Chemical defense of the Caribbean ascidian

Didemnum conchyliatum

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ABSTRACT: Field and laboratory experiments were performed to investigate the palatability to predatory fishes of organic extracts of the Caribbean ascidian Didemnum conchyliatum. This tan-colored compound ascidian grows as an epibiont on seagrass blades. A dichloromethane/methanol extract of the ascidian incorporated into carrageenan food strips at the same volumetric concentration as the extract occurred in the ascidian tissues deterred feeding of a natural assemblage of consumers in the same seagrass beds from which the ascidians were collected. Bioassay-directed fractionation of this extract revealed that the deterrent property was restricted to fractions containing novel indole-maleimide-imidazole alkaloids, didemnimides A to D. Laboratory assays of purified metabolites revealed that didemnimides C and D deterred feeding by a generalist predatory reef fish, but that didemnimides A and B were not deterrent. Only didemnimide D deterred feeding in the field; neither didemnimide C nor the fraction containing didemnimides A and B were deterrent when assayed in the natural environment of the ascidian. Antipredatory activity of the didemnimides was highly dependent on compound structure; only didemnimide D, which bears both a bromine on the indole ring and a methyl group on the imidazole ring, inhibited feeding in both laboratory and field assays. Didemnimide D deterred feeding at approximately 0.035 mg ml⁻¹, and is among the most potent antipredatory chemical defenses described from tunicates to date.

KEY WORDS: Ascidian · Chemical defense · Caribbean · Predation · Structure-function relationship

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 1992, 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, McClintock et al. 1994, Pennings et al. 1994).

Most studies of marine invertebrate chemical defenses have focused on species from tropical reef habitats, where predation by fishes is intense (Hixon 1983, Grigg et al. 1984, Huston 1985). Less well studied are the chemical defenses of invertebrates from tropical, shallow soft-sediment environments: seagrass beds and mangroves. These habitats, which are geographically much more expansive than coral reefs, are under the influence of many of the same consumer species of fishes, in addition to a set of distinct fish and invertebrate predators (Thayer et al. 1987, Blaber et al. 1992, Worthington et al. 1992, Connolly 1994). Soft-sediment environments serve as nursery grounds for juveniles of many fishes that move off-shore as they mature.

Among the fouling invertebrates of tropical soft-sediment environments are several genera of ascidians, especially of the Didemnidae (Por 1984). Benthic ascidians appear to suffer little predation by generalist
predators (Millar 1971, Goodbody & Gibson 1974, Stoecker 1980), although they are consumed by some predatory molluscs (Young 1986, Dalby 1989, McClintock et al. 1994). Although didemnid ascidians have calcitic spicules in their tissues, available evidence suggests that they do not function as a structural defense against fish predators (Lindquist et al. 1992). In addition, some ascidians possess acidic inclusions or high concentrations of heavy metals in their tissues (e.g. vanadium, Hawkins et al. 1980); these substances were thought to inhibit fouling or predation (e.g. Stoecker 1978), but subsequent investigations have disputed this assessment (Parry 1984, Davis & Wright 1989).

In general, the tissues of ascidians are a rich source of alkaloids and amino acid-derived secondary metabolites (reviewed in Davidson 1993, Molinski 1993), although the ecological functions of these compounds are largely unknown. Only very few studies have been performed to examine the feeding deterrent properties of ascidian metabolites, with some evidence for chemical defenses of both larvae and adults (Young & Bingham 1987, Paul et al. 1990, Davis 1991, McClintock et al. 1991, Lindquist et al. 1992).

For the present study, we assessed the chemical defenses of Didemnum conchyliatum, a tan-colored didemnid ascidian from shallow Caribbean soft-sediment environments. A large population of D. conchyliatum was found encrusting the blades of the seagrass Thalassia testudinum in shallow (<3 m) seagrass meadows between mangrove islands at Sweetings Cay, Grand Bahama Island, Bahamas. Evidence of predation on this conspicuous soft-bodied animal was not observed. Preliminary chemical analysis of the organic extract of D. conchyliatum indicated the presence of large amounts of relatively polar, orange- and yellow-colored, secondary metabolites. The great abundance of D. conchyliatum at this site provided sufficient material for the isolation and characterization of these compounds (Vervoort et al. 1997). At the same time, we addressed the following questions concerning the ecological functions of these metabolites: first, do these compounds function as chemical defenses against generalist predators? Second, is there a relationship between the structure of the metabolites and their function as a feeding deterrent?

**MATERIALS AND METHODS**

**Ascidian collection.** This study was largely completed during an expedition to the mangroves surrounding Grand Bahama Island, Bahamas, on board the research vessel 'Seward Johnson' in September 1994. Approximately 2100 ml wet volume of the compound ascidian Didemnum conchyliatum was collected at 1 to 3 m depth from a seagrass meadow between mangrove islands at Sweetings Cay. The collection included many hundreds of colonies from a large area (approx. 0.25 km²). The ascidians were immediately extracted for ecological and chemical investigations.

**Extraction and isolation.** The volume of the collected ascidians was determined by displacement in methanol. The tissue was then fully extracted with methanol and twice with a 1:1 mixture of dichloromethane (CH₂Cl₂) and methanol. The resulting extracts were combined and divided into aliquots, each from a known volume of ascidian tissue.

One aliquot of the crude extract of Didemnum conchyliatum was used to separate the constituent metabolites of the extract by partitioning them between solvents of different polarities. The crude extract was successively partitioned between water and isooctane, dichloromethane, ethyl acetate, and 2-propanol. The resulting 5 partitions were subjected to aquarium assays (see below).

The isooctane, dichloromethane, and ethyl acetate partitions each had some feeding deterrent activity, and contained metabolites in common when subjected to silica gel thin-layer chromatography (TLC) on Merck plastic-backed plates impregnated with fluorescent indicator. These 3 partitions were combined for subsequent fractionation by vacuum flash chromatography on silica gel employing a 0 to 50% methanol in dichloromethane gradient. The least polar fractions yielded a mixture of hydrocarbons, which were combined into a single fraction designated I. The next more polar fractions contained bright yellow-colored compounds, later identified as didemnimides C and D, which were combined into a single fraction designated II. The next more polar fractions contained bright orange-colored compounds, later identified as didemnimides A and B, which were combined and designated III. The 2 most polar, colorless fractions were designated IV and V. Volumetric portions of all 5 fractions were tested for feeding deterrence in aquarium assays and field assays (see below).

A portion of fraction II was further separated by reverse phase liquid chromatography on an open column with 30% water in methanol as eluent, followed by normal phase liquid chromatography on an open column with 5% methanol in dichloromethane as eluent. This procedure yielded the pure didemnimides C and D, in order of decreasing polarity, and a remaining mixture of less polar compounds that were not further identified. Didemnimides C and D and the remaining mixture were tested separately for feeding deterrence in aquarium assays and were used in field assays.

**Structural assignments of secondary metabolites.** A portion of fraction III was further separated by reverse
Phase liquid chromatography on an open column with 35% water in methanol as eluent, followed by normal phase liquid chromatography on an open column with 5% methanol in dichloromethane as eluent. This procedure yielded the pure didemnimides A and B, in order of decreasing polarity. Didemnimide A was subjected to single crystal X-ray analysis to identify the carbon skeleton of the didemnimides (Vervoort et al. 1997). Didemnimides A to D were subsequently fully assigned using 1- and 2-dimensional NMR (nuclear magnetic resonance) spectroscopy, UV/VIS (UV and visible light) and infrared spectroscopy, and mass spectrometry (Vervoort et al. 1997).

Field assays. Feeding experiments were conducted in the field on each of 4 samples: the crude extract, purified didemnimide C and D, and a combination containing fractions III, IV, V, and the remains of fraction II after separation of didemnimides C and D. In each case, the sample was volumetrically reconstituted in a matrix of carrageenan at the concentrations as they occurred in the ascidian. Strips of this matrix were then used in field experiments (see methods in Fenical & Pawlik 1991). The matrix was made by combining 1.5 g carrageenan (Gelcarin, FF961L: FMC Corp., Philadelphia, PA, USA) and 3 g lyophilized, macerated squid with deionized water to a total volume of 65 ml. The mixture was heated to boiling in a microwave oven (about 1 min on 'cook'), then the sample, in a minimal volume of solvent (treated matrix) or solvent alone (control matrix), was added. 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 cooled and gelled, 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, colored ink spot.

Field assay methods are described in Pawlik & Fenical (1989). One treatment and 1 control strip each were tied to a 50 cm length of 3-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 in the same seagrass meadow from which the ascidians had been collected, with the end of each rope opposite the food strips attached to a large nail that was pushed into the sandy bottom. Ropes were left in the field for approximately 14 h, overnight. After that time, 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 (1-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.

Laboratory assays. Aquarium assays were performed as described in Pawlik et al. (1995) on the crude extract, each of the 5 solvent partitions, each of fractions I to V, purified didemnimides C and D, and the remains of fraction II after separation of didemnimides C and D. Assays were performed on board the research vessel ‘Seward Johnson’ 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 3 fish (1 terminal phase male, 2 females) were held in each of 15 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 treated 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. Pellets were considered rejected if not eaten after a minimum of 3 attempts, by 1 or more fish, to take them into their mouth cavity, or if the pellets were 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 4 or more of the pellets were rejected (p ≤ 0.043, 1-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 lyophilized, macerated squid with 3 g alginic acid and 100 ml 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 CaCl₂ 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.

RESULTS

Assay food strips containing a crude dichloromethane/methanol extract of Didemnum conchyliatum at the same volumetric concentration as the extract occurs in
the tissues of the ascidians significantly deterred feeding of a natural assemblage of consumers present in the seagrass bed (p < 0.0005, Wilcoxon paired-sample test; Fig. 1). For this and for subsequent feeding assays, food strips were observed being consumed by clown and dwarf wrasse (Halichoeres maculipinna and Dorytonotus megalepis, respectively) and bandtail pufferfish (Sphaeroides spengleri), although several species of juvenile and adult parrotfishes and damselfishes were observed near the assay ropes.

In laboratory aquarium assays employing the reef fish Thalassoma bifasciatum the crude extract of Didemnum conchyliatum and the isoctane and dichloromethane partitions of the crude extract significantly deterred feeding (Fig. 2). The ethyl acetate partition did not significantly deter feeding, but TLC analysis revealed that this partition contained some of the same metabolites as the isoctane and dichloromethane partitions, albeit in lower concentrations. The 2-propanol and water partitions of the crude extract were distinct from the others by TLC, and did not deter feeding in aquarium assays (Fig. 2).

The combined isoctane, dichloromethane, and ethyl acetate partitions were further separated by vacuum flash chromatography into 5 fractions containing hydrocarbons (fraction I), didemninides C and D (fraction II), didemninides A and B (fraction III), and more polar colorless compounds (fractions IV and V). Of these, fractions II and V significantly deterred feeding of Thalassoma bifasciatum in laboratory assays (Fig. 3).

Fraction II was further separated into 2 compounds, didemninides C and D, and a remaining mixture, by open column liquid chromatography. These were subjected to laboratory assays at concentrations volumetrically equivalent to those found in the tissues of Didemnum conchyliatum. Both didemninides C and D deterred feeding of Thalassoma bifasciatum, but the remaining mixture did not (Fig. 4).

Both didemninides C and D, and the remaining mixture of fraction II combined with fractions III, IV and V were all subjected to field assays in the seagrass meadows of the mangrove channel where the ascidians were collected. Although didemnimide D also detere

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**Fig. 1.** Field assay. Feeding by seagrass bed consumers of paired control food strips and strips containing a crude extract of Didemnum conchyliatum at the same concentration as found in the tissues of the ascidians. 1 SD above the mean is indicated. N= no. of paired treatment and control strips retrieved of the 20 deployed (no. of pairs used in statistical analysis). Probability calculated using the Wilcoxon paired-sample test.

**Fig. 2.** Aquarium assay. Consumption by Thalassoma bifasciatum of control food pellets and pellets containing natural concentrations of the crude extract and the solvent partitions (1 = isoctane, 2 = dichloromethane, 3 = ethyl acetate, 4 = 2-propanol, 5 = water) of the crude extract of Didemnum conchyliatum. *p < 0.05, Fisher exact test.

**Fig. 3.** Aquarium assay. Consumption by Thalassoma bifasciatum of control food pellets and pellets containing natural concentrations of fractions resulting from the flash column separation of the combined isoctane, dichloromethane and ethyl acetate fractions of the crude extract of Didemnum conchyliatum. *p < 0.05, Fisher exact test.
feeding of a natural assemblage of mangrove consumers in field assays (p < 0.0005, Wilcoxon paired-sample test; Fig. 5), didemnimide C did not (p = 0.81; Fig. 6), and neither did the combination of fractions III, IV, V and the remaining mixture of fraction II (p = 0.14; Fig. 7).

Didemnimides A to D were reisolated and identified once the samples were brought back to the Scripps Institution of Oceanography (Fig. 8) (Vervoort et al. 1997). They are the first representatives of a novel class of indole-maleimide-imidazole alkaloids. The yellow didemnimides C and D were identified as methylated derivatives of the carbon skeleton. Didemnimide D, the less polar of the two, was found to be brominated. The more polar, orange didemnimides A and B were identified as non-methylated derivatives of this carbon skeleton. Didemnimide B, the less polar of the two, was brominated (Fig. 8).

Calculations of compound concentrations were all based on large collections of many hundreds of colonies of *Didemnum conchyliatum* because individual colonies were too small to be analyzed separately. As a mean value from 10 replicate samples, 100 ml volume of ascidian tissue yielded 28.68 g of dry tissue and 2.68 g of crude organic extract. After solvent partitioning and liquid chromatography, the yields of pure
in this study, didemnimide D, is one of the most potent feeding deterrent metabolites isolated to date, with an active, natural concentration estimated at 0.035 mg ml⁻¹. Among previously studied ascidian metabolites, didemnimide D deters feeding at concentrations lower by an order of magnitude or more, rivaled only by the cyclic peptide nordidemnin B from another Caribbean didemnid, *Trididemnum solidum* (Lindquist et al. 1992). Although the active concentration of didemnimide D is reported as the natural concentration in this paper, the value is likely a conservative one, because of loss of compound during the purification process. In point of fact, the crude extract of *D. conchyliatum* was more deterrent in both field and laboratory assays than subsequently purified fractions or compounds. This may result not only from a loss of active metabolites during purification steps but also from the separation of didemnimide C from the mixture; the latter compound was deterrent in laboratory assays, but not field assays, at natural concentrations. Didemnimide C may enhance the deterrent effects of didemnimide D in an additive or synergistic manner, resulting in the enhanced deterrent capacity of the crude extract. Nevertheless, didemnimide D is clearly the major deterrent compound in the crude extract, because field assays of both didemnimide C and the mixture containing fractions III to V were not deterrent (Figs. 6 & 7).

It is possible that the contrasting tan pigmentation of colonies of *Didemnum conchyliatum* on green seagrass blades serves as aposematic or warning coloration. There is evidence that the orange color of larvae of *Ecteinascidia turbinata* deters predation by fishes that have previously sampled the tasteful larvae (Young & Bingham 1967). Similarly, fish that sample and reject adult colonies of *D. conchyliatum* from seagrass blades may learn to avoid, this potential predator; in the latter case, these compounds may play other ecological roles. The tropical Pacific ascidian *Sigillina signifera* (= *Atapazoa* sp.) contains a series of bipyrole metabolites, the tambilamines, some of which deterred feeding at or below natural concentrations in field assays conducted on Guam reefs (Paul et al. 1990, Davis 1991, Lindquist et al. 1992, Lindquist & Hay 1995). Like the present study, earlier investigators have found that unusual metabolites may or may not deter potential predators; in the latter case, these compounds may play other ecological roles. The tropical Caribbean didemnid *Trididemnum solidum* and its tadpole larvae both contained a suite of didemnin and nordidemnin peptides, which inhibited feeding of reef fishes at concentrations below those found in the ascidian tissues. (Lindquist et al. 1992, Lindquist & Hay 1995). A related species from the same area, *Trididemnum cyanosphorum*, contained a mixture of didemnenones that were not deterrent at natural concentrations (Lindquist et al. 1992).

The deterrent metabolite isolated from *Didemnum conchyliatum* in this study, didemnimide D, is one of the most potent feeding deterrent metabolites isolated to date, with an active, natural concentration estimated at 0.035 mg ml⁻¹. Among previously studied ascidian metabolites, didemnimide D deters feeding at concentrations lower by an order of magnitude or more, rivaled only by the cyclic peptide nordidemnin B from another Caribbean didemnid, *Trididemnum solidum* (Lindquist et al. 1992). Although the active concentration of didemnimide D is reported as the natural concentration in this paper, the value is likely a conservative one, because of loss of compound during the purification process. In point of fact, the crude extract of *D. conchyliatum* was more deterrent in both field and laboratory assays than subsequently purified fractions or compounds. This may result not only from a loss of active metabolites during purification steps but also from the separation of didemnimide C from the mixture; the latter compound was deterrent in laboratory assays, but not field assays, at natural concentrations. Didemnimide C may enhance the deterrent effects of didemnimide D in an additive or synergistic manner, resulting in the enhanced deterrent capacity of the crude extract. Nevertheless, didemnimide D is clearly the major deterrent compound in the crude extract, because field assays of both didemnimide C and the mixture containing fractions III to V were not deterrent (Figs. 6 & 7).

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Field assays conducted in earlier studies of invertebrate chemical defenses were performed on coral reefs over short periods of time (<1 h) during daylight hours (e.g. Pawlik & Fenical 1992, Chanas & Pawlik 1995), whereas the assays performed in the present study were conducted for 14 h or more, overnight. Assays strips left in seagrass beds for shorter periods of time during the day exhibited little evidence of feeding activity. Because the treatment and control food strips could not be observed during the preponderance of the nighttime assay period, the identities of the consumers is unknown, and may have included invertebrates. Bite marks on food strips, however, were identical to those
we have seen in the past resulting from feeding by
fishes, and it is presumed that the majority of feeding
on food strips took place as fishes moved through the
garbs during their crepuscular migrations (Jones

A surprisingly large number of metabolites from
marine invertebrates that have been subjected to
relevant assays do not appear to play a role in anti-
predatory defense, and seemingly minor changes in
stereochemistry, structure, or functionality of deterrent
compounds renders them inactive (Pawlik 1993). Of
the 4 didemnimides isolated from the tissues of Didem-
num conchylatum, only the fully substituted didem-
nimide D, which bears both a bromine on the indole ring
and a methyl group on the imidazole ring (Fig. 8), was
deterrrent in both laboratory and field feeding assays.
While didemnimide C may also play a role in chemical
defense (see earlier discussion), the functions of di-
 demnimides A and B remains unresolved. One possi-
bility is that these compounds are precursors of the
more highly substituted didemnimides C and D and
that the biosynthetic process of bromination and
methylation requires an excess of precursor molecules
to proceed. Alternatively, the biosynthetic pathway
simply may not be optimized for the production of this
chemical defense, and the inactive metabolites pro-
duced as side products may be regarded as so much
'biochemical baggage' (Haslam 1986). Finally, the pos-
sibility remains that didemnimides A and B function in
some other role, defensive or otherwise, which might
include antimicrobial, antifouling, or UV-protective
(Pawlik 1993), but evidence of these possible functions
awaits further experimentation.

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