8 mg/ml. The concentration of **3** in all type B specimens is always above the minimum concentration required for the feeding deterrence (0.9 mg/ml). Whereas for type A specimens the concentration of **1** and **2** is sometimes below the required concentration (0.8 mg/ml for **1** and 0.7 mg/ml for **2**). The results obtained for type A sponges do not show any compensation (*e.g.* a lower concentration of one metabolite is compensated by a higher concentration of the other metabolite). Because the feeding deterrence of type A sponges is probably caused by both secondary metabolites (**1** and **2**), mixtures of **1** and **2** were also tested for their feeding deterrence. The mixtures used are realistic values because the 70 samples of type A individuals served as standard. These results indicate that if the concentration of (**1** or **2**) or (**1** and **2**) is below the required concentration for feeding deterrence for the single compound, the mixture of **1** and **2** is still feeding deterrent. One tested mixture, with concentrations of 0.6 mg/ml for **1** and 0.3 mg/ml for **2** is still feeding deterrent, although the single concentrations are 0.2 (for **1**) or 0.4 mg/ml (for **2**) below the required concentration for feeding deterrence.

All three secondary metabolites were active in the settlement inhibition of fouling organisms at natural concentration. The natural concentration was determined as average for all concentrations obtained within each sponge type for the major metabolites: 1.5 mg/ml for **1**, 0.8 mg/ml for **2**, and 4.9 mg/ml for **3**. Scepttrin (**3**) inhibited the settlement of *Vibrio harveyi* by 97 % and by 59 % at half of the natural concentration, oroidin (**2**) by 73 % and 4,5-dibromopyrrole-2-carboxylic acid (**1**) by 65 %. For type A sponges the result of the 1:1 mixture of 4,5-dibromopyrrole-2-carboxylic acid (**1**) and oroidin (**2**) is of interest. The settlement is inhibited by 88 % using this mixture (this above the single results for **1** and **2**) which is reduced to 67 % at half of the natural concentration and to 44 % at one-fourth of the natural concentration. The settlement of two unknown fouling bacteria is inhibited by 97 % and 98 %, respectively, for scepttrin (**3**) at natural concentration. Although, an antimicrobial activity of **3** was already described by WALKER *et al.* in 1981, the investigations here were focused on microorganisms which play a potential ecological role (antifouling).

To examine the potential allelopathic function of brominated pyrrole alkaloids, natural concentrations of a crude extract of *A. conifera* were incorporated into gels and allowed to be overgrown by the sponge *Tedania ignis* in the field using the technique of ENGEL & PAWLIK (2000). In this assay, which was conducted in Florida Bay, Key Largo, Florida, USA, the overgrowth by *T. ignis* was significantly less on extract-treated gels relative to the overgrowth on control gels (Fig. 3). Crude extract of *A. conifera* inhibited overgrowth of *T. ignis*.



**Fig. 3.** Sponge overgrowth assay results using *Tedania ignis* as overgrowing sponge, and whole sponge volumetric concentration of crude extract from *Agelas conifera*. Percentage overgrowth was calculated by dividing the number of growth squares by the total number of squares, and multiplied by 100. A paired t-test, performed on arcsine-transformed data, was used to assess the significance of the difference in mean overgrowth of the sponge *Tedania ignis* over paired control gels and gels treated with the dichloromethane/methanol crude extract of the sponge *Agelas conifera* (Mean + 1 SD indicated) (ZAR, 1999).

## DISCUSSION AND CONCLUSIONS

For the first time such a comprehensive investigation was carried out for one genus (8 *Agelas* species, including in total 121 individuals). According to their HPLC profiles the chemistry of *Agelas cervicornis*, *A. dispar* and *A. wiedenmayeri* is very similar to *A. clathrodes* (type A). The same is true for *Agelas cerebrum*, *A. dilatata* and *A. sceptrum* which are very similar to *A. conifera* (type B). This example demonstrates the importance of chemotaxonomy for this genus. The variation in the average concentration of the major secondary metabolites, **1**, **2**, and **3**, within the eight examined *Agelas* species is relatively small, however, the intraspecific variation is quite large. Most interestingly in this investigation is that the major metabolites of *Agelas* sponges have two ecological functions for the sponge (feeding deterrence and inhibition of the settlement of fouling microorganisms). So far, the description of multiple ecological functions for pure compounds or extracts are rare in the literature (e.g. SCHMITT *et al.*, 1995; BECERRO *et al.*, 1997; NEWBOLD *et al.*, 1999; WILSON *et al.*, 1999, KUBANEK *et al.*, 2002). One example of a secondary metabolite was described in 1995 for the brown seaweed *Dictyota menstrualis* (SCHMITT *et al.*, 1995). The diterpene alcohol dictyol E was shown to be feeding deterrent against herbivores and prevent fouling organisms from colonizing. Results of the overgrowth assay might indicate that the bromopyrrole alkaloids have a third ecological function for *Agelas* sponges. The crude extract of *A. conifera* was active to inhibit the growth of the sponge *Tedania ignis*. This is important for intra- and interspecific enforcement of the restricted space on the reef (PORTER & TARGETT, 1988). Further investigations with pure sceptrin (**3**) have to confirm this hypothesis. So far, we were not able to perform this assay, since approximately 1 g of purified **3** is necessary. The presented data suggest that bromopyrrole alkaloids fulfill multiple ecological functions in the defense of the common and diverse genus *Agelas*.

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