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Chemical defense of the Caribbean sponge *Agelas clathrodes* (Schmidt)

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Abstract

Marine sponges have been a rich source of natural products, but only in recent years have studies been undertaken to evaluate the ecological functions of these compounds. Previously, we found that crude organic extracts of the tissues of 6 species of the genus *Agelas* from the Caribbean were all strongly unpalatable to a predatory reef fish in aquarium assays. In this study, we used bioassay-directed isolation techniques to identify the deterrent metabolites in one of these species, *Agelas clathrodes* (Schmidt). Crude organic extracts of *A. clathrodes* at natural concentrations deterred feeding of the reef fish *Thalassoma bifasciatum* (Bloch) in aquarium assays, and of a natural suite of reef fishes in field assays. Separation of the crude extract by column chromatography yielded a series of fractions, of which only the polar fractions were deterrent in both aquarium and field assays. Two previously described compounds, oroidin (**1**) and 4,5-dibromopyrrol-2-carboxylic acid (**2**), were identified by high resolution mass spectrometry and NMR spectrometry as the deterrent metabolites in the active fractions. Purified samples of both compounds deterred feeding in aquarium assays, both separately and in combination, at concentrations found in the sponge tissue. In addition, both compounds were identified by analytical thin-layer chromatography as constituents of the crude extracts of *A. conifera* (Schmidt), *A. dispar* Duchassaing and Michelotti, *A. inaequalis* Pulitzer–Finali, *A. sceptrum* (Lamarck), and *A. wiedenmeyeri* Alcolado. These results suggest that sponges of the genus *Agelas* share a common chemical defense against fish predators.

Keywords: *Agelas*; Alkaloids; Chemical defense; Coral reef sponges; Predator–prey interaction

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1. Introduction

Sponges are the single richest source of marine natural products (Braekman et al., 1989; reviewed in Faulkner, 1995); these compounds have exhibited potentially important activity in pharmacological studies (e.g., Kitagawa and Kobayashi, 1993; Munro et al., 1994), but their ecological functions remain to be determined. Compounds isolated from sponges vary widely in structural complexity; compound classes include sterols, terpenoids, amino acid derivatives, saponins, and macrolides (Sarma et al., 1993; Faulkner, 1995). Many of these compounds have complex carbon skeletons that are nitrogen- or halogen-rich (Paul, 1992a; Faulkner, 1995). Concentrations of secondary metabolites in sponge tissues can be quite high; for example, scalaradial constituted 2.4% of the total dry mass in the Pacific sponge *Hyrtios erecta* (Rogers and Paul, 1991).

The structural complexity and high concentration of the secondary metabolites isolated from sponges suggest they play an important ecological function (Paul, 1992a; Pawlik, 1993). Among the proposed roles, sponge secondary metabolites have been implicated in sponge-coral allelopathic interactions (Sullivan et al., 1983; Porter and Targett, 1988), in the inhibition of settlement of larval fouling organisms (Davis et al., 1991; Henrikson and Pawlik, 1995), and in the protection of sponges from micro-organisms and ultraviolet radiation (Paul, 1992a).

The most prevalent theory regarding the function of sponge secondary metabolites is that they act to deter potential predators (e.g., Paul, 1992b; Pawlik, 1993). Sponges are soft-bodied and sessile, and thus appear to be physically vulnerable to predation. Caribbean demosponges, for example, represent a rich protein source (mean of 20.7 mg soluble protein per ml of tissue, $n = 71$; Chanas and Pawlik, 1995) in an environment noted for intense grazing activity by fishes (Hixon, 1983). Nevertheless, very few fish species are known to feed on Caribbean sponges (Randall and Hartman, 1968; Wulff, 1994). Only recently have ecologically relevant methods been used to test whether sponge secondary metabolites deter consumption by predatory fishes (Thompson et al., 1985; Pawlik et al., 1988; Herb et al., 1990; Rogers and Paul, 1991; Bobzin and Faulkner, 1992; Duffy and Paul, 1992; Pennings et al., 1994; Albrizio et al., 1995).

In our recent survey of the chemical antipredatory defenses of 73 species of Caribbean sponges (Pawlik et al., 1995), we discovered that all of the species within the genus *Agelas* yielded crude organic extracts that strongly deterred the feeding of a predatory reef fish in aquarium assays. Surprisingly, neither spicules, nor the spiculated spongin skeleton of *Agelas clathrodes*, deterred fish feeding in aquarium or field assays (Chanas and Pawlik, 1995, 1996). Therefore, secondary metabolites alone appear to provide *A. clathrodes* with a defense against predatory reef fishes. The purpose of the study reported herein was to isolate and identify the metabolite(s) responsible for the chemical defense of *A. clathrodes* using bioassay-directed fractionation techniques (see Pawlik and Fenical, 1992).

2. Methods

2.1. Collections

Agelas clathrodes (Schmidt) and other *Agelas* species were collected from locations in both the Florida Keys and the Bahamas. Portions of sponges were collected by cutting

tissue with a sharp knife. Replicate collections at one location were taken from distant sites (>10 m) to avoid collecting asexually produced clones. Tissue samples were extracted immediately or stored at -20°C until used in extractions.

2.2. Crude extracts

For aquarium assays of crude extracts, sponge ectosome (within 1 cm of surface) was chopped into small pieces and added to 40 ml of 1:1 dichloromethane:methanol (DCM:MeOH) in a graduated centrifuge tube to a final volume of 50 ml (10 ml of tissue extracted as in Pawlik et al., 1995). Sponge ectosome was used in these experiments because surface tissues would be most subject to possible predation by fishes. The tissue was allowed to extract at 4°C for 24 h. The DCM:MeOH extract was filtered and the tissue re-extracted in 40 ml of MeOH at 4°C for 1 h. The MeOH extract was filtered and combined with the DCM:MeOH extract; the combined extract was concentrated by vacuum evaporation to an organic residue (with <2 ml of residual water). For field assays of crude extracts, ectosomal sponge tissue was minced and added to 100 ml of 1:1 DCM:MeOH in a graduated cylinder to a final volume of 160 ml (60 ml of tissue extracted). The tissue was allowed to extract at 4°C for 24 h, then MeOH for 1 h. The 1:1 DCM:MeOH and MeOH extracts were filtered, combined, and concentrated by vacuum evaporation.

2.3. Aquarium assays

For aquarium assays, crude extracts or purified compounds were mixed with 10 ml of alginate-based food (see Pawlik et al., 1995) until all organic and water-soluble components were distributed uniformly throughout the paste. Food coloring was added to both treated and control foods to make them the same color. The alginate food was then dispensed with a 10 ml syringe into a 0.25 M calcium chloride solution forming a strand that was allowed to harden for 2 min. The hardened strand was rinsed in filtered seawater and cut into 3 mm pellets. Control pellets were prepared identically but without the addition of crude extract. Control and treated pellets were presented to groups of three bluehead wrasses (*Thalassoma bifasciatum* (Bloch); 1 blue-head phase, 2 yellow phase) held in each of 10 separate, opaque-sided compartments in laboratory aquaria (see Pawlik et al., 1995). Groups of fish were haphazardly chosen during feeding assays and offered either a treated or control food pellet, followed by the other choice. If the second pellet was treated and was rejected by the fish, another control pellet was offered to determine whether the fish had ceased feeding; groups of fish that would not eat control pellets were not used in assays. A pellet was considered rejected if not eaten after a minimum of three attempts by one or more fish to take it into their mouth cavity, or if the pellet was approached and ignored after one such attempt. The significance of differences in the consumption of treated vs. control pellets was evaluated with the Fisher exact test (Zar, 1984). For any single assay of 10 replicates, an extract was significantly deterrent if 4 or more of the pellets were rejected ($p \leq 0.043$, one-tailed test); therefore, in judging the mean deterrency of multiple samples from the same

species, extracts were considered deterrent if the mean number of pellets eaten was less than or equal to 6. Uneaten, treated pellets were frozen for later analysis to confirm the presence of deterrent compounds using analytical thin-layer chromatography (TLC). Aquarium assays were conducted aboard the R/V 'Columbus Iselin', the R/V 'Seward Johnson', or in the wet laboratory of UNC-Wilmington at Wrightsville Beach, North Carolina.

2.4. Field assays

For field assays, crude extracts or purified compounds from a 60-ml volume of sponge tissue were dissolved in a minimal volume of MeOH and combined with 60 ml of preheated carrageenan-based food (see Chanas and Pawlik, 1995). Food coloring was added to both treated and control foods to make them the same color. The mixture was then poured into molds crossed by lengths of cotton string and allowed to harden. After hardening, 20 string-embedded strips were cut from the molds. Control strips were prepared identically but without the addition of crude extracts. Field assays were conducted on shallow water reefs off Key Largo, Florida (see Chanas and Pawlik, 1995). One treated and one control strip each were tied to a 50 cm length of 3-strand nylon rope at a distance of ~4 and 12 cm from one end of the rope (the order was haphazard). Twenty ropes were deployed on the reef for each experiment, with the end of each rope opposite the food strips unwound and clamped onto a piece of coral or rock. Within 1 h, the ropes were retrieved and the percentage decrease in the strip length recorded to the nearest 5%. The Wilcoxon paired-sample test (two-tailed) was employed to analyze the results after excluding pairs for which both control and treated strips had been either completely eaten, or not eaten at all (Zar, 1984). Uneaten treated strips were frozen for later analysis to confirm the presence of deterrent compounds using TLC.

2.5. Isolation and identification of deterrent compounds

Approximately 500 ml of frozen tissue of *Agelas clathrodes* were diced into 1 cm^3 pieces and sequentially extracted for 24 h each in MeOH (repeated 3 times), 1:1 DCM:MeOH (3 times), and DCM (once). The extracts were filtered, concentrated by vacuum evaporation, and combined to yield an orange-colored crude extract. An aliquot of crude extract representing 250 ml of tissue was separated by vacuum flash chromatography over silica gel (Whatman) employing a solvent gradient of DCM in MeOH (200 ml volumes of DCM, followed by 90:10 DCM:MeOH, 85:15, 70:30, 50:50, 40:60, 25:75, and 100% MeOH). Compounds contained in the fractions were subjected to TLC and visualized (silica with fluorescent indicator in 80:20 DCM:MeOH; detection by spraying with 50% sulfuric acid and heating), then recombined based on compound similarities. Aquarium assays were performed with each of the 5 recombined fractions using a volume of extract equivalent to 10 ml of tissue. Based on aquarium assay results, fractions were pooled into non-polar and polar groups and subjected to field assays.

Aquarium and field assay results indicated that the deterrent activity was restricted to the more polar fractions. Due to the complex mixture of salts and free bases in the crude

extract, the separation scheme was modified to isolate the deterrent compound(s) from the polar components. An aliquot of crude extract representing 250 ml of tissue was partitioned between *n*-butanol and water. The *n*-butanol fraction was evaporated under vacuum and separated by column chromatography (gravity) on silica gel using 40:20:1 chloroform: MeOH: ammonium hydroxide (NH_4OH) as the eluant, followed by a final rinse using MeOH. Eight fractions were collected and monitored using TLC (silica in 40:20:1 chloroform:MeOH:NH_4OH). Aquarium assays were performed with the water soluble material and the eight fractions using a volume of extract equivalent to 10 ml of tissue. From the deterrent fraction(s), purified compounds were assayed at various concentrations.

Samples from three different specimens of *Agelas clathrodes* from each of four different reefs (1 Florida, 3 Bahamas) were analyzed in an attempt to characterize quantitative variability of the deterrent compounds within and between reef populations. Frozen samples (10 ml) of *Agelas clathrodes* were exhaustively extracted as before; the extracts were then individually filtered, dried by vacuum evaporation, and weighed (extract mass). The crude extract from each sample was dissolved in MeOH and sonicated for 15 min. The supernatant MeOH was filtered and evaporated under vacuum; the residue was partitioned between *n*-butanol and water. The upper (*n*-butanol) phase was removed, vacuum evaporated, and the residue separated by high-performance liquid chromatography (HPLC) on an analytical silica gel column with 3:1 chloroform:MeOH (saturated with ammonia) as the eluant. Compounds were detected by monitoring both refractive index and UV absorption of the eluant. The mass of collected compounds was determined gravimetrically (± 0.3 mg) and reported as dry mass per volume of tissue; mass data were compared between locations using a one-way ANOVA (Bonferroni pairwise comparison, $\alpha = 0.05$).

Qualitative analysis of deterrent compounds in crude organic extracts of five other species of the genus *Agelas* (*A. conifera* (Schmidt), *A. dispar* Duchassaing and Michelotti, *A. inaequalis* Pulitzer–Finali, *A. sceptrum* (Lamarck), and *A. wiedenmeyeri* Alcolado) was accomplished using analytical TLC and comparing crude organic extracts to purified standards (as in Braekman et al., 1992). Three replicate colonies of each of the five *Agelas* species were collected from reefs in the Florida Keys or Bahamas Islands. Frozen samples (10 ml) were extracted individually as before; the extracts were dried by vacuum evaporation and individually partitioned between *n*-butanol and water. The upper (*n*-butanol) phase was removed, vacuum evaporated, and compared to purified standards of deterrent compounds using analytical TLC (silica in 40:20:1 chloroform:MeOH:NH_4OH).

3. Results

3.1. Deterrency of crude organic extracts

The crude organic extracts from colonies of *Agelas clathrodes* deterred feeding of *Thalassoma bifasciatum* in aquarium assays (mean deterrency = 0.9 of 10 pellets eaten;

Table 1
Consumption by *Thalassoma bifasciatum* of food pellets containing crude extracts and purified compounds from *Agelas clathrodes*

Sample	Conc. (mg/ml)	Treated pellets eaten
Crude extract (natural concentration)		0.9±0.9 ($N = 10$)
Oroidin (**1**)	4.0	4
	2.0	4
	1.0	5
	0.5	5
Pyrrole carboxylic acid (**2**)	2.0	1
	1.0	2
	0.5	10
Oroidin + carboxylic acid	2.0 + 1.0	1

Concentrations given as mg/ml of artificial food. Fish consumed all 10 control pellets in all cases. For any individual assay, samples were considered deterrent if the number of pellets eaten was less than or equal to 6 ($p \leq 0.043$, Fisher exact test, one tailed, see Section 2).

SE = 0.87; $N = 10$; $p \leq 0.0004$; Fisher exact test; Table 1). The crude organic extracts of colonies of *Agelas clathrodes* deterred feeding of a natural assemblage of reef fishes in field assays (Fig. 1).

3.2. Isolation and identification of deterrent compounds

In aquarium assays of the vacuum flash column fractions, the combined fractions 1–9, the combined fractions 5–6 and 7–8, and fraction 9 deterred feeding (number of treated pellets eaten ≤ 4; $p < 0.005$) while combined fractions 1–4 did not deter feeding (Fig. 2A). Thin-layer chromatography revealed that fractions 1–4 mostly consisted of

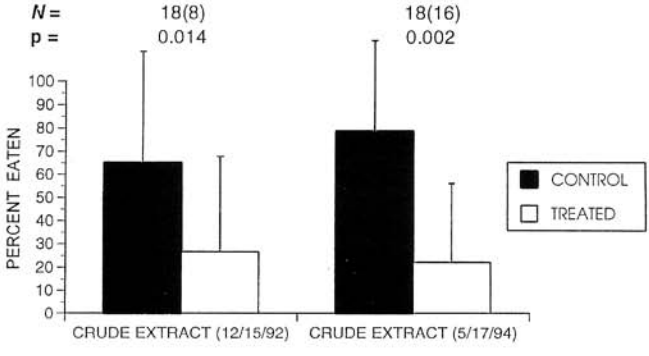


Fig. 1. Field assays of crude extracts of *Agelas clathrodes*. 1 SD above the mean percentage of foods strips eaten is indicated. Date of assay indicated under bars. p-values computed using Wilcoxon paired sample test (two-tailed). N = number of ropes retrieved out of 20 ropes deployed (number of ropes used in statistical analysis).

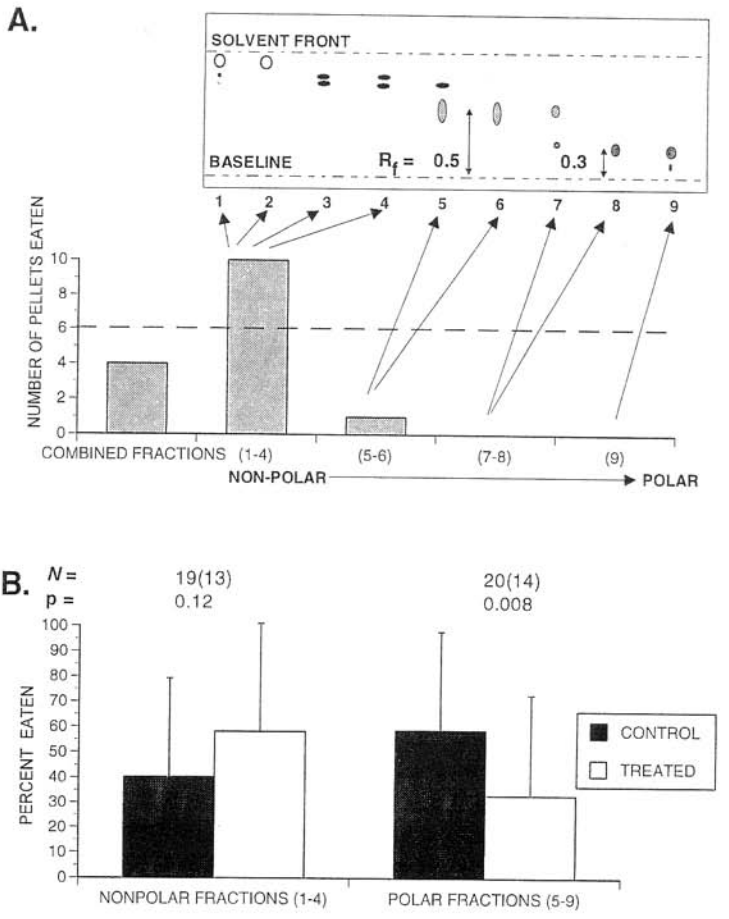
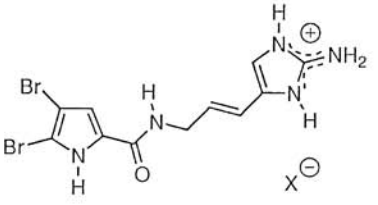


Fig. 2. Aquarium and field assays of vacuum flash column fractions of extracts of *Agelas clathrodes*. (A) Aquarium assays of vacuum flash column fractions. Fractions were assayed in combination, based on compound similarities indicated by the diagram of the TLC plate. All control pellets were eaten in all assays. For any individual assay, a treatment was considered deterrent if the number of treated pellets eaten was less than or equal to 6 ($p < 0.043$, Fisher exact test, one-tailed), as indicated by the dotted line. R_f-values of oroidin **(1)** and 4,5-dibromopyrrol-2-carboxylic acid **(2)** are illustrated in the diagram of the TLC plate. (B) Field assays of vacuum flash column fractions. Fractions were combined on the basis of compound polarity. Data presented as in Fig. 1.

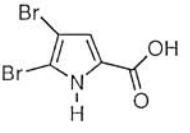
pigments while fractions 5–9 contained UV-active compounds. The non-polar combined fractions 1–4 did not deter feeding in a field assay ($p = 0.12$), but the polar combined fractions 5–9 deterred feeding ($p = 0.008$; Fig. 2B).

Of the 8 fractions separated by column chromatography, only 3 deterred feeding in aquarium assays. From one of the active fractions the alkaloid oroidin **(1)** was identified by nuclear magnetic resonance (NMR) spectrometry and high resolution mass spectrometry (HRMS). Using the same techniques, the major component of the other two

active fractions was identified as 4,5-dibromopyrrol-2-carboxylic acid (**2**). Oroidin deterred feeding in aquarium assays at concentrations of 0.5, 1.0, 2.0, and 4.0 mg/ml prepared food ($p < 0.016$; Table 1). In aquarium assays of the acid, feeding was deterred at 1.0 and 2.0 mg/ml food, but not at 0.5 mg/ml food (Table 1). In combination, oroidin and the carboxylic acid (2.0 and 1.0 mg/ml, respectively) also deterred feeding (Table 1).



(1)



(2)

The mean mass of oroidin in 12 sample colonies of *Agelas clathrodes* was 1.4 (±1.1; S.D.) mg/ml tissue; there were no significant differences in mean oroidin content between colonies from the four reefs (one-way ANOVA, $p = 0.817$; Table 2). Due to the complexity of the mixtures, quantitative measures of the carboxylic acid were not possible; however the average mass ratio of oroidin to the pyrrole carboxylic acid was estimated to be about 3:1. Analytical TLC of extracts of the five additional *Agelas* species revealed the presence of both oroidin and the carboxylic acid in all colonies sampled. Oroidin is visible on fluorescent TLC plates under short-wave UV light (254 nm), has an $R_f = 0.5$ (Fig. 2A), and stains violet after treatment with sulfuric acid and

Table 2
Oroidin content in colonies of *Agelas clathrodes* (±0.3 mg/ml).

Collection site	Sample #	Oroidin (mg/ml)	Mean ± SD
Molasses Reef	1	2.7	
(Florida)	2	0.3	
	3	1.2	1.4±1.2
Acklins Island	1	0.9	
(Bahamas)	2	0.6	
	3	0.9	0.8±0.3
Eleuthera Island	1	1.8	
(Bahamas)	2	1.5	
	3	1.5	1.6±0.3
Little San Salvador	1	0.6	
(Bahamas)	2	3.9	
	3	0.3	1.6±2.0
MEAN, all samples		1.4±1.1	

heat; the carboxylic acid is also visible on fluorescent TLC plates under short-wave UV light, has an $R_f = 0.3$ (Fig. 2A), and stains red.

4. Discussion

The study reported herein follows a recent survey of the crude extracts of 73 species of Caribbean sponges for antipredatory chemical defenses (Pawlik et al., 1995); the task of this and subsequent studies is to isolate and identify the secondary metabolites responsible for the activity of unpalatable crude extracts. Deterrent metabolites appear to be the principal defensive strategy of Caribbean sponges against predatory reef fishes, because no evidence has been found for structural or nutritional defenses in a similarly broad survey (Chanas and Pawlik, 1995). In a more in-depth study, neither the spicules, nor the intact spiculated spongin skeleton of *Agelas clathrodes* deterred feeding of reef fish in aquarium or field assays using prepared foods of nutritional qualities that were similar to those of sponge tissue (Chanas and Pawlik, 1996). Isolation and identification of oroidin **(1)** and its hydrolysis product, the pyrrol carboxylic acid **(2)** as the chemical defenses of *Agelas* species follows the discovery of a new polymeric pyridinium alkaloid, amphitoxin, which functions as the antipreditory defense of the sponge *Amphimedon compressa* (Albrizio et al., 1995).

The sponge genus *Agelas* includes several species (Zea, 1987), some of which are locally abundant on Caribbean reefs. Alvarez et al. (1991) reported that *Agelas dispar* and *A. conifera* ranked 9th and 19th, respectively, in their survey of a Venezuelan coral reef. Our own observations of sponge distributions on Mollases Reef, Key Largo, Florida, (3–10 m depth) indicate that *A. wiedenmeyeri* and *A. clathrodes* are among the most common sponges at that site. It appears that some of the success of the species within this genus is due to the elaboration of a taxonomically conserved chemical defense mechanism. Brominated-pyrrole derivatives are common in members of the family Agelasidae, as well as in the closely-related families Axinellidae and Halichondriidae, and have been used as chemotaxonomic indicators (Braekman et al., 1992). These compounds have exhibited a wide range of biological effects in pharmacological assays, including antiviral and antibacterial activity (e.g., Keifer et al., 1991). Oroidin-type alkaloids, in particular, are known from the genera *Agelas* (Agelasidae), *Phakellia* (Axinellidae), and *Hymeniacidon* (Halichondriidae) (Jiménez and Crews, 1994). Although other secondary metabolites have been isolated from *A. clathrodes* (Braekman et al., 1989; Morales and Rodríguez, 1991, 1992), only oroidin and the pyrrol carboxylic acid were identified as the deterrent metabolites in the present study. The presence of these two compounds in species of *Agelas* from locations other than the Caribbean (Forenza et al., 1971; Braekman et al., 1989, 1992) suggests that this chemical defense against fish predators may be common across the genus. Whether these compounds also serve as chemical defenses against potential invertebrate predators is the subject of ongoing research.

In the past, ecological functions of sponge extracts were often inferred from their activity in toxicity assays, which usually involved placing fresh-water fishes in aqueous

solutions of sponge extract and monitoring fish behavior (Bakus and Green, 1974; Green, 1977). As has been demonstrated in other studies (Schulte and Bakus, 1992; Pawlik et al., 1995), the results of feeding deterrency assays do not correlate with those of toxicity assays. For example, Bakus and Thun (1978) reported that *A. clathrodes* yielded a non-toxic crude extract, yet the crude extract of *A. clathrodes*, and the compounds isolated from it, were strong feeding deterrents in the present study.

Previous feeding studies have indicated that *Agelas* spp. possess a chemical defense that is effective against sponge-eating fishes as well as generalist predators. Hoppe (1988) demonstrated that whole or cut pieces of *Agelas clathrodes* were never consumed when offered to three species of spongivorous angelfishes (*Pomacanthus paru*, *Holacanthus ciliaris*, and *H. tricolor*). Additionally, when specimens of *H. tricolor* were force-fed pieces of *A. clathrodes*, they suffered "spasmodic and convulsive movements....[which] mostly resulted in vomiting of the fed piece of sponge" (Hoppe, 1988). Stomach content analyses revealed that *Agelas* spp. were absent or constituted a very small portion of the diet of most sponge-eating fishes (Randall and Hartman, 1968). This suggests that most spongivorous fishes avoid *Agelas* spp. because of the defensive metabolites in their tissues.

The majority of sponges on Caribbean reefs elaborate secondary metabolites that serve as defenses against predatory reef fishes (Pawlik et al., 1995). Discovery of the compounds responsible for chemical defenses has only just begun. Further isolation and identification will allow for comparisons of the deterrent chemistry to reveal structure-function and chemotaxonomic relationships. Also interesting is the metabolic cost of these chemical defenses, and its impact on sponge growth and fecundity, particularly because not all reef sponges employ deterrent metabolites, and these chemically undefended species are often eaten by spongivorous fishes (Pawlik et al., 1995). In addition, it is likely that many identified sponge natural products will not play a role in deterring predatory reef fishes. Other defensive functions of secondary metabolites (or additional functions of identified deterrent compounds) may include deterring invertebrate predators, antifouling, anti-overgrowth, or protection from UV-radiation (Paul, 1992b; Pawlik, 1993).

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