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CHEMICAL DEFENSE OF THE CARIBBEAN REEF SPONGE *Axinella corrugata* AGAINST PREDATORY FISHES

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Abstract—Field and laboratory experiments were performed to investigate the palatability to predatory fishes of organic extracts and purified compounds from the Caribbean reef sponge *Axinella corrugata* (=*Teichaxinella morchella*). When incorporated into artificial foods at the same volumetric concentration as found in sponge tissue, crude extracts of the sponge, as well as a butanol-soluble partition of the crude extract, deterred feeding of the Caribbean reef fish *Thalassoma bifasciatum* in laboratory aquarium assays and deterred feeding of a natural assemblage of fishes in assays performed on reefs where *A. corrugata* is found. Bioassay-directed fractionation of the butanol-soluble partition led to the isolation of a single compound responsible for feeding deterrency, stevensine, a previously described dibrominated alkaloid. The mean concentration of stevensine in *A. corrugata*, as determined by quantitative NMR analysis, was 19.0 mg/ml (N = 8, SD = 7.2 mg/ml). Stevensine deterred feeding in laboratory aquarium assays at concentrations >2.25 mg/ml, and deterred feeding in field assays at ~12 mg/ml. Stevensine represents another in the oroidin class of brominated pyrrole derivatives that function as chemical defenses of sponges in the families Axinellidae and Agelasidae.

Key Words—Chemical defense, sponges, predation, Caribbean, *Teichaxinella, Axinella, Agelas*, brominated metabolites.

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INTRODUCTION

Marine invertebrates that lack physical protection may use alternative defensive strategies such as chemical defense. Many of the unusual secondary metabolites that have been isolated from soft-bodied marine invertebrates are proposed to function as agents that deter predation (reviews in Paul, 1992a; Pawlik, 1993). Only recently have ecologically relevant field and laboratory experiments been designed to test this hypothesis, and our knowledge of the ecological roles of invertebrate secondary metabolites has increased rapidly over the last decade (e.g., Pawlik et al., 1987; Harvell et al., 1988; Becerro et al. 1994; McClintock et al., 1994; Pennings et al., 1994; Hay, 1996).

Sponges are the single most diverse source of marine natural products (Braekman et al., 1989; reviewed in Faulkner, 1998, and previous reviews cited therein); 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, 1998). Many of these compounds have complex carbon skeletons that are nitrogen- or halogen-rich (Faulkner, 1998; Paul, 1992a). 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 and Faulkner, 1983; Porter and Targett, 1988), the inhibition of settlement of larval fouling organisms (Davis et al., 1991; Pawlik, 1992; Henrikson and Pawlik, 1995, 1998), and in the protection of sponges from microorganisms 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 milliliter of tissue, $N = 71$ (Chanas and Pawlik, 1995)] in an environment noted for intense grazing activity by fishes (Hixon, 1983; Jones et al., 1991). Nevertheless, very few fish species are known to feed on Caribbean sponges (Randall and Hartman, 1968). 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; Duffy and Paul, 1992; 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 five species within the family Axinellidae yielded crude organic extracts that deterred the feeding of predatory reef fish in aquarium assays. One species, *Axinella corrugata* (previously *Teichaxinella morchella*) consistently yielded a highly deterrent crude extract (0 of 10 food pellets eaten). The purpose of the study reported herein was to isolate and identify the metabolite(s) responsible for the chemical defense of *A. corrugata* using bioassay-guided fractionation techniques.

METHODS AND MATERIALS

Sponge Collection. Samples of *Axinella corrugata* used for assays were collected in June and July 1996 on shallow (depth <25 m) reefs surrounding the Bahamas Islands while on board the *R/V Seward Johnson.* Additional samples obtained for analysis of intraspecific variability of metabolite concentration were collected from reefs off Key Largo, Florida, in July and August 1996. Individual sponges were collected by cutting the narrow base of the sponge from the substratum with a sharp knife. Sponges were extracted immediately or stored at −20°C until used.

Extraction and Isolation. For preliminary field and aquarium assays, sponge samples were processed immediately after collection, while later analyses used frozen material. Fresh or frozen sponge was cut into small pieces and placed in a 1000-ml graduated cylinder containing 500 ml of 1 : 1 dichloromethane–methanol (DCM/MeOH). Sponge tissue was added until the displaced volume reached 1000 ml. The tissue was thoroughly extracted twice in 1 : 1 DCM/MeOH and once in MeOH, each for >12 hr. The resulting extracts were combined and divided into aliquots, each from a known volume of sponge tissue. Some of these aliquots were used for assays of the crude extract or for later quantification, while the majority were used for bioassay-directed fractionation.

Aliquots of crude extract representing a known volume of sponge were combined and successively partitioned with organic solvents of increasing polarity. The crude extract was initially partitioned between water and 2,2,4-trimethylpentane (TMP), then between water and DCM, and finally between water and butanol (~500 ml of each solvent), leaving four solvent partitions: TMP, DCM, butanol, and aqueous. Each partition was filtered through Celite and dried by rotary evaporation before being subjected to feeding assays and further separation.

Using aquarium assays, fish feeding deterrent activity was largely isolated in the butanol partition. Partitions were analyzed using C_{18}-reverse phase thin-layer chromatography (TLC; methanol elution, UV visualization). Subsequent

purification of constituent compounds from the butanol partition employed size-exclusion chromatography using lipophilic Sephadex LH-20, with methanol elution. Resulting fractions were analyzed using TLC, nuclear magnetic resonance (NMR) spectrometry, high-resolution mass spectrometry (HR-MS), and fractions were subjected to aquarium assays. Comparison of the NMR and HR-MS data with the literature enabled structural assignment of the active metabolite.

To determine the concentration of the active metabolite in butanol partitions of crude extracts from sponge samples from different locations, quantitative proton NMR analysis was used. Integration of proton signals arising from both pure samples of the active compound and a known amount of an internal standard (cyclooctatetraene) provided an estimate of the mass of the active compound in the butanol partition from a known volume of sponge tissue.

Laboratory Feeding Assays. Aquarium assays were performed as described in Pawlik et al. (1995) on the crude extract, each solvent partition, each fraction, and the purified active metabolite from *Axinella corrugata*. Assays were performed on board the research vessel *Seward Johnson* or in aquaria at the University of North Carolina at Wilmington by employing a common predatory reef fish, the bluehead wrasse *Thalassoma bifasciatum*. The advantages of using this species for aquarium bioassays have previously been detailed (Pawlik et al., 1987, 1995). Groups of three fish (one terminal phase male, two females) were held in each of 15–20 separate, opaque-sided compartments in flow-through laboratory aquaria. Groups of 10 fish were chosen out of 15 at random during feeding assays and randomly offered either a treated or control food pellet, followed by the other choice. When the second pellet was a treated pellet and was rejected, a third pellet was offered as a control to determine whether the fish had ceased feeding. Groups of fish that did not eat control pellets were considered satiated and were not used in the experiment. A food pellet was considered rejected if one or more fish took the pellet into their mouths and expelled it at least three times or if the pellet was approached and ignored. The significance of differences in the consumption of treated versus control pellets was evaluated with the Fisher exact test (Zar, 1984). For any single assay of 10 replicates, an extract was significantly deterrent if four or more of the pellets were rejected ($P \geq 0.043$, one-tailed test); therefore, a sample was considered deterrent if the number of pellets eaten was less than or equal to 6.

Food pellets for laboratory assays were made by mixing a volume of 5 g of lyophilized, macerated squid with 3 g of alginic acid and 100 ml of water, followed by addition of the volumetric equivalent of a fraction or compound in a carrier solvent (for treated pellets) or solvent alone (for control pellets). The mixture was vigorously stirred to remove lumps and then loaded into a 5-ml syringe. The tip of the syringe was then dipped into a beaker filled with a 0.25 M solution of $CaCl_2$ and the contents of the syringe slowly expelled to form a long strand. After a few minutes, the strand was removed, rinsed in seawater,

and chopped into 4-mm-long pieces with a razor blade to form uniform pellets.

Field Feeding Assays. Feeding experiments were conducted in the field on each of three samples: the crude extract, the butanol partition, and the purified active compound. In each case, the sample was volumetrically reconstituted in a matrix of carrageenan at the concentrations as they occurred in the sponge. Strips of this matrix were then used in field experiments (see methods in Fenical and Pawlik, 1991). The matrix was made by combining 1.5 g of carrageenan (Gelcarin, FF961L; FMC Corp, Philadelphia, Pennsylvania) and 3 g of lyophilized, macerated squid with deionized water to a total volume of 65 ml. The mixture was heated to boiling in a microwave oven (about one minute on "cook"), then the sample was added with a minimal volume of solvent (treated matrix) or solvent alone (control matrix). Food dye was added to the control matrix so as to color-match the treated matrix. The mixture was stirred and heated again to boiling (pure compounds were later judged to be stable to this treatment). The molten mixture was then poured into plastic molds crossed by lengths of cotton string that protruded from the ends of the molds. After the matrix had cooled and set, 1.0- × 0.5- × 5.0-cm strips were sliced to size with a razor blade and removed from the mold. For each experiment, 20 treated strips and 20 control strips were prepared. To distinguish treated from control strips, the cotton string attached to each strip was marked with a small ink spot.

Field assay methods are described in detail in Pawlik and Fenical (1989, 1992). One treatment and one control strip each were tied to a 50-cm length of three-strand nylon rope at a distance of approximately 4 and 12 cm from one end of the rope (the order was haphazard). Twenty ropes were deployed on the same reefs from which *Axinella corrugata* had been collected, with the end of each rope opposite the food strips attached to the substratum by inserting a piece of coral or rock through the rope twines. Within 1 hr, the ropes were retrieved and the amount of each strip eaten was recorded as a percentage decrease in the strip length (to the nearest 5%). The Wilcoxon paired-sample test (one-tailed) (Zar, 1984) was employed to analyze the results after excluding pairs for which both control and treatment slices had been either completely eaten or not eaten at all.

RESULTS

Assay food strips containing a crude extract of *Axinella corrugata* at the same volumetric concentration as the extract occurs in the tissues of the sponge significantly deterred feeding of a natural assemblage of consumers present on the reef ($P < 0.001$, Wilcoxon paired-sample test; Figure 1). For this and for subsequent field feeding assays, food strips were observed being consumed by

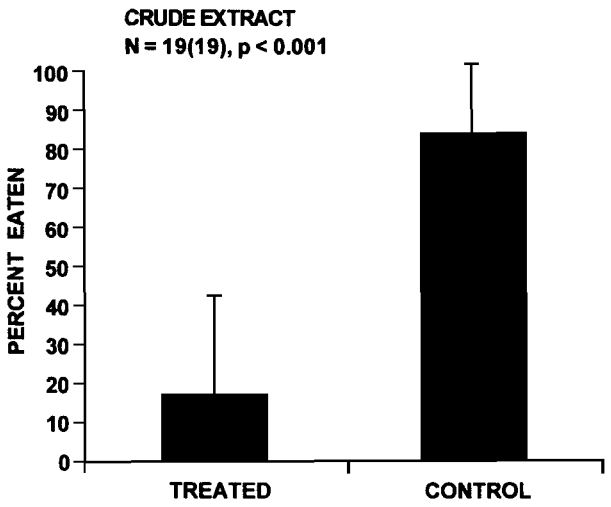


FIG. 1. Field assay. Feeding by reef fishes on paired control food strips and strips containing a crude extract of *Axinella corrugata* at the same concentration as found in the tissues of the sponge; 1 SD above the mean is indicated; N = number of paired treatment and control strips retrieved of the 20 deployed (number of pairs used in statistical analysis). Probability calculated using the Wilcoxon paired-sample test.

a wide variety of reef fishes, particularly wrasses (*Thalassoma* and *Halichoeres* spp.), snappers (*Ocyurus chrysurus*), parrotfishes (*Scarus* and *Sparisoma* spp.), grunts (*Haemulon* spp.), tilefish (*Malancanthus plumieri*), porgy (*Calamus* spp.) and angelfishes (*Pomacanthus* and *Holacanthus* spp.).

In laboratory aquarium assays employing the reef fish, *Thalassoma bifasciatum*, the crude extract of *Axinella corrugata* and the butanol partition of the crude extract significantly deterred feeding (<2 pellets eaten of 10 offered). Weak feeding deterrent activity was observed in the aqueous partition. The butanol partition also significantly deterred feeding of reef fishes in a field assay ($P < 0.005$; Figure 2).

Bioassay-guided fractionation of the butanol partition of the crude extract using LH-20 Sephadex chromatography resulted in the isolation of active fractions containing a single metabolite. This metabolite was identified as stevensine (Figure 3) by NMR spectroscopy and high-resolution mass spectroscopy and comparison with data reported in the literature for both naturally occurring and synthetically produced stevensine (Albizati and Faulkner, 1985; Wright et al., 1991; Xu et al., 1997).

Volumetric concentrations of stevensine in the tissues of individual specimens of *Axinella corrugata* from eight locations in the Bahamas and Florida

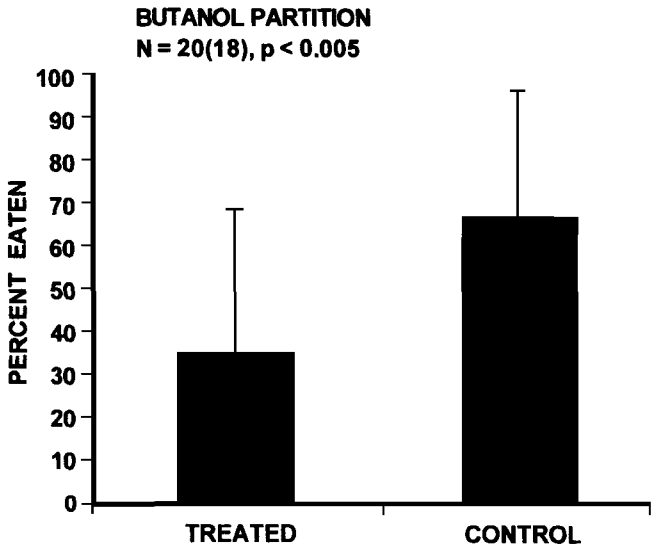
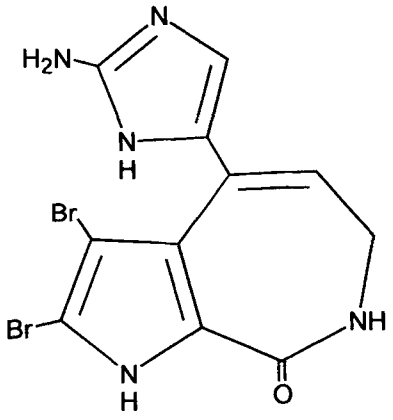


FIG. 2. Field assay. Feeding by reef fishes on paired control food strips and strips containing a butanol partition of the crude extract of *Axinella corrugata*. Details as in Figure 1.



STEVENSINE

FIG. 3. The structure of stevensine, defensive metabolite present in the tissues of *Axinella corrugata*.

TABLE 1. VOLUMETRIC CONCENTRATION OF STEVENSINE IN TISSUES OF INDIVIDUAL SPECIMENS OF *Axinella corrugata* COLLECTED IN BAHAMAS ISLANDS AND FLORIDA AS DETERMINED BY QUANTITATIVE NMR ANALYSIS

Location	Date (in 1996)	Depth (m)	Concentration (mg/ml tissue)
Bahamas Islands			
Sweetings Cay	Jun 18	22	12.0
Sweetings Cay	Jun 19	15	17.0
Little San Salvador Is., reef	Jun 25	22	30.0
Little San Salvador Is., pinnacles	Jun 25	22	27.0
Andros Island	Jul 2	18	25.0
Chubb Cay	Jul 1	28	12.0
Key Largo, Florida Keys			
Conch Reef	Aug 2	15	13.0
Molasses Reef	Jul 31	15	16.0
Mean concentration			19.0 ± 7.2

Keys were determined using quantitative proton NMR (Table 1). Four proton signals from stevensine (δ 6.23, 6.85, 7.39, 8.15 ppm) were integrated, and the mean was compared with the proton signal derived from the internal standard (COT; δ 5.7 ppm). Stevensine concentrations ranged from 12 to 30 mg/ml sponge tissue (mean = 19.0 ± 7.2 mg/ml).

Purified stevensine deterred feeding of a natural assemblage of reef fishes in a field assay in which the compound was assayed at the low end of the concentration range exhibited in the tissues of *Axinella corrugata* (~12 mg/ml; $P < 0.05$; Figure 4). Stevensine was also subjected to a series of laboratory assays at decreasing concentrations (Figure 5). The minimally active concentration at which stevensine deterred feeding of the wrasse *Thalassoma bifasciatum* was 2.0–2.25 mg/ml.

DISCUSSION

This study is part of a broader program that developed from a recent survey of the crude extracts of 73 species of Caribbean sponges for antipredatory chemical defenses (Pawlik et al., 1995). 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, 1996). Identification of the metabolites responsible for sponge chemical defenses will provide a better understanding of structural components required for distastefulness, as well as the evolution of chemical defenses. To date, a new polymeric pyridinium alkaloid, amphitoxin,

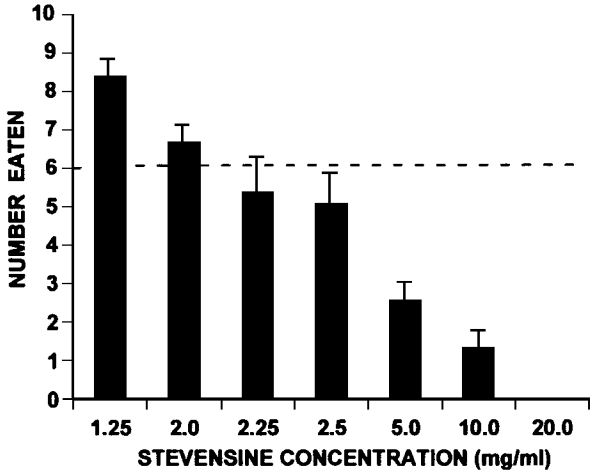


FIG. 4. Aquarium assay. Consumption by *Thalassoma bifasciatum* of control food pellets and pellets (mean + SE) containing a range of concentrations of stevensine. Fish consumed all 10 control pellets in all cases. Three replicate assays were performed at each concentration. For any individual assay, the treatment was considered deterrent if the number of pellets eaten was less than or equal to 6 ($P < 0.04$, Fisher exact test, one-tailed), as indicated by the dotted line on the graph.

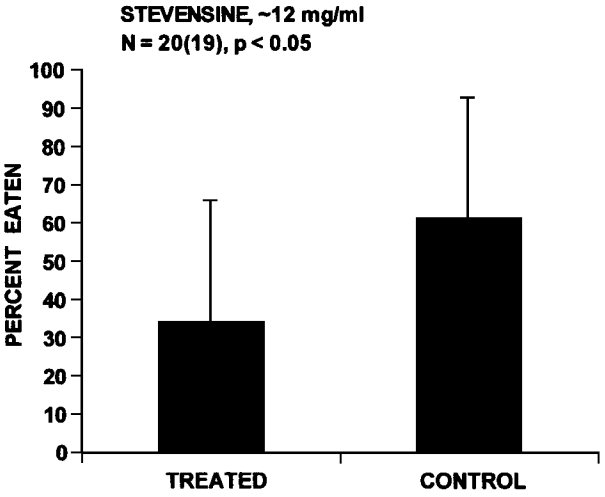


FIG. 5. Field assay. Feeding by reef fishes on paired control food strips and strips containing purified stevensine from *Axinella corrugata* at ~12 mg/ml. Details as in Figure 1.

has been isolated as the chemical defense of *Amphimedon compressa* (Albrizio et al., 1995), and oroidin and its carboxypyrrole hydrolysis product have been identified as the primary defensive agents of Caribbean sponges of the genus *Agelas* (Cahans et al., 1996).

Brominated pyrrole derivatives are common secondary metabolites in sponges of the families Axinellidae and Agelasidae, with *Axinella corrugata* a member of the former. These two families were once included in the order Axinellida, partially on the basis of similar secondary metabolite chemistry (Bergquist, 1978). The two families have since been separated at the ordinal level due to marked differences in skeletal structure (Hartman, 1982), and the family Axinellidae has been allocated to different orders and the genera reassigned based on morphological characteristics (Alvarez and Crisp, 1995; Alvarez et al., 1998). Brominated pyrroles have exhibited a wide range of biological effects in pharmacological assays, including antiviral and antibacterial activity (e.g., Keifer et al., 1991) and have been used as chemotaxonomic indicators (Braekman et al., 1992). Stevensine is closely related to the compound oroidin, which is commonly extracted from the tissues of sponges of the genus *Agelas* (Forenza et al., 1971; Braekman et al., 1989, 1992). Oroidin from Caribbean species of *Agelas* has been demonstrated to act as a chemical defense against fish predators (Chanas et al., 1996). Whether these compounds also serve as chemical defenses against potential invertebrate predators, or function as antimicrobial agents or as inhibitors of overgrowth or fouling, remains to be investigated.

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 meabolites, and these chemically undefended species are often eaten by spongivorous fishes (Pawlik et al., 1995; Pawlik, 1998). 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, 1992a; Pawlik, 1993). Stevensine has recently been shown to have weak antimicrobial activity against a test panel of marine bacteria that included strains isolated from necrotic sponges (Newbold et al., 1999), providing evidence that some secondary metabolites may serve multiple defensive roles.

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