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Chemical and structural defenses of Caribbean gorgonians (*Pseudopterogorgia* spp.). I. Development of an in situ feeding assay

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ABSTRACT: An in situ method was developed to assess the deterrent effects of various bioactive terpenes and calcitic sclerites which are hypothesized to function as chemical and structural defenses in gorgonian corals. Assays on coral reefs demonstrated that the crude lipid extracts of the Caribbean gorgonians *Pseudopterogorgia acerosa* and *P. rigida*, as well as the purified terpenoids curcuhydroquinone and curcuquinone from *P. rigida*, deterred natural predators at concentrations below their normal levels in gorgonian tissues. Assays conducted with purified sclerites from *P. acerosa* showed that the sclerites, alone, also function effectively to reduce predation on otherwise palatable foods. This in situ bioassay allows for precise dosage control and its utility in separating chemical and structural aspects of defense is demonstrated.

INTRODUCTION

Gorgonians are known to contain high levels of a variety of novel and highly bioactive compounds which have been hypothesized to function in chemical defense (Fenical 1982, Bakus et al. 1986). In the last 2 decades more than 100 papers have appeared describing the isolation of novel sesquiterpenes, diterpenes, and other potentially defensive products of fatty acid metabolism from gorgonians (see reviews by Tursch et al. 1978, Faulkner 1984, 1986). These secondary compounds are of interest due to their novel structures and potential pharmaceutical and agrichemical applications. The functions of these secondary compounds remain controversial, however, and various functions have been hypothesized including ichthyodeterrence (Gerhart 1984, 1985, 1986, Pawlik et al. 1987), competitor deterrence (Coll et al. 1982, Sammarco et al. 1983, Rittschof et al. 1985), and microbial as well as algal deterrence (Targett et al. 1983, Bandurraga & Fenical 1985, Bakus et al. 1986). It is likely that many secondary compounds have multiple functions and thus form the basis of a complex chemical defense system (Burkholder 1973, Standing et al. 1982, Rittschof et al. 1985, Gerhart 1986).

In most analyses of gorgonian defenses, researchers have concentrated on the chemical elements of gorgonians and the potential ichthyodeterrent properties of their secondary metabolites. Chemical defenses provide an intuitively appealing explanation for how sessile long-lived colonies, without extensive calcium carbonate skeletons, can survive the intense predation characterizing most tropical reef environments. In their pursuit of chemical defenses, however, researchers have tended to overlook the potential defensive importance of the calcium carbonate sclerites which are embedded in the coenenchyme and can comprise up to 90 % of the dry weight in some species (see Carey 1918, Kinzie 1971, Koehl 1982, Harvell & Suchanek 1987). A more balanced approach, such as that employed by Sammarco et al. (1987) with alcyonaceans, would consider the integrated chemical and structural defensive adaptations of gorgonian colonies.

To begin the task of unravelling the function of gorgonian secondary compounds and their structural elements, we need realistic bioassays which allow assessments of their relative toxicities and deterrent properties in natural systems. Despite the large number of studies designed to assay deterrent or toxic properties of marine natural products (Bakus & Thun 1979,

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Bakus 1981, Coll et al. 1982, 1986, Coll & Sammarco 1983, LaBarre et al. 1986), there are few realistic assessments of the dosage properties of these compounds applied to predators in their natural environments (but see Gerhart 1984). In addition, techniques are needed for separating the deterrent properties of secondary compounds from the deterrent properties of structural elements such as the sclerites.

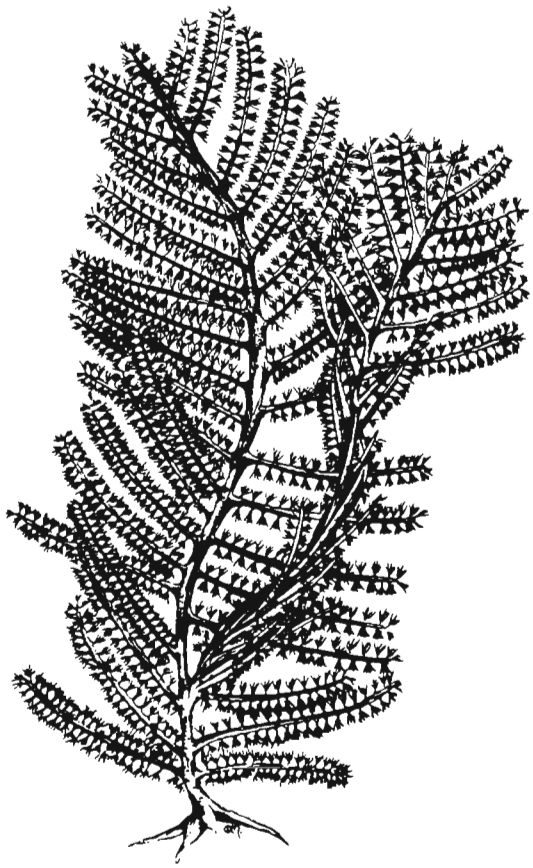


Fig. 1 Diagram of *Pseudopterogorgia* spp.

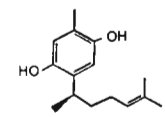
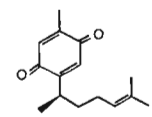
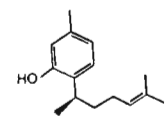
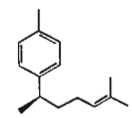
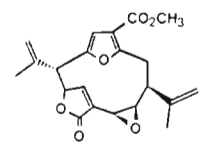
In this paper we introduce an experimental field technique for assessing the deterrent properties of pure compounds, crude extracts, and structural elements derived from gorgonians. The bioassay described is specifically intended for carnivorous fishes and involves preparation of a carageenan-based artificial food which can be rapidly deployed in a replicated concentration series in appropriate gorgonian-rich habitats. We worked with crude extracts from 2 species of gorgonians, *Pseudopterogorgia rigida* and *P. acerosa*, and assessed the relative deterrence of the 2 dominant secondary compounds from *P. rigida* (Fig. 1). These species were chosen because their lipid extracts showed significant deterrent properties at low concentrations in previous laboratory experiments (Pawlik et al. 1987). Although the 2 species are almost indistinguishable morphologically, and are very similar in spicular characteristics, their chemical components are distinct and represent different chemical classes.

MATERIALS AND METHODS

Study sites. We collected samples and conducted experiments at 2 distant sites representing the eastern and western Caribbean: (1) the Grenadine Islands, which are in the windward southern Antilles just north of Venezuela (12°43′ N, 61°20′ W) (our primary collecting and study sites were Union and Canouan Islands); and (2) Carrie Bow Cay, a Smithsonian Research Station located directly on the barrier reef in Belize.

Isolation of compounds and extract composition. We conducted all palatability experiments with crude dichloromethane extracts and purified terpenoid compounds derived from *Pseudopterogorgia rigida* and *P. acerosa*. The lipid soluble compounds of these 2 closely related species can easily be distinguished using Thin

Table 1. Composition of the chloroform extracts from 2 species of *Pseudopterogorgia* by dry weight. Estimates are derived from measurement of the average composition of 20 colonies. Minor elements (•) were estimated qualitatively

Compound	Structure	% Composition
P. rigida		
Curcuhydroquinone		18%
Curcuquinone		6–8%
Curcuphenol		4–6%
•Curcumene		4%
P. acerosa		
Pseudopterolide		7–9%

Layer Chromatography (TLC) (Fenical 1982). In Table 1, we list the major secondary compounds comprising the dichloromethane extract from *P. rigida* and *P. acerosa*. We measured dry weights of gorgonian tissue with the proteinaceous axial support rod removed. The maximum average weight of crude extract per total dry weight was 35 % from *P. rigida* and 7.5 % from *P. acerosa* (measured on samples from the Grenadines; Harvell & Fenical 1989). Since predatory fishes appeared to perceive the concentration of defensive compounds volumetrically rather than on a dry weight basis, we converted our dry weight to volumetric compositions. We used a conversion factor of 0.9 $ml\,g^{-1}$ since this is an average specific gravity of virtually all lipid compounds (CRC Handbook). An estimate of volumetric compositon of extracts was 14 % for *P. rigida*. We will assume that the scaling of dry weight to volumetric is similar for *P. acerosa*, in which case the volumetric concentration of the extract is ca 3.5 %. To calculate volumetric composition of sclerites, we used a conversion factor of 2.710 for calcite (CRC Handbook).

Curcuquinone and curcuhydroquinone are the major terpenoid metabolites found in *Pseudopterogorgia rigida* (McEnroe et al. 1978; Table 1). An extract of *P. rigida* was prepared by macerating the fresh gorgonian in chloroform, filtering the undissolved animal parts, and removing the volatile solvent under reduced pressure. The extract was chromatographed, by vacuum flash methods, over TLC grade silica gel to yield fractions containing curcuquinone and curcuhydroquinone eluted with 25 % EtOAc in isooctane. Preparative silica HPLC (high-performance liquid chromatography) with 15 % EtOAc/isooctane yielded purified curcuquinone while 25 % EtOAc/isooctane yielded purified curcuhydroquinone. Both purity and identity of these terpenoids were confirmed by 360 MHz 'H magnetic resonance.

Laboratory palatability assays. Our initial experiments were conducted in the laboratory of the research vessel 'Columbus Iselin' to determine the concentrations of compounds to which the fishes would be sensitive. All laboratory experiments were conducted with freshly captured blue head wrasse *Thalassoma bifasciatum* in flow-through aquaria. Fish were contained in 3 aquaria, with 5 to 7 male and initial-phase *T. bifasciatum* per aquarium. *T. bifasciatum* were conditioned for 3 d and trained to consume brine shrimp particles released from a pipet. Our protocol for the assays is as described by Pawlik et al. (1987): Standard weight pieces of dried brine shrimp (0.025 g) were saturated with an appropriate concentration of extract in diethyl ether and then offered to fish alternately with control shrimp pieces which had been coated only with ether. Since 16 fishes were used in 4 separate aquaria, each trial was assumed independent of all others. Particles were scored as consumed if they were ingested and not subsequently expelled. Particles were scored as rejected either (1) if they were taken into the mouth and then rejected, or (2) if they sank to the bottom of the tank without being ingested. Since the fish actively ingested most particles, a rejection response was obvious.

Field palatability assays. Once we had detected a range of concentrations that the fish were sensitive to in the laboratory, we then conducted field experiments to test their sensitivity to varying concentrations in nature. Field experiments were conducted in the Grenadines and in Belize. In the Grenadines, we used 4 % agar (supplemented with urchin roe to make it attractive to fish) as a matrix for the artificial food. We

Table 2. Composition of the artificial diet in experiments conducted in the Grenadines and Belize. CMPD: compound; % Vol: volumetric percentage; % Dry: dry weight percentage

Treatment	Urchin rose (ml)	Shrimp (g)	Carag/Agar (g)	Fresh water (ml)	EtOH (ml)	CMPD (g)	% Vol	% Dry wt
Grenadines								
P. rigida crude	20	—	2.5	10	1	0.10	0.29	5.0
Curcuhydroquinone	20	—	2.5	10	1	0.05	0.14	2.0
Curcuquinone	20	—	2.5	10	1	0.05	0.14	2.0
Belize								
P. rigida crude	—	0.20	0.33	20	1	0.25	1.12	52
P. rigida crude	—	0.20	0.33	20	1	0.50	2.14	48
Curcuhydroquinone	—	0.20	0.33	20	1	0.10	0.43	16
Curcuhydroquinone	—	0.20	0.33	20	1	0.20	0.86	27
Curcuhydroquinone	—	0.20	0.33	20	1	0.30	1.28	36
P. acerosa crude	—	0.20	0.33	20	1	0.25	1.07	52
P. acerosa sclerites	—	0.20	0.33	20	0	0.05	0.67	—
P. acerosa sclerites	—	0.20	0.33	20	0	2.5	33.87	—
P. acerosa sclerites	—	0.20	0.33	20	0	5.0	67.77	—

Table 3. Results of laboratory assays with crude extracts of *Pseudopterogorgia* and *P. acerosa* and pure compounds from *P. rigida*. % dry wt: concentration in dry weight equivalents used in the experiment; % rejection: percentage of trials in which fish rejected the food

Extract	Experimental			Control		p	X^2
	% dry wt	% reject	(*n*)	% reject	(*n*)		
P. acerosa crude	8	25	(8)	0	(8)	0.01	6.667
P. rigida crude	12	100	(6)	0	(6)	0.004	8.333
	6	100	(6)	16	(6)	0.019	5.486
	3	83	(6)	0	(6)	0.019	5.486
	1.5	83	(6)	0	(6)	0.019	5.486
P. rigida compounds							
Curcumene mixture	0.04	60	(10)	0	(10)	0.015	5.952
	0.08	40	(5)	0	(5)	NS	
Curcuquinone	0.09	80	(10)	0	(10)	0.001	10.21
Curcuhydroquinone	2.2	100	(5)	0	(5)	0.011	6.400
	1.1	100	(5)	0	(5)	0.011	6.400
	0.6	80	(5)	0	(5)	NS	
	0.3	80	(5)	0	(5)	NS	
	0.1	80	(10)	10	(10)	0.007	7.270
Curcuhydroquinone monoacetate	0.005	20	(5)	0	(5)	NS	

initially added each compound to the agar diet, with 2% ethanol as carrier, in the same concentration as the effective concentration from the laboratory trials. The composition of the artificial diet in different experiments is given in Table 2. In Belize, pure compounds, crude extracts, and sclerites were added to a 2% carageenan (Gelcarin FF961L #434, lot #581015) mixture. Carageenan was found to be a superior matrix for these experiments because the strips were flexible, not easily torn, yet sheared readily when bitten by a fish. We calculated the proportion of each additive per unit volume of gorgonian tissue (ml compound/ml hydrated gorgonian tissue). This solution could be poured into a mold which produced strips 0.2 × 0.5 × 4.0 cm attached to cotton strings. Targett et al. (1986) employed a similar technique with agar 'popsicles' to assess the deterrent effects of plant secondary compounds on herbivorous fish feeding in laboratory trials. Once our strips were constructed, they were tied to 1 m polypropylene lines (1 experimental and 1 control per rope) and haphazardly deployed on the reef. Experiments were conducted on shallow 3 to 9 m fore-reef habitats. Before running our experiments, we surveyed several reefs by setting out ropes with carageenan strips to locate areas with high fish predation. When running experiments, we deployed ropes onto the reefs at both locations at ca 10:00 h and collected them after 14:00 h each day. We distributed between 15 and 30 replicates of each treatment per trial and conducted between 1 and 3 trials per experiment (see 'Results' for actual sample sizes). Since the success of the assay is highly dependent upon adequate predation on the controls, we analyzed each experiment separately and relative to its controls. Using this type of assay, comparisons of trials run at different time intervals or in different locations would be difficult not only because compounds might slowly leach from the carageenan strips at different rates but also because fish predation rates vary with factors such as time of day, water visibility, and current velocity (Harvell pers. obs.). Thus it is important to compare only trials made under similar conditions. Hay et al. (1987) employed a similar technique to assess deterrence of algal extracts using cut *Thalassia* blades deployed similarly on lines. In their studies, soluble extracts were coated on the surface of *Thalassia* blades and consumption was monitored for each of the different treatments.

Sclerites were incorporated into the carageenan mixture to determine if structural elements affected fish feeding preferences. Sclerites were obtained from *Pseudopterogorgia acerosa* by soaking colonies in bleach overnight, washing the residue away, repeatedly rinsing sclerites and soaking in fresh water and sodium thiosulfite (3%) for 1 to 2 d to remove all traces of bleach, then air drying for 1 to 2 d.

RESULTS

Palatability

The compositions of extracts of *Pseudopterogorgia rigida* and *P. acerosa* are described in Table 1. Although we suspect that there will be minor geographic variation in the quantitative composition of these extracts, we will refer to the composition of the

Table 4. Results of in situ field assays in the Grenadines. *N*: no. of strips with a least 1 bite/no. of strips total. Only replicates with at least 1 bite removed from either control or experimental treatment were analyzed. Significance test with Mann-Whitney *U* test. –: insufficient replicates for test

Compound	Mean number of bites taken				*p*
	Experimental	(*N*)	Control	(*N*)	
Curcuhydroquinone	1.2 ± 1.3	(13/13)	4.8 ± 4.5	(13/13)	< 0.05
Curcuquinone	0.3 ± 0.7	(7/12)	8.0 ± 4.6	(7/12)	< 0.05
Curcumene mixture	3.7 ± 2.5	(3/7)	3.7 ± 5.5	(3/7)	—

extract from the Grenadines as the ambient composition. In our laboratory palatability experiments we found that crude extracts of *P. rigida* effectively deterred fish even at a concentration of 1.5 % (or ⅛ normal concentrations; Table 3). Subsequently, we analyzed each fraction of the crude extract for deterrence. A hydrocarbon mixture consisting mainly of curcumene, a triglyceride mixture, and the monoacetate of curcuhydroquinone (Table 1), which are minor components of *P. rigida*, did not significantly deter consumers. Curcuhydroquinone and curcuquinone had significant deterrent effects. Curcuhydroquinone was still deterrent at 1/16 of its ambient concentration (Table 3). An ambient concentration of *P. acerosa* crude extract also was deterrent (Table 3).

The same compounds also were deterrent in field experiments. In the Grenadines, curcuhydroquinone and curcuquinone significantly deterred fish at about half normal concentrations, but the curcumene mixture did not (Table 4). In Belize, we repeated the experiments with *Pseudopterogorgia rigida* extract. The crude extract showed significant deterrence at a concentration of 2.5 % by volume; there was no significant deterrent effect at 1 % by volume even though the dry weight composition exceeded that previously measured in colonies by almost an order of magnitude (Fig. 2). This suggests that consumers are sensitive to changes in volumetric concentrations and not to changes in dry weight concentrations. The approximate concentration of the extract in normal tissue is 14 % by volume. The hydroquinone fraction (Fig. 3) showed no deterrent activity at 0.4 % but did at 0.9 and 1.3 %.

Crude extracts of *Pseudopterogorgia acerosa* were deterrent to fishes in field experiments at ca 1 % by volume in artificial foods (Fig. 4). The normal concent-

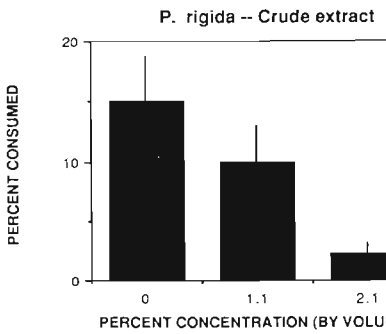


Fig. 2. Carnivory assay. Mean percent of carageenan strips consumed by fish in field trials in Belize. Error bars are 1 standard error. Two concentrations of *Pseudopterogorgia rigida* extract were incorporated into the strips. Probabilities that differences between treatments and controls arose due to chance were calculated with a Mann-Whitney *U* test: control and 1.1 %, $p = 0.510$; control and 2.1 %, $p = 0.006$

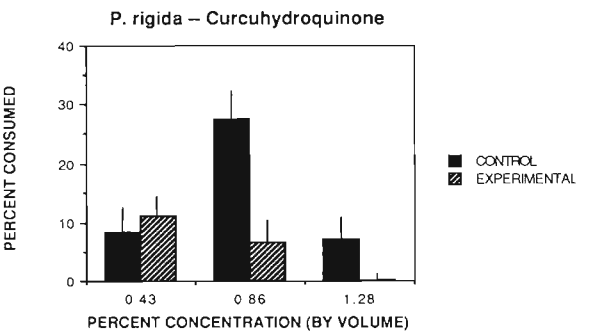


Fig. 3. Carnivory assay. Mean percent of carageenan strips consumed by fish in field trials in Belize. Error bars are 1 standard error Three concentrations of pure curcuhydroquinone were incorporated into treatment strips. Mann-Whitney *U* test statistic for 0.43, 0.86, and 1.28 %: $p = 0.474$, $p = 0.009$, $p = 0.010$ respectively

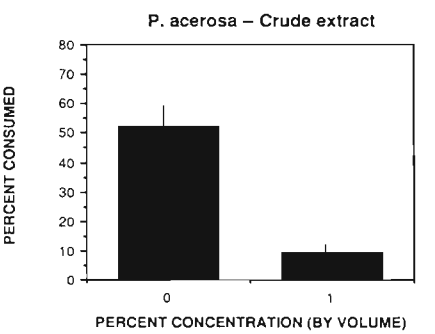


Fig. 4. Carnivory assay. Mean percent of carageenan strips consumed by fish in field trials in Belize. Error bars are 1 standard error. One concentration of *Pseudopterogorgia acerosa* crude extract was incorporated into treatment strips. Mann-Whitney *U* test statistic: $p = 0.001$

ration in colonies is ca 3.2% by volume. Thus, at concentrations of ½ ambient, these extracts have a deterrent effect.

Sclerites deterred fish at concentrations of 34 and 68% by volume. There was no deterrent effect of sclerite controls at 0.7% (Fig. 5).

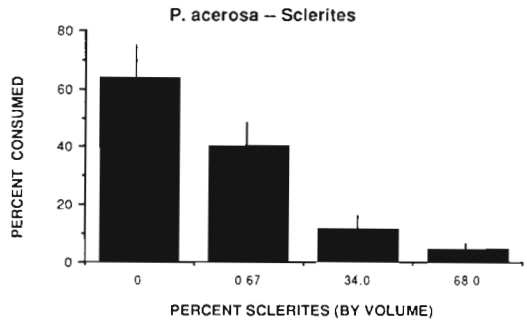


Fig. 5. Carnivory assay. Mean percent of carageenan strips consumed by fish in field trials in Belize. Error bars are 1 standard error. Three concentrations of sclerites were incorporated into the treatment strips. Kruskal-Wallis test for differences among ranks of 4 categories: $p<0.001$. Multiple comparisons among all groups except 34 and 68% are significant ($p<0.05$) with a Scheffe multiple comparisons test

A number of fish species frequented the sites of the field palatability assay. Carnivorous or omnivorous fish observed in the vicinity were: blue head wrasse *Thalassoma bifasciatum*, yellow head wrasse *Halichoeres garnoti*, clown wrasse *Halichoeres maculipinna*, Sargent majors *Abudefduf saxatilus*, Beau Gregory *Eupomacentrus lecoster*, bicolor damsel *Eupomacentrus partitus*, foureye butterflyfish *Chaetodon capistratus*, banded butterflyfish *Chaetodon striatus*, and rock beauty *Holocanthus tricolor*. We also observed herbivorous fishes, including parrotfishes (redband parrot fish: *Sparisoma aurofrenatum*) and surgeonfishes. Of these fishes, we observed wrasses *T. bifasciatum* and surgeonfishes feeding on our assay. In Belize, we additionally observed grey angel fish *Pomacanthus arcuatus* feeding on experiments. At both sizes, *T. bifasciatum* was the dominant consumer. However, the assays were sampled by a variety of fishes as indicated by a range of different sized and shaped bite marks on the strips.

We also observed several species of fish feeding naturally on gorgonians in Belize. As previously noted by Randall (1967), Birkeland & Neudecker (1981), and Lasker (1985), *Chaetodon capistratus* is a regular consumer of a variety of gorgonian species. We also observed scrawled file fish *Alutera scripta* and *Thalassoma bifasciatum* consuming polyps occasionally in Belize (Harvell pers. obs.).

DISCUSSION

The *Pseudopterogorgia rigida* extract is composed of 4 potentially deterrent secondary compounds (Table 1). The 2 comprising ca 26% of the extract, curcuhydroquinone and curcuquinone, are each independently deterrent to fishes. Crude extracts of *P. acerosa* also effectively deterred fish predation on artificial foods. The curcumene was not as active as the major compounds.

From our results, it appears more useful to measure dosage of compounds volumetrically than by dry weights. This is likely to be important with animals like gorgonians which are composed of water-filled tissue where large volumes can be occupied by small dry weights. An advantage of employing the carageenan assay is that volume and dry weight can be manipulated independently. For example, we show that a 1% volumetric change can be detected by fishes in field experiments, while a 20% dry weight change was not detected.

Both crude extracts and dominant secondary compounds from *Pseudopterogorgia rigida* such as curcuquinone and curcuhydroquinone are extremely active as fish feeding deterrents at low concentrations. Terpenes of terrestrial plants have been implicated in producing significant biological and physiological effects in a number of cases (Rosenthal & Janzen 1979). In particular, quinones have been implicated in redox reactions in which they are capable of binding proteins such as major metabolic enzymes (Thomson 1971). Hydroquinones and quinones are well known as secondary metabolites of terrestrial plants and as defensive compounds of insects in terrestrial systems (Aneshansley et al. 1969, Eisner 1970, Thomson 1971).

We have based our estimates of compound concentrations on the levels contained within polyps. These were determined not by a bulk colony approach, but by subsampling different regions of colonies. Maximum concentrations occur in the polyps (Harvell & Fenical 1989). Since fishes are largely consuming polyps, this is the approximate dosage they ingest. Some fishes, such as *Alutera scripta*, consume gorgonian coenenchyme (Randall 1967) and may ingest a lower dose.

Our bioassay is straightforward to employ and is an advance over previous assays in 3 respects:

(1) *Precise dosage control:* Since we incorporate a known quantity of compound into a known volume of matrix, we control exactly the dose any consumer receives (to the extent to which diffusion of compounds does not reduce the dosage through time). Dosage is critical since many potentially deterrent compounds may occur naturally at levels which have no deterrent capability. A secondary compound can only be consid-

ered a defense if it is deterrent at the same concentrations in which it occurs in the organism. Thus it is critical to both assess normal concentrations of compounds and conduct experiments that bracket the range.

(2) *Ingestive assay:* Most chemical defenses against consumers probably act after ingestion. Thus assays should be applied in the same way. It is difficult to assess the potential deterrent properties of an organism's constituent compounds by running experiments in which a fish is exposed to a high dosage of the compounds released into the water by macerating the organism's tissues. This approach has been useful in screening for compounds with extremely toxic properties, but provides little information about deterrent and nontoxic compounds. To assess the deterrent potential of a compound or mixture of compounds it is critical to conduct an ingestive assay.

(3) *Natural consumers under field conditions:* The use of non-marine fish to assess deterrence of marine defensive compounds is not useful. To understand how these compounds function, we ideally want to assess their effect on natural consumers behaving in their natural environment. Hence field assays with resident fish are the most meaningful approach. In studies of the adaptation of resident fish to deterrent compounds, it may be useful to contrast their behavior with non-marine fish, which may be unable to consume even very low dosages of some of these compunds.

This technique will allow us to address general questions in chemical ecology that have been intractable previously, but now are more straightforward to assess: (1) How does relative deterrence vary among different classes of compounds? (2) Are chemically rich species more deterrent than species with single secondary compounds? (3) Is there a trade-off between effort dedicated to chemical and structural defense? (4) Does increased food value reduce the deterrent properties of defensive compounds? (5) What are the mechanisms of action of these terpenoid compounds? (6) Are there synergistic effects of compounds (cf. Berenbaum 1986)?

This study also indicates the potentially important role that structural elements may play in the defense of gorgonian corals. Most fishes that prey on gorgonians (e.g. chaetodontids) are reported to consume only the polyps and not coenenchyme (Birkeland & Neudecker 1981, Lasker 1985, Harvell pers. obs.). Most of the species that are regularly attacked by fishes (plexaurids, gorgoniids, pseudopterogorgiids) do not have polyps heavily armored with sclerites, in contrast to other groups such as the muriciids. However, the coenenchyme in these groups is heavily invested with sclerites. The distribution of sclerites within colonies may play as important a role in defense as the distribution of chemical compounds (cf. Harvell & Fenical 1989). In work with sponge spicules, Koehl (1982) suggested that spicules in sponges may serve an important role in defense as well as support. Similarly, Sammarco et al. (1987) have emphasized the importance of spicules in Australian soft corals. The role of sclerites in gorgonians has not been previously investigated experimentally.

The demonstration that the major secondary terpenoid compounds of *Pseudopterogorgia* spp. have strong ichthyodeterrent properties is only the first step in unravelling the complex chemical and defensive adaptations of gorgonians. However, before we can assess issues important to understanding the evolution of defenses such as spatial, temporal, and genetic variation in levels of secondary compounds, we need to calibrate the effective concentrations of deterrent compounds. We favor the use of in situ field assays as an approach to assessing the relative deterrence of various chemical and structural elements because they provide a measure of the effectiveness of these elements against consumers in nature.

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