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CHEMICAL DEFENSES OF THE SACOGLOSSAN MOLLUSK *Elysia rufescens* AND ITS HOST ALGA *Bryopsis* sp.

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Abstract— Sacoglossans are a group of opisthobranch mollusks that have been the source of numerous secondary metabolites; however, there are few examples where a defensive ecological role for these compounds has been demonstrated experimentally. We investigated the deterrent properties of the sacoglossan *Elysia rufescens* and its food alga *Bryopsis* sp. against natural fish predators. *Bryopsis* sp. produces kahalalide F, a major depsipeptide that is accumulated by the sacoglossan and that shows in vitro cytotoxicity against several cancer cell lines. Our data show that both *Bryopsis* sp. and *Elysia rufescens* are chemically protected against fish predators, as indicated by the deterrent properties of their extracts at naturally occurring concentrations. Following bioassay-guided fractionation, we observed that the antipredatory compounds of *Bryopsis* sp. were present in the butanol and chloroform fractions, both containing the depsipeptide kahalalide F. Antipredatory compounds of *Elysia rufescens* were exclusively present in the dichloromethane fraction. Further bioassay-guided fractionation led to the isolation of kahalalide F as the only compound responsible for the deterrent properties of the sacoglossan. Our data show that kahalalide F protects both *Bryopsis* sp. and *Elysia rufescens* from fish predation. This is the first report of a diet-derived depsipeptide used as a chemical defense in a sacoglossan.

Key Words—Antipredatory role, herbivore–prey relationship, depsipeptides, kahalalide F, sacoglossan mollusks, green algae, *Elysia rufescens*, *Bryopsis* sp.

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INTRODUCTION

Sacoglossans (=ascoglossans) are a group of opisthobranch mollusks that feed primarily on siphonaceous green algae (Williams and Walker, 1999). They are highly specialized herbivores that can sequester functional chloroplasts from their diet and use them as a source of photosynthetic energy (Clark et al., 1990; Williams and Walker, 1999). Sacoglossans can also sequester secondary metabolites from their diets or use the sequestered chloroplasts to convert bicarbonate into a variety of carbohydrates that can be used for the synthesis of secondary metabolites (Ireland and Scheuer, 1979; Ireland and Faulkner, 1981; Paul and Van Alstyne, 1988; Gavagnin et al., 2000). It has been hypothesized that these compounds defend sacoglossans against predators. These putative chemical defenses are throught to provide ecological advantages and may function as a driving force in the evolution of this group (Cimino and Ghiselin, 1998).

Although marine natural products chemists have isolated numerous secondary metabolites from sacoglossans (Faulkner, 1992; Avila, 1995), their ecological roles remain largely uninvestigated. Many species synthesize polypropionates (Ireland and Scheuer, 1979; Ireland and Faulkner, 1981; Ksebati and Schmitz, 1985; Dawe and Wright, 1986; Roussis et al., 1990; Di Marzo et al., 1991; Vardaro et al., 1991; Gavagnin et al., 1996). However, these compounds do not show an antipredatory role when they have been experimentally tested (Hay et al., 1989; Roussis et al., 1990). Both the chemically defended sacoglossan *Cyerce nigricans* and its chemically defended host alga *Chlorodesmis fastigiata* contain chlorodesmin (Hay et al., 1989). Although chlorodesmin significantly deters feeding by herbivorous fishes (Paul, 1987; Wylie and Paul, 1988), it does not account for the antipredatory properties of the mollusk (Hay et al., 1989). Two propionate-derived metabolites isolated from the same mollusk species also lacked the deterrent properties of the extracts (Roussis et al., 1990).

Our study focused on the sacoglossan *Elysia rufescens* and its food alga *Bryopsis* sp. Kahalalide F is the major metabolite of a series of amino and fatty acid-derived depsipeptides produced by the green alga *Bryopsis* sp. that are accumalated by *Elysia* spp. (Hamman and Scheuer, 1993; Hamman et al., 1996). Kahalalide F shows a series of in vitro activities against tumor cell lines, viruses, and fungi (Hamman and Scheuer, 1993), but no ecological roles have been investigated. In our study, we investigated whether or not *Bryopsis* sp. and *Elysia rufescens* are chemically defended against generalist fish predators and, if so, whether or not kahalalide F is the compound responsible for the deterrent properties.

METHODS AND MATERIALS

Extraction and Isolation. We collected 5.4 kg wet mass of *Elysia rufescens* and 4.67 kg wet mass of *Bryopsis* sp. by snorkeling at low tide near Black Point, Oahu, Hawaii, during February 1995. Marilyn Dunlap and Alison Kay identified the animals. A voucher specimen is deposited at the Bernice P. Bishop Museum, Honolulu, BPBM 247679. *Bryopsis* sp. is an undescribed *Bryopsis* species highly abundant in the reef flat at Black Point. In contrast to *Bryopsis* sp. at other sites (Kan et al., 1999, personal observation), the specimens at Black Point lacked macroepophytes. We used bioassay-guided fractionation to isolate and identify ecologically active compounds. We used various solvents to extract and fractionate the extracts from both species along gradients of polarity (Figures 1 and 2). The general goal and approach was the same for the two species, but the specifics of the process varied between *E. rufecens* and *Bryopsis* sp. The sacoglossans were extracted with ethanol (5 × 3 liters) and dichloromethane (CH_2Cl_2, 2 liters). Ethanol and CH_2Cl_2 extracts from *E. rufescens* were combined (282.3 g) and partitioned between CH_2Cl_2 and water (Figure 1). The aqueous layer was extracted with *n*-BuOH, leaving 216.4 g of aqueous extract after evaporation. The *n*-BuOH layer was combined with the CH_2Cl_2 layer, concentrated (65.9 g), and partitioned between hexanes (30.5 g) and MeOH–H_2O (9 : 1) (35.4 g). The methanol layer was collected and water was added to adjust the MeOH concentration to 60%. Extraction with CH_2Cl_2 and concentration yielded 23 g of CH_2Cl_2 extract and 12.4 g of the aqueous methanol extract. The CH_2Cl_2 fraction was subjected to ODS flash column chromatography, by using a stepwise aqueous methanol gradient (50% MeOH = ODS1, 70% MeOH = ODS2, 90% MeOH = ODS3, 100% MeOH = ODS4, $CHCl_3$–MeOH–H_2O (7 : 3 : 0.5) = ODS5). Fraction ODS3 (12.2 g), was subjected to ODS flash column chromatography by using a stepwise aqueous acetonitrile gradient [50% MeCN, 60% MeCN, 70% MeCN, 80% MeCN, 100% MeOH, $CHCl_3$–MeOH–H_2O (CMW, 7 : 3 : 0.5)]. The peptide-containing fractions (monitored by TLC) were combined (8.3 g) and purified on an ODS column by using a stepwise aqueous MeCN gradient solvent system (61%, 62%, 70% aqueous MeCN and 100% MeOH). The kahalalide F-containing fraction eluted with 61% MeCN (4.1 g), and it was passed through an ODS BondElut short column. A reverse-phase HPLC separation (Ultracarb 10 ODS PO; MeOH–H_2O–TFA, 65 : 35 : 0.05) led to 2 g of pure kahalalide F. All the non-kahalalide F-containing fractions and the HPLC side fractions were recombined to reconstitute what was called fraction ODS6 (Figure 1).

The methanol (2 × 3 liters) and $CHCl_3$–MeOH (1 : 1) (2 × 3 liters) extracts from *Bryopsis* sp. were combined (159.82 g) and partitioned between $CHCl_3$ and water (Figure 2). The aqueous layer was extracted with *n*-BuOH (4.23 g). The $CHCl_3$ layer was concentrated (11.09 g) and partitioned between hexanes (4.71 g) and MeOH–H_2O (9 : 1) (6.87 g). The methanol layer was collected (2.67 g), and water was added to adjust the MeOH concentration to 60%. Extraction with $CHCl_3$ and concentration yielded 4.2 g of chloroform fraction.

We used TLC and proton nuclear magnetic resonance (NMR spectra measured on a General Electric QE-300 or GN Omega 500 instrument) to detect kahalalide

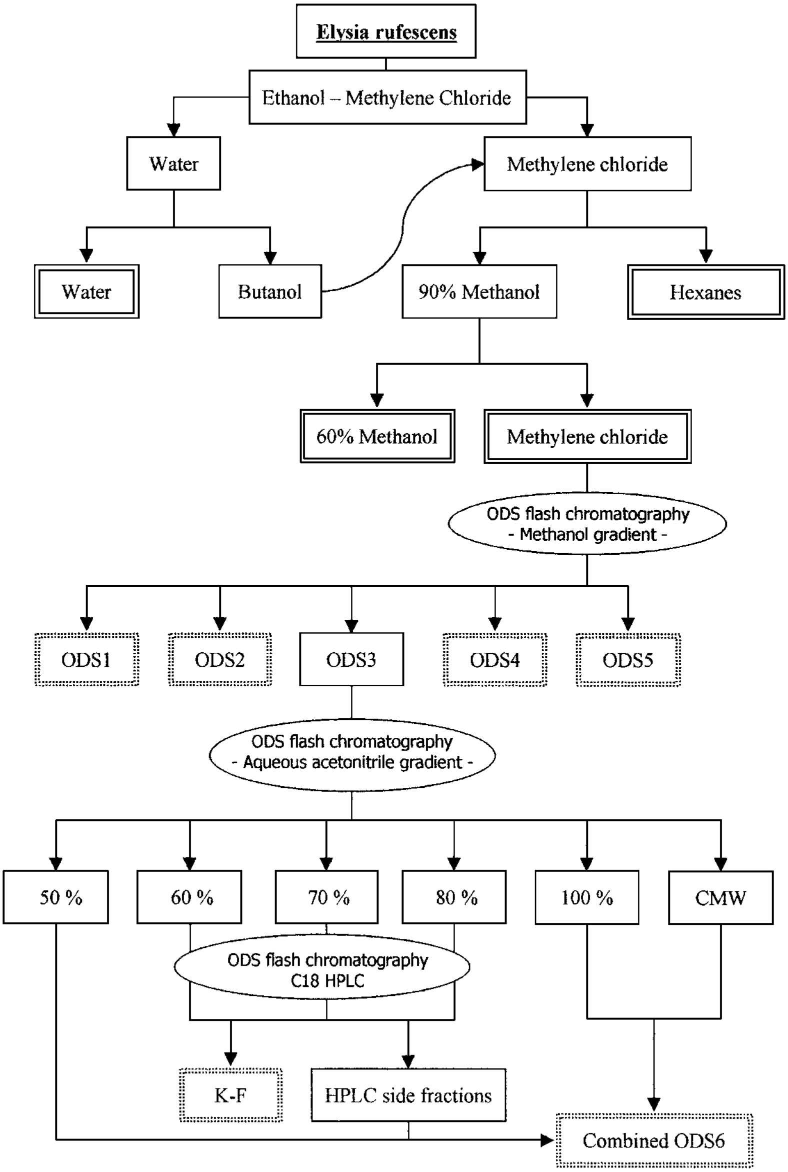

FIG. 1. Scheme of the extraction and partitioning procedure used to obtain secondary metabolites from the sacoglossan *Elysia rufescens*. Fractions enclosed in a double frame were tested at their naturally occurring concentrations against natural fish predators in the field. Fractions sharing the same double frame were tested in the same feeding experiment. See text for more information on the isolation procedure and assay techniques.

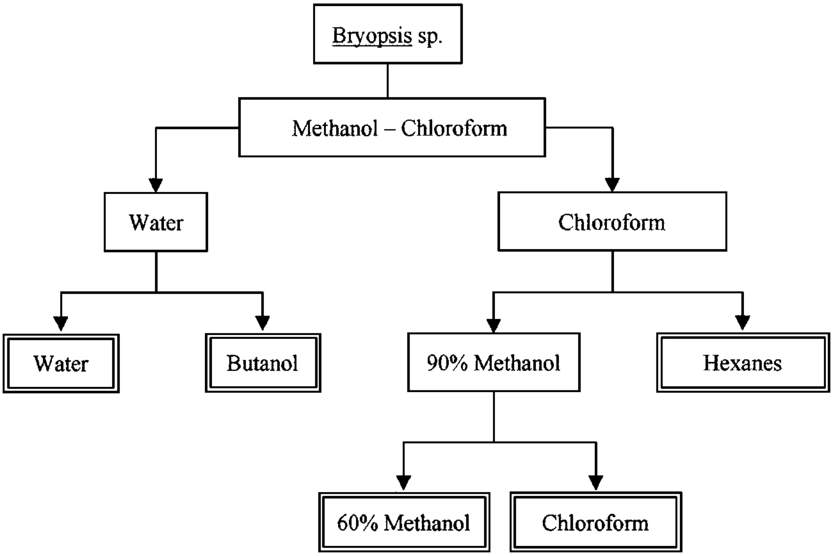

FIG. 2. Scheme of the extraction procedure used to obtain secondary metabolites from the green alga *Bryopsis* sp. Fractions sharing the same double frame were tested at their naturally occurring concentrations against natural fish predators in the same field feeding experiment. See text for more information on the extraction procedure and assay techniques.

F in the fractions and in the mucus produced by *Elysia*. The presence of secondary metabolites in the mucus has been traditionally considered as indirect evidence of the ecological role of the compound (Faulkner, 1992). To collect mucus, we put slugs with seawater in zip-lock bags during collection and then transferred the bags with slugs to containers with ice for transportation to the laboratory. Once in the laboratory we took the slugs out of the seawater, which had taken on a mucilaginous consistency due to the production of mucus by the slugs. We freeze-dried the water–mucus solution, extracted with DCM, and then partitioned using the *Elysia* scheme. Presence of kahalalide F was monitored in the fractions by proton NMR analysis.

Antipredatory Experiments. We used field experiments to determine whether *Bryopsis* sp. and *Elysia rufescens* are chemically defended against a natural coral reef fish assemblage. Methods were similar to those of Becerro et al. (1998). We added extracts from *Bryopsis* sp. and *E. rufescens* to an artificial diet consisting of 5 g of ground food, 2.5 g of carrageenan, and 80 ml of water. We used ground material of the green alga *Enteromorpha* sp. or ground catfish pellets (Kruse's Perfection Brand) in an attempt to better mimic the nutritional characteristics of *Bryopsis* sp. and *E. rufescens* in our artificial diets. We added the necessary amount of extracts, fractions, or compounds relative to wet mass of the food to match the natural concentration of extracts, fractions, or compounds relative to the wet mass of *Bryopsis* or *E. rufescens*. The actual amount of extract (dissolved in 2 ml of dichloromethane–methanol 1 : 1) added to the mixture varied according to the percent yield (per wet mass) of the particular extract or fraction tested. Control foods were prepared by adding 2 ml of solvent to the carrageenan-food diet. The mixture was poured into 1-cm^3 molds containing a rubber O-ring, so that safety pins could be used to attach cubes to ropes (40 cm long). Each rope contained either four control or four treated food cubes. We placed 20 pairs of control and treated ropes on the reef of Western Shoals, Apra Harbor, Guam. Pairs were removed when approximately half of the cubes were eaten in any of the treatments. We used Wilcoxon signed-ranks test for paired comparisons to test for significant differences in the number of control and treatment cubes eaten.

RESULTS

In field experiments, extracts from *Bryopsis* sp. and *Elysia rufescens* significantly deterred fish predators at naturally occurring concentrations. The antipredatory properties of *Bryopsis* sp. are associated with the butanol and $CHCl_3$ fractions ($P < 0.001$ and $P = 0.01$ respectively, Figure 3). TLC analysis showed that kahalalide F is exclusively present in the active fractions.

The antipredatory properties of *Elysia rufescens* were associated with the methylene chloride fraction ($P = 0.002$, Figure 4), a combination of the butanol and methylene chloride fractions from the first fractionation procedure (Figure 1). Kahalalide F was the only compound responsible for the antipredatory properties of the extract ($P = 0.02$, Figure 5). Kahalalide F was also present in the mucus, as observed by proton NMR, although the concentration at which it occured is unknown.

DISCUSSION

Secondary chemistry seems to play a major role in the biology, ecology, and evolution of mollusks. Sea hares, cephalaspideans, and nudibranchs either sequester secondary metabolites from their diet or synthesize them de novo to use them as chemical defenses (Faulkner, 1992; Avila, 1995). Although the data available for sacoglossan mollusks seem to support a defensive role for these compounds (Gavagnin et al., 1994a), experimental evidence is still scarce and does not always support this hypothesis. Our study provides evidence that the sacoglossan *Elysia rufescens* and its host alga *Bryopsis* sp. are chemically defended against generalist fish predators. Kahalalide F, a major depsipeptide sequestered by *E. rufescens* from the green alga *Bryopsis* sp., is the compound responsible for

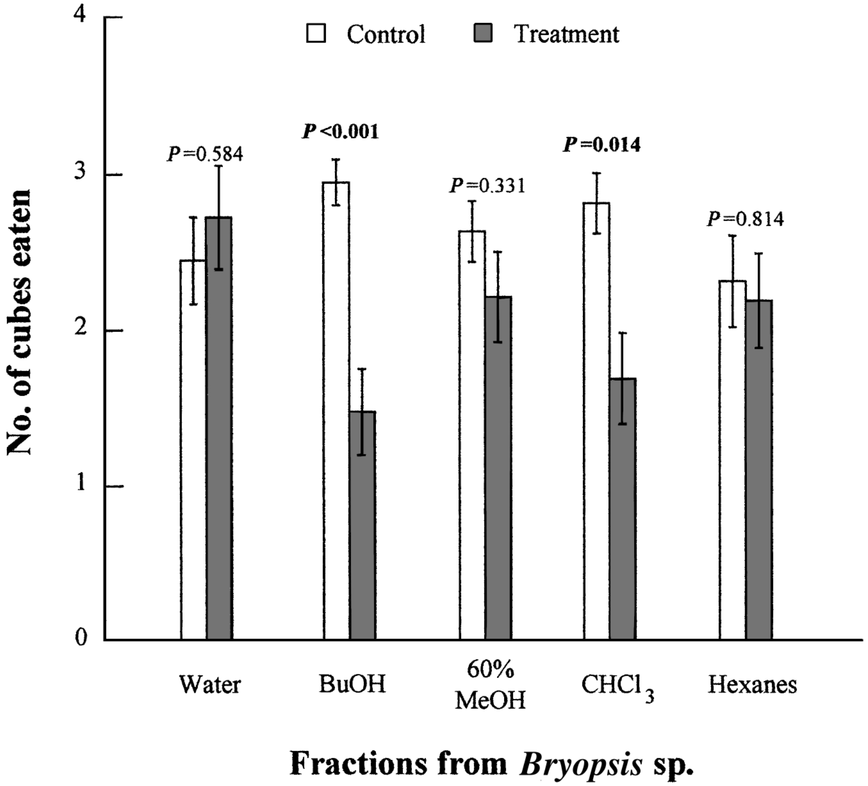

FIG. 3. Feeding deterrence of fractions from the green alga *Bryopsis* sp. towards natural fish consumers in the field. Bars represent average (mean ± 1 SE) number of control (empty bars) and treated (filled bars) cubes eaten in the field by natural fish predators. Mean differences between treatments and their respective controls were tested with Wilcoxon signed-ranks test for paired comparisons. The aqueous (water), butanol (BuOH), 60% methanol (MeOH), chloroform ($CHCl_3$), and hexanes fractions were all tested at their naturally occurring concentrations.

this activity. To our knowledge, this is the first report of a diet-derived depsipeptide used as a chemical defense by sacoglossans.

Sequestration of secondary metabolites is widespread among opisthobranch mollusks. There is ample evidence that sea hares and nudibranchs incorporate biologically active compounds in their tissues and use them for their own defense (Faulkner, 1992; Avila, 1995). Similarly, sacoglossans also accumulate or modify secondary metabolites from their diets. *Elysiella pusilla* (=*Elysia halimedae*) sequesters diterpenoids from its food alga *Halimeda macroloba* and uses them as defense against fish predators (Paul and Van Alstyne, 1988). The sacoglossan

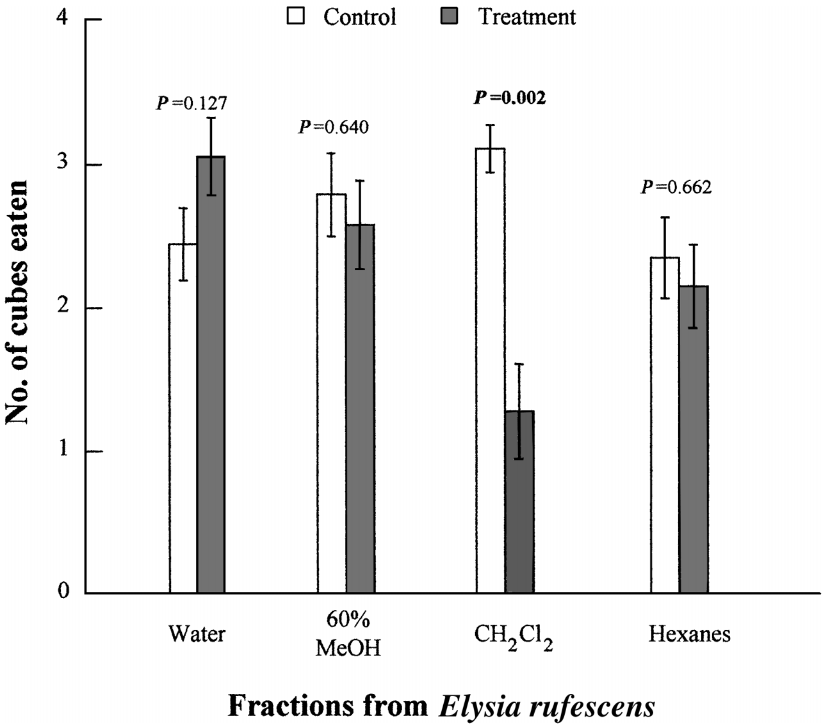

FIG. 4. Feeding deterrence of fractions from the sacoglossan *Elysia rufescens* towards natural fish predators in the field. Bars and statistical values as in Figure 3. The aqueous (water), 60% methanol (MeOH), methylene chloride (CH_2Cl_2), and hexanes fractions were all tested at their naturally occurring concentrations.

Oxynoe panamensis feeds on the chemically defended alga *Caulerpa sertularoides*, from which it incorporates caulerpin and caulerpicin (Doty and Aguilar-Santos, 1970). Chlorodesmin, a diterpenoid from the green alga *Chlorodesmis fastigiata*, significantly deters feeding by some herbivorous fishes (Paul, 1987; Wylie and Paul, 1988). However, the sacoglossan *Cyerce nigricans* specializes on feeding on *Chlorodesmis fastigiata* (Hay et al., 1989), from which it incorporates chlorodesmin. Although the sacoglossan is chemically defended, chlorodesmin does not account for the deterrent properties of the mollusk (Hay et al., 1989). Our data support a deterrent role for the metabolites ingested by *Elysia rufescens* from *Bryopsis* sp. Similarly, the sacoglossan *Costasiella ocellifera* sequesters the brominated compound avrainvilleol from its diet alga *Avrainvillea longicaulis* and uses it as a defense against fish predators (Hay et al., 1990).

The production of polypropionate metabolites by marine mollusks is well documented (Cimino and Sodano, 1993), including that by many sacoglossan

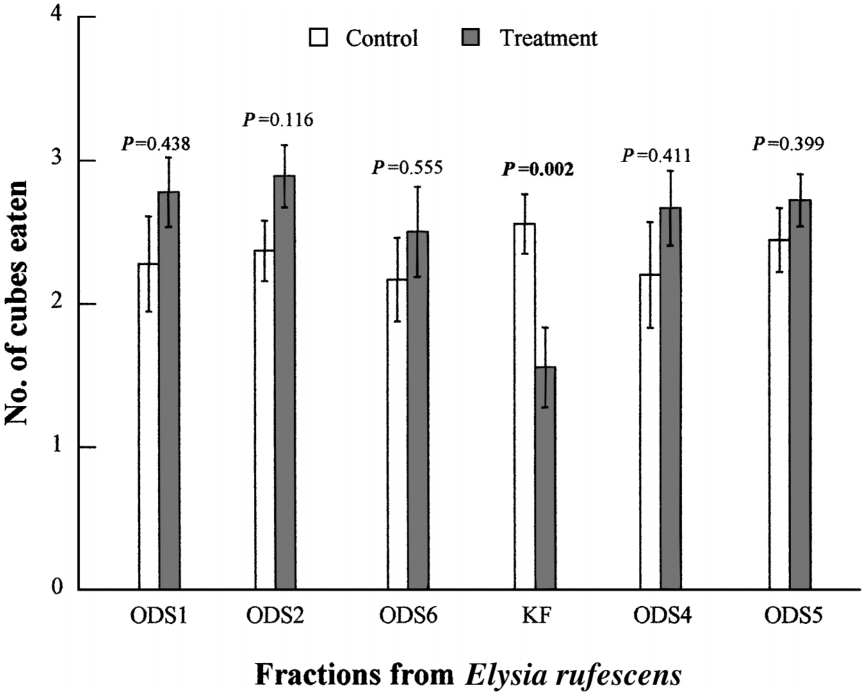

FIG. 5. Feeding deterrence of methylene chloride subfractions from the sacoglossan *Elysia rufescens* towards natural fish predators in the field. Bars and statistical values as in Figure 3. All subfractions were tested at their naturally occurring concentrations. Subfraction labels: ODS1 = 50% methanol, ODS2 = 70% methanol, ODS6 = all compounds (except kahalalide F) obtained from the 90% methanol subfraction, KF = kahalalide F, ODS4 = 100% methanol, ODS5 = chloroform–methanol–water (7 : 3 : 0.5), methylene chloride (CH_2Cl_2), See text and Figure 1 for more information on the partitioning procedure and isolation of kahalalide F).

species (Ireland and Scheuer, 1979; Ireland and Faulkner, 1981; Ksebati and Schmitz, 1985; Dawe and Wright, 1986; Roussis et al., 1990; Di Marzo et al., 1991; Vardaro et al., 1991; Gavagnin et al., 1996). Many polypropionates isolated from mollusks have strong antibacterial (Biskupiak and Ireland, 1983; Dawe and Wright, 1986), cytotoxic (Ksebati and Schmitz, 1985), and ichthyotoxic (Gavagnin et al., 1994b; Vardaro et al., 1991) properties, and crude extracts from species containing polypropionates significantly inhibit feeding by fish predators (Hay et al., 1989). Polypropionates are also present in the mucus of sacoglossans (Di Marzo et al., 1991; Vardaro et al., 1991). Deterrent secretions have been described in many opisthobranchs including sacoglossans (see review by Avila, 1995), and the presence of secondary metabolites in the mucus may be considered as indirect evidence for the role of these compounds as a defensive mechanism against predators (Faulkner, 1992). The sacoglossan *Elysiella pusilla* (= *Elysia halimedae*) incorporates from its diet *Halimeda macroloba* the aldehyde halimedatetraacetate, which the sacoglossan reduces to its corresponding alcohol and incorporates in high concentrations in its body, mucus, and egg masses (Paul and Van Alstyne, 1988). The alcohol deters feeding by fish predators at natural concentrations (Paul and Van Alstyne, 1988). Polypropionates might function similarly, although experimental data on the sacoglossan *Cyerce nigricans* failed to support a deterrent role for polypropionates (Hay et al., 1989; Roussis et al., 1990), and further research is necessary to establish their role against predation. Whether polypropionates play other biological or ecological roles is uncertain. There is evidence supporting the involvement of cyercenes in the regenerative processes after autotomy of body parts in the sacoglossan *Cyerce cristallina* (Di Marzo et al., 1991), a process widely distributed among sacoglossans that has received little attention (Lewin, 1970; Di Marzo et al., 1991; Trowbridge, 1994).

Our bioassay-guided fractionation procedure shows that kahalalide F is the only compound responsible for the antipredatory properties of *Elysia rufescens*. In the mollusk, kahalalide F is the major metabolite out of a group of several compounds isolated from the mollusk and its dietary alga (kahalalides A–J) (Hamman and Scheuer, 1993; Hamman et al., 1996; Goetz et al., 1997). Kahalalide F is found in *E. rufescens* at concentrations between 0.4% (this study) to 1% (Hamann et al, 1996), which are several orders of magnitude higher than the concentration found in the alga (0.0005%, this study). Kahalalide F shows remarkable clinical bioactivity while the rest of the kahalalides lack significant cytotoxicity (Hamman et al., 1996; Goetz et al., 1997). Although toxicity and deterrent activity are not necessarily related (Pawlik et al., 1995), the diverse biological activity of kahalalide F shows that the same compound may exhibit both clinically oriented and ecologically oriented activities. Since we only performed bioassay-guided fractionation with the extracts from *E. rufescens*, the possibility that the minor, nontoxic kahalalides may help deter predators in *Bryopsis* sp. cannot be completely ruled out. Detailed TLC analyses of every fraction showed that all of the active algal fractions contained kahalalide F, while we found no traces of kahalalide F in the nonactive fractions.

It is worth noting that the concentration at which kahalalide F deters predators in the alga is very low (0.0005% of the algal wet mass). Even if all of the kahalalides contribute to this effect, their total percentage of the algal biomass is 0.0032%. Halimedatetraacetate, the major metabolite in *Halimeda macroloba*, is about 0.2% of the algal wet mass (Paul and Van Alstyne, 1988). Both the sacoglossan *Cyerce nigricans* and its host alga *Chlorodesmis fastigata* contain the cytotoxic diterpenoid chlorodesmin (Hay et al., 1989). Although the sacoglossan is chemically defended against fish predators, chlorodesmin is not responsible for the antipredatory properties of the crude extract when tested at natural concentrations (less than 1% of the mollusk dry mass) (Hay et al., 1989). Two pyrones from the same sacoglossan species accounted for 0.9% and 0.45% of the dry mass of the mollusk and also failed to account for the repellent properties of the crude extract (Roussis et al., 1990). Because of their low concentrations, minor compounds may be easily overlooked in marine chemical ecology, yet they may play a determinant role in the biology and ecology of benthic organisms. The killer sponge *Dysidea* sp. is chemically defended against fish predators (Thacker et al., 1998). However, the major compound, 7-olepupuane, does not account for the total activity of the extract, suggesting that either addition or synergism of other minor compounds enhances the activity of the major compound (Thacker et al., 1998). Our study may be an example of how minor compounds (kahalalide F, 0.0005% of the algal mass) account for the activities detected in the whole extract.

Elysia rufescens is a highly cryptic but chemically defended species. In fact, *E. rufescens* may have evolved a variety of defensive mechanisms to reduce the chances of predation. We showed that *Bryopsis* sp. is a chemically defended alga, that may provide the sacoglossan an associational refuge (Hay et al., 1990; Hay, 1992; Duffy and Hay, 1994). By feeding on *Bryopsis* sp., *E. rufescens* sequesters algal chloroplasts and makes itself highly cryptic. However, predation may be high on cryptic organisms (Trowbridge, 1994), so the acquisition of other defensive strategies may expand the benefits of crypsis. *E. rufescens* sequesters the antipredatory compound from *Bryopsis*, accumulates the compound up to several times above the concentration in the alga, and becomes chemically defended itself. Moreover, *E. rufescens* releases the antipredatory compound into its mucus, which may be considered as a defensive mechanism to deter predators (Lewin, 1970; Jensen, 1984; Trowbridge, 1994). Predation is an important factor influencing mortality in benthic systems (Lubchenco and Gaines, 1981), and several mechanisms may work together with the same goal. Predator–prey relationships are important processes in marine benthic communities. Many of these relationships are chemically mediated interactions between predators and their prey. By investigating these associations, we will broaden our understanding of the biology and ecology of benthic organisms and the factors that affect the evolution of these predator–prey interactions.

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