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DOES THE ODOR FROM SPONGES OF THE GENUS *Ircinia* PROTECT THEM FROM FISH PREDATORS?

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Abstract—Caribbean sponges of the genus *Ircinia* contain high concentrations of linear furanosesterterpene tetronic acids (FTAs) and produce and exude low-molecular-weight volatile compounds (e.g., dimethyl sulfide, methyl isocyanide, methyl isothiocyanate) that give these sponges their characteristic unpleasant garlic odor. It has recently been suggested that FTAs are unlikely to function as antipredatory chemical defenses, and this function may instead be attributed to bioactive volatiles. We tested crude organic extracts and purified fractions isolated from *Ircinia campana*, *I. felix*, and *I. strobilina* at naturally occurring concentrations in laboratory and field feeding assays to determine their palatability to generalist fish predators. We also used a qualitative technique to test the crude volatile fraction from *I. felix* and *I. strobilina* and dimethylsulfide in laboratory feeding assays. Crude organic extracts of all three species deterred feeding of fishes in both aquarium and field experiments. Bioassay-directed fractionation resulted in the isolation of the FTA fraction as the sole active fraction of the nonvolatile crude extract for each species, and further assays of subfractions suggested that feeding deterrent activity is shared by the FTAs. FTAs deterred fish feeding in aquarium assays at concentrations as low as 0.5 mg/ml (fraction B, variabilin), while the natural concentrations of combined FTA fractions were >5.0 mg/ml for all three species. In contrast, natural mixtures of volatiles transferred from sponge tissue to food pellets and pure dimethylsulfide incorporated into food pellets were readily eaten by fish in aquarium assays. Although FTAs may play other ecological roles in *Ircinia* spp., these compounds are effective as defenses against potential predatory fishes. Volatile compounds may serve other defensive functions (e.g., antimicrobial, antifouling) but do not appear to provide a defense against fish predators.

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INTRODUCTION

Sponges produce a wide variety of marine natural products that often exhibit potent activity in pharmacological assays (Faulkner, 2000, and previous reviews cited therein). Yet, the roles that these metabolites play in sponge biology remain largely unknown. Because secondary metabolites in sponges are structurally complex and frequently present in high concentrations, it has been presumed their synthesis occurs at some metabolic cost to the organism, and for this cost, they should provide some benefit (Paul, 1992; Pawlik, 1993; McClintock and Baker, 2001). Sponge secondary metabolites may act to inhibit biofouling and overgrowth (Henrikson and Pawlik, 1995, 1996), inhibit the growth of microorganisms (Newbold et al., 1999; Zea et al., 1999), prevent damage caused by UV radiation (Paul, 1992), or act as allelopathic agents (Sullivan et al., 1983; Porter and Targett, 1988; Engel and Pawlik, 2000), but the most commonly hypothesized function is that of predator deterrence (Pawlik, 1993; Pawlik et al., 1995; Chanas et al., 1996; McClintock, 1997; Wilson et al., 1999; Waddell and Pawlik, 2000a,b).

In a previous study, we surveyed the crude organic extracts of 71 species of Caribbean sponges (Pawlik et al., 1995) and found that 63.4% contained metabolites that were unpalatable to the generalist predatory fish *Thalassoma bifasciatum* in aquarium assays. Among the least palatable were extracts from sponges of the genus *Ircinia*. Three species of *Ircinia—I. felix, I. strobilina*, and *I. campana*—are commonly found on tropical coral reefs, grassbeds, and in mangroves throughout the Caribbean (Zea, 1987; Schmahl, 1991).

Sponges of the genus *Ircinia* are well known for their strong, unpleasant garlic odor, which has recently been traced to a mixture of low-molecular-weight, volatile nitrogen- or sulfur-containing compounds, including dimethylsufide, methyl isocyanide, and methyl isothiocyanate (Duque et al., 2001). These volatile compounds exhibit antimicrobial activity in laboratory assays (Duque et al., 2001). In addition, furanosesterterpene tetronic acids (FTAs) have been isolated from *Ircinia* spp. (Cimino et al., 1972; Martínez et al., 1997) (Figure 1). These compounds reportedly have a variety of biological activities in various assay systems, including antimicrobial activity and the inhibition of Ca^{2+} transport (Beveridge et al., 1995), but their potential ecological functions have never been experimentally addressed.

Recently, Zea et al. (1999) reported that concentrations of FTAs in *Ircinia felix* were lower in the sponge surface than in internal tissues, greater in sponges found or transplanted to areas of lower light intensities, and greater in sponges that had been intentionally injured. Moreover, they reported that, when injured, *I. felix*

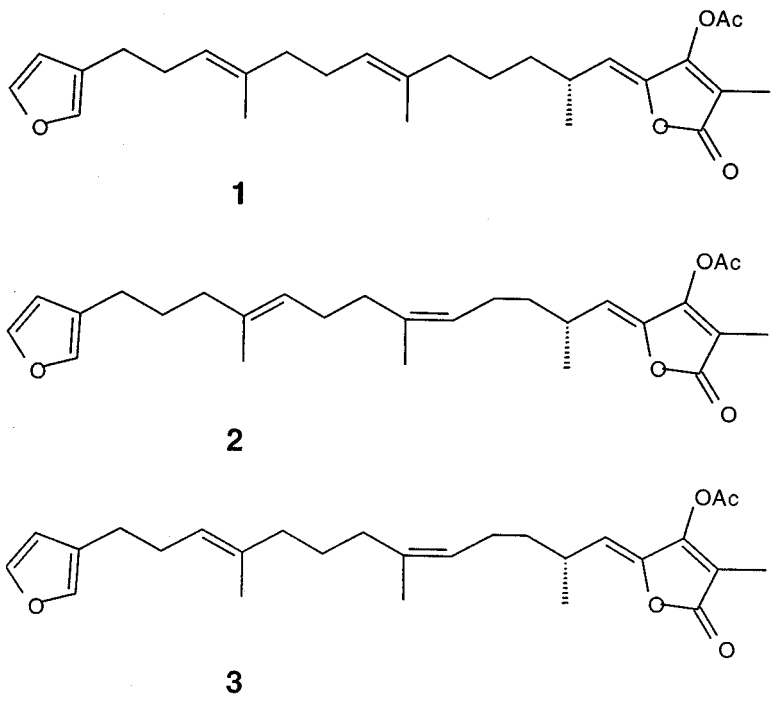

FIG. 1. Furanosesterterpene tetronic acids (FTAs, shown as acetate esters) from *Ircinia* spp. **1**, variabilin; **2**, strobilinin; **3**, felixinin.

did not release FTAs into the surrounding water column, suggesting that these nonpolar compounds were not involved in external ecological interactions such as chemical defense against potential predators, antifouling, or antiovergrowth (Zea et al., 1999). Instead, they suggested that the odor-causing, volatile compounds were responsible for chemical defense, either by direct action, or at a distance by signaling invasive organisms or potential predators of the presence of FTAs within the sponge tissue (Zea et al., 1999; Duque et al., 2001).

Considering the foregoing, we became interested in the relative antipredatory role of volatiles and FTAs from *Ircinia* spp. Standard techniques for isolating crude organic extracts, such as those used in Pawlik et al. (1995), result in the loss of highly volatile constituents, and to our knowledge, volatile metabolites of sponges have not previously been subjected to feeding assays. Specifically, we wanted to answer these questions: (1) Which nonvolatile components of the crude organic extracts of *Ircinia* spp. are responsible for the antipredatory effects described previously (Pawlik et al., 1995)? (2) Do the odor-causing volatile metabolites of *Ircinia* spp. also contribute to the antipredatory chemical defenses of these sponges?

METHODS AND MATERIALS

For experiments with nonvolatile crude organic extracts of sponges, samples of *Ircina felix* and *I. strobilina* were collected in August 1995 from reefs, mangroves, and seagrass beds from Acklins Island, Long Island, and Sweetings Cay, Bahamas, at 1–30 m depth. Samples of *I. campana* were collected October 1995 at Rodriguez Cay, Key Largo, Florida, at 1–2 m depth. Replicate specimens were obtained from geographically distinct locations to avoid collection of asexually reproduced clones. Samples were removed from the substrate by cutting the tissue with a sharp knife. Sponges were identified according to Zea (1987). Specimens were extracted immediately or frozen at -20°C until extraction.

Sponge tissue was chopped into cubes, tissue volume was measured by displacement, and the tissue was serially extracted in methanol and a 1:1 mixture of dichloromethane (DCM) and methanol (MeOH) (details in Pawlik et al., 1995; Chanas et al., 1996). The resulting crude extract was resuspended and divided into aliquots that were stored under nitrogen and kept at -20°C until use in aquarium or field assays. To separate compounds based on polar affinity, an aliquot of each crude extract was successively partitioned against water, hexanes, DCM, and *n*-butanol. The four partitions were tested for feeding deterrency in aquarium and field assays (see below).

Aquarium assays indicated that antifeedant activity was present in the hexanes and DCM partitions. Subsequent reverse-phase (C_{18}) thin-layer chromatography (TLC) of all the partitions using 9:1 MeOH–H_2O as the mobile phase revealed that these two partitions contained the same compounds. The hexanes and DCM partitions were combined as a nonpolar partition, and the *n*-butanol and aqueous partitions were likewise combined to form a polar partition, and both combined partitions were subjected to aquarium and field assays.

The combined nonpolar partition was dried and repartitioned between hexanes and acetonitrile. TLC analysis (as above) indicated nearly complete separation of at least two compounds into the acetonitrile repartition, with two pink-stained spots having R_f values of approximately 0.45 and 0.55, respectively. Both repartitions were subjected to aquarium assays, and the acetonitrile repartition was also assayed in the field.

Reverse-phase preparative TLC was used to isolate two fractions (A and B) from the acetonitrile repartition using 7:3 MeOH–H_2O as the mobile phase. Fractions A and B were further purified by using high-performance liquid chromatography (Spherisorb ODS-2 C_{18} column with a mobile phase of 65:34.7:0.03 acetonitrile–water–trifluoroacetic acid). Fraction A was a mixture of compounds, but fraction B was a single metabolite and was subjected to mass spectrometry (HP 5890 series II gas chromatograph coupled with an HP 5971 mass detector) and to ^{1}H and ^{13}C NMR spectroscopy using a 300-MHz Varian model NMR.

For assays of the naturally occurring volatile metabolites from *Ircinia* spp., individual specimens of *I. felix* and *I. strobilina* were collected in July 2000 at Sweetings Cay and Little San Salvador, Bahamas, from 14–18 m depth. For each species, sponge samples were drained of excess seawater and pureed in a blender. The resulting sponge slurry (125 ml) was stirred and slightly heated in a stoppered Erlenmeyer flask through which air was bubbled from an aquarium air pump. The air emerging from the flask was passed by airline tubing through an air-stone in a test tube containing distilled water (20 ml) chilled in a bath of ice in seawater. After several trials, the ratio of 125 ml of sponge slurry to 20 ml of distilled water produced an odor from the distilled water at 25°C that was approximately as strong as that of the slurry from which it was obtained. The distilled water containing the mixture of volatile metabolites was immediately used to make alginic acid food (see below) and subjected to aquarium fish feeding assays. Pure dimethylsulfide, at concentrations equivalent to 1, 10, and 100 times those found in the tissues of *Ircinia felix* was also subjected to aquarium fish feeding assays (Duque et al., 2001) (natural concentration was $\sim$0.3 μg/g of ash-free tissue dry weight, equivalent to 0.29 μl/liter, calculated from an average ash-free weight of 82 mg/ml of sponge; cf. Chanas and Pawlik, 1995).

Aquarium assays using the generalist reef predator, *Thalassoma bifasciatum*, were performed according to the methods detailed in Pawlik et al. (1995). Briefly, crude extracts, partitions, fractions, or pure compounds were incorporated at natural volumetric concentrations (or higher, for fractions A and B and for dimethylsulfide) into a food matrix composed of alginic acid and freeze-dried, powdered squid mantle. The matrix was extruded through a syringe into a 0.25 M solution of $CaCl_2$ to produce a hardened noodle that was then cut into food pellets. Treated and control pellets (lacking addition of extract or compounds) were offered to 10 replicate sets of three fish. A treated pellet was considered deterrent if it was not eaten after three or more attempts to take it into their mouths, by one or more fish or if it was ignored. Control pellets were eaten in all assays, as fish that would not eat control pellets were considered satiated and were not used in assays. For any single assay of 10 replicates, an extract was significantly deterrent if four or more pellets were rejected ($P < 0.043$, Fisher exact test, one-tailed).

Field assays were conducted as described in Chanas and Pawlik (1995) and Chanas et al. (1996) on nonvolatile crude extracts and all partitions of all three *Ircinia* spp. Field assays were conducted on shallow reefs near Key Largo, Florida, in May 1996, or in the Bahamas (most at Sweetings Cay) during July–August 1996 or 1997. Briefly, crude extracts, partitions, or fractions were incorporated at natural volumetric concentrations into a heated food matrix composed of carrageenan and freeze-dried, powdered squid mantle. The heated 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 $\times$ 0.5 $\times$ 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. 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 reef, with the end of each rope opposite the food strips attached to the substratum by inserting a piece of nonliving substratum 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) 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

Food pellets containing natural concentrations of crude organic extracts of *Ircinia campana*, *I. felix*, and *I. strobilina* deterred feeding of *Thalassoma bifasciatum* in aquarium assays (Figure 2), and deterred feeding of a natural assemblage of reef fishes (Figure 3). Food pellets containing the water and *n*-butanol (BuOH)

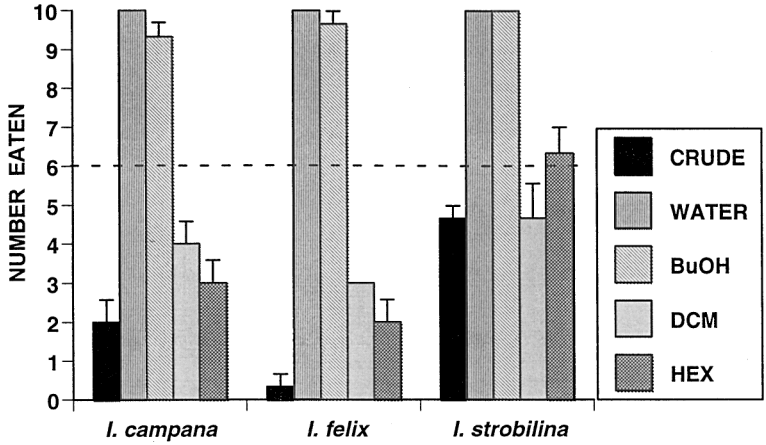

FIG. 2. Aquarium assay. Consumption by *Thalassoma bifasciatum* of food pellets containing natural concentrations of crude extracts and solvent partitions of the crude extracts from three *Ircinia* spp. For each species, assays were replicated with extracts and partitions from 3 sponge samples ($N = 3$: mean + SD is shown). BuOH = butanol partition, DCM = dicholormethane partition, HEX = hexane partition. For each assay, all 10 control food pellets were eaten. For any individual assay, extracts were considered deterrent if the number of pellets eaten was ≤ 6 ($P \leq 0.043$, Fisher exact test) as indicated by the dotted line on the graph.

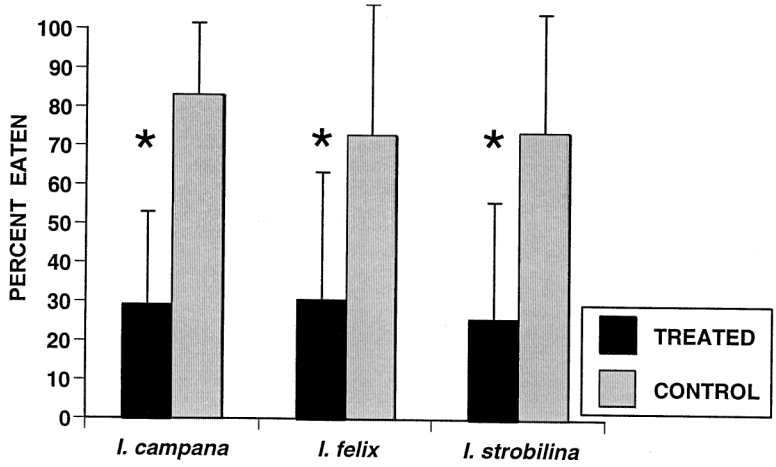

FIG. 3. Field assay. Consumption by reef fishes of paired control food strips and strips containing the natural concentration of the crude organic extracts from three *Ircinia* spp. Mean + 1 SD is indicated. For each assay, 20 pairs of food strips were deployed in the field. $^{*}P < 0.05$, Wilcoxon paired-sample test.

partitions of the crude extracts of all three species were palatable, and the compounds responsible for antifeedant activity were restricted to the dichloromethane and hexanes partitions (Figure 2). Field assays of the combined polar (water and BuOH) partitions and combined nonpolar (DCM and hexanes) partitions confirmed the results of the aquarium assays, with the activity restricted to the nonpolar partitions (Figure 4). Repartitioning of the combined nonpolar partitions between acetonitrile and hexanes further separated the activity: the acetonitrile repartition deterred feeding in field assays for each species, while the reconstituted remainder of the crude extract was not deterrent (Figure 5).

The acetonitrile repartition yielded two spots by TLC analysis, and these were separated and quantified by HPLC into fractions A and B for each of the three species of *Ircinia* (Table 1). Fractions A and B from *Ircinia felix* were subjected to aquarium assays at natural concentrations and at lower concentrations in order to ascertain the minimal effective concentration (Table 2).

Analysis of fractions A and B by HPLC revealed that fraction A was a mixture of compounds, but that fraction B was a single metabolite. Analysis of fraction B by GC-MS yielded a M′ of 429 with a base peak of 73. NMR data revealed the structure of fraction B to be that of the known FTA variabilin (**1**). Fraction A was not further separated or analyzed, but was most likely a mixture of the other FTAs present in the mixture isolated from *Ircinia* spp. (Cimino et al., 1972; Martínez et al., 1997) (Figure 1).

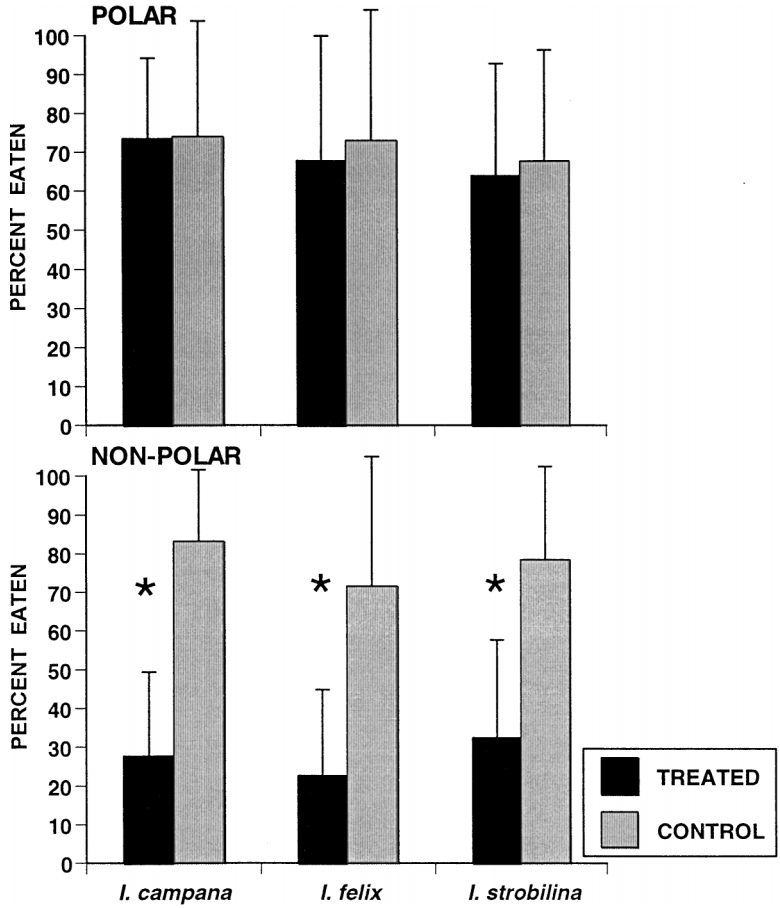

FIG. 4. Field assay. Consumption by reef fishes of paired control food strips and strips containing natural concentrations of recombined polar (water and BuOH) and nonpolar (DCM and hexanes) solvent partions of the crude organic extracts of three *Ircinia* spp. Data presented as in Figure 2.

Food pellets containing roughly natural concentrations of the volatile metabolites from *I. felix* and *I. strobilina* were readily eaten by *Thalassoma bifasciatum* in aquarium assays. Pellets exhibited odor at the same level of intensity as sponge slurry when presented to the fish. Food pellets containing 1, 10, and 100 times the natural concentration of dimethylsulfide as tissue of *Ircinia* spp. were also readily eaten by fish in aquarium assays, although these pellets did not have a noticeable smell at the time of the assays.

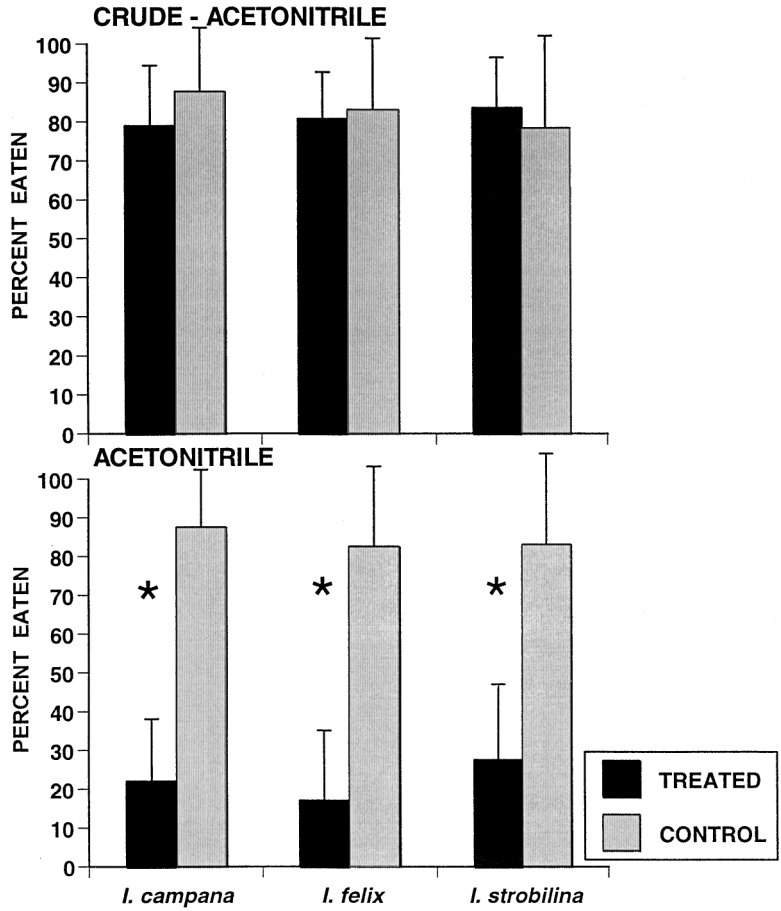

FIG. 5. Field assay. Consumption by reef fishes of paired control food strips and strips containing natural concentrations of the recombined crude extract minus the acetonitrile repartition and the acetonitrile repartition from the crude organic extracts of three *Ircinia* spp. Data presented as in Figure 2.

DISCUSSION

Since the initial survey of crude extracts of 73 species of Caribbean sponges for their capacity to deter feeding of the generalist predatory fish *Thalassoma bifasciatum* in aquarium assays (Pawlik et al., 1995), bioassay-guided fractionation using aquarium and field assays has resulted in the isolation and identification of several metabolites responsible for sponge chemical defenses against fish predators: amphitoxin from *Amphimedon compressa* (Albrizio et al., 1995), oroidin and

TABLE 1. CONCENTRATION OF FRACTIONS FROM *Ircinia* spp. THAT DETERRED FISH FEEDING IN AQUARIUM AND FIELD ASSAYS[a]

Species	Concentration (mg/ml)		
	Fraction A	Fraction B	Total
Ircinia campana	4.17	3.08	7.52
Ircinia felix	5.23	3.84	9.07
Ircinia strobilina	3.35	2.43	5.78

[a] Fraction B was identified as the FTA variabilin **(1)**, while fraction A contains a mixture of the other FTAs.

related metabolites from *Agelas* spp. (Chanas et al., 1996; Assmann et al., 2000), stevensine from *Axinella corrugata* (Wilson et al., 1999), and triterpene glycosides from *Erylus formosus* (Kubanek et al., 2000). The present study confirms that the metabolites of *Ircinia* spp. that deter feeding by predatory fishes are the FTAs and not the foul-smelling volatile metabolites, as suggested by Zea et al. (1999) and Duque et al. (2001). Because the technique for transferring the volatile metabolites from macerated sponge tissue to the assay food did not rely on a precise quantitative method, but rather a comparison of the intensity of the odor, it is possible that the concentration of volatiles transferred to the assay food were below natural levels or that some oxidation of the volatiles had occurred in the process. However, it is just as likely that volatile concentrations exceeded natural levels in replicate experiments. In any case, we observed no hesitation on the part of the assay fish

TABLE 2. AQUARIUM ASSAY: CONSUMPTION OF FOOD PELLETS BY *Thalassoma bifasciatum* CONTAINING EXTRACTS AND ISOLATED METABOLITES FROM *Ircinia felix*[a]

Sample	Concentration (mg/ml)	Treated pellets eaten
Crude extract		0.33 (±0.33)
Crude extract minus fractions A + B		9
Fraction A	5.23 (natural)	0
	4.0	2
	2.0	4
	1.0	7
Fraction B	3.84 (natural)	0
	2.0	0
	1.0	2
	0.5	6
	0.25	9

[a] Fish consumed all 10 control pellets in each assay. $N = 10$ replicates for assays of the crude extract. For any individual assay, samples are considered deterrent if the number of pellets eaten is ≤ 6 ($P \leq 0.043$, Fisher exact test).

in consuming food pellets made from volatile-treated assay food in replicate experiments performed with two *Ircinia* spp., and these pellets retained the sponge odor throughout the assay process. We have used *Thalassoma bifasciatum* as an assay fish for testing the defensive properties of secondary metabolites from marine organisms for over 15 years (Pawlik et al., 1987) and have found that deterrent metabolites will elicit avoidance behavior in assay fishes (repetitive mouthing, opercular flaring, followed by subsequent consumption) at concentrations that are a fraction of those that result in immediate rejection. In this study, fish responses to volatile-treated pellets were no different from those to control pellets (immediate consumption). Given that these bad-smelling volatiles do not deter fish feeding in aquarium assays, it appears that they neither act as direct natural deterrents nor serve to signal the fish of the presence of FTAs in the sponge tissue (Duque et al., 2001).

Concentrations of FTAs were greatest in *I. felix*, followed by *I. campana* and *I. strobilina* (Table 1), as was also reported by Martínez et al. (1997) for the same species from the Colombian Caribbean. Levels of FTAs for all three species were several-fold higher than required to deter predation by generalist fishes (Table 2). Although concentrations of FTAs were lower in the surface tissues than in the sponge interior of *Ircinia* spp. (Zea et al., 1999), surface concentrations exceeded those necessary to deter fish predation, and it is likely that any bites taken by generalist predatory fishes would include both surface and internal sponge tissues.

Despite the presence of FTAs, *Ircinia* spp. are subject to predation by specialist sponge predators. In their survey of the diets of Caribbean fishes, Randall and Hartman (1968) reported that *I. strobilina* was found in the guts of five spongivorous species and was the most abundant sponge in the guts of three, comprising 30%, 9%, and 5.6% of the gut contents of the tilefishes *Cantherhines macrocerus* and *C. pullus* and the queen angel, *Holacanthus ciliaris*, respectively. *Ircinia* spp. were also consumed by some seastars (Wulff, 1995; Waddell and Pawlik, 2000a), although avoided by hermit crabs (Waddell and Pawlik, 2000b). Clearly, some fish and invertebrate predators have circumvented the chemical defenses of *Ircinia* spp.

Although other possible roles of the odor-producing volatile compounds from *Ircinia* spp. (e.g., antifouling, antiovergrowth) have not been studied and cannot be ruled out, it may be possible that these compounds are produced as metabolic by-products. For example, dimethylsulfide is an apparent waste product of the digestion of phytoplankton by zooplankton (e.g., Dacey and Wakeham, 1986). Similarly, volatile metabolites may be waste products of the digestion of phytoplankton (Pile, 1997) or of endosymbiotic bacteria (Simpson, 1984) by *Ircinia* spp. Nevertheless, the broad spectrum of bioactivities of known marine isocyanide and isothiocyanate compounds (Duque et al., 2001) and the high level at which they are produced and released from *Ircinia* spp. is certainly suggestive of a functional role for these metabolites.

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