

UNPALATABLE COMPOUNDS IN THE MARINE  
GASTROPOD *Dolabella auricularia*: DISTRIBUTION AND  
EFFECT OF DIET

STEVEN C. PENNINGS,<sup>1,\*</sup> VALERIE J. PAUL,<sup>1</sup> D. CHUCK DUNBAR,<sup>2</sup>  
MARK T. HAMANN,<sup>2</sup> WILFRED A. LUMBANG,<sup>1</sup> BRANDIE NOVACK,<sup>1</sup>  
and ROBERT S. JACOBS<sup>3</sup>

<sup>1</sup>*Marine Laboratory  
University of Guam  
Mangilao, Guam 96923*

<sup>2</sup>*Department of Pharmacognosy  
University of Mississippi  
University, Mississippi 38677*

<sup>3</sup>*Department of Biological Sciences  
University of California  
Santa Barbara, California 93106*

(Received May 5, 1998; accepted November 18, 1998)

**Abstract**—Sea hares are a rich source of novel secondary metabolites, most of which are derived from their algal diet, but the natural function(s) of these metabolites are largely unknown. We used field and laboratory assays to measure the palatability of extracts from the tissues, ink, and eggs of *Dolabella auricularia*. Digestive-gland extracts contained a wide variety of secondary metabolites, including the red algal compound prepacifinol epoxide and its derivative johnstonol, and they were unpalatable to reef fishes. Skin extracts were moderately unpalatable, but our bioassay-guided fractionation led us to (–)-7-dehydrocholesterol, rather than to an algal secondary metabolite. Ink extracts were consistently unpalatable to reef fishes only at high concentrations, suggesting either that ink must be concentrated to deter predators, that unpalatable components of ink rapidly decompose, or that ink has other functions. Unpalatability of ink was traced to a purple fraction, consistent with the hypothesis that the active compound is aplysiocyanin, a known ink constituent modified from a red algal pigment. Egg extracts were moderately unpalatable; however, we could not trace this activity to any algal-derived secondary metabolite. Body-wall extract was highly palatable. Our results suggest that dietary-derived secondary metabolites play a role in chemical defense of *D.*

\*To whom correspondence should be addressed at: University of Georgia Marine Institute, Sapelo Island, Georgia 31327.

*auricularia* via the ink, but are not responsible for unpalatability of skin or eggs. Accumulation of dietary-derived metabolites in the digestive gland may occur to detoxify a chemically rich diet, rather than or in addition to deterring predators.

**Key Words**—Anaspidea, chemical defense, dehydrocholesterol, *Dolabella*, johnstonol, prepacifinol epoxide, sea hare, secondary metabolites, sequester.

#### INTRODUCTION

Sea hares of the genera *Aplysia*, *Stylocheilus*, *Dolabella*, and *Bursatella* have provided natural-products chemists with a rich source of secondary metabolites, most of which are derived from the animals' algal diets (reviewed in Faulkner, 1992, 1997; Avila, 1995; Pettit, 1996; Yamada and Kigoshi, 1997). Despite interest in sea-hare chemistry dating back at least to Flury (1915), the function(s) of these secondary metabolites remains unclear (Pennings and Paul, 1993; Pennings, 1994; Nolen et al., 1995).

Since many algal metabolites deter feeding by herbivores, an obvious hypothesis is that sequestered compounds protect sea hares from potential predators (Carefoot, 1987; Faulkner, 1992). Although this hypothesis has some experimental support (Pennings, 1990; Paul and Pennings, 1991; de Nys et al., 1996), four lines of evidence indicate that the situation is more complex. First, sequestered secondary metabolites are located primarily in an internal organ, the digestive gland, rather than in the skin, where they could function more effectively in defense (Winkler, 1969; Faulkner, 1992; Pennings and Paul, 1993). Second, sea hare species that eat algae lacking in secondary metabolites appear to be just as unpalatable as species that eat chemically rich algae (Pennings, 1994). Third, individual sea hares raised on chemically rich and chemically poor algae do not differ greatly in palatability (Pennings, 1990; Nolen et al., 1995). Fourth, although sea-hare eggs appear to be unpalatable to predators (Faulkner, 1992; Pennings, 1994), attempts to isolate algal-derived compounds from them have failed (Faulkner, 1992; but see Carefoot et al., 1998), suggesting that eggs contain alternate lines of defense, perhaps compounds produced de novo by sea hares.

This evidence suggests that additional research is needed before an adaptationist explanation of sea-hare chemical defenses can be accepted. Here, we pursued three lines of inquiry. First, we compared the palatability of extracts from *Dolabella auricularia* tissues, ink, and eggs. Second, we compared the palatability of animals fed different algal diets. Third, we tried to identify unpalatable compounds to determine if they had an algal origin.

## METHODS AND MATERIALS

*Study Organism*

*Dolabella auricularia* is a large (to ~1 kg wet mass), herbivorous, opisthobranch gastropod with a circumtropical distribution and a lifespan of ~16 months (Switzer-Dunlap and Hadfield, 1977, 1979). On the island of Guam (13°25'N, 144°55'E), *D. auricularia* occurs in shallow (<2 m) subtidal areas of mixed sand and boulders. Two morphotypes occur: a rare, relatively slender, agile morph and a common, relatively blocky morph, which we studied. *D. auricularia* is a generalized, nocturnal feeder (Pennings and Paul, 1992; Pennings et al., 1993) that sequesters secondary metabolites from algae (Faulkner, 1984, 1988; Pennings and Paul, 1993). *D. auricularia* and egg masses were collected by hand from Apra Harbor, Guam.

*General Extraction and Feeding Assay Methods*

Samples were extracted four times in 1 : 1 methanol-dichloromethane, and the organic layer was evaporated to dryness in a rotary evaporator. Residual solids were dried and weighed to calculate extract yields. Ink collected mixed with water was partitioned between methanol and dichloromethane to yield water-soluble and organic-soluble extracts.

Most feeding assays were conducted in the field on shallow (<5 m) reefs in Apra Harbor (Table 1). Fish that fed during our assays included: the damselfishes *Abudefduf sexfasciatus* and *Amblyglyphidon curacao*; the wrasses *Cheilinus fasciatus*, *Coris gaimard*, *Thalassoma hardwickii*, and *T. lutescens*; the triggerfish *Balistipus undulatus*; and the unicornfish *Naso vlamingii*. Extracts were incorporated into artificial diets consisting either of carageenan (4.0 g), paraffin wax (3.0 g), pureed frozen squid (2.0 g), and tap water (110 ml) (the squid diet) or of carageenan (2.5 g), wax (2.0 g), Kruse Catfish Food (Kruse Grain and Milling, 5.0 g), and tap water (80 ml) (the pellet food diet). Ingredients were mixed, heated to boiling, and poured into a mold to form small (~1 cm<sup>3</sup>) cubes. Experimental foods contained extracts or secondary metabolites dissolved in small amounts of solvent and added to the recipe after heating but before pouring; concentrations were based on the dry mass of the diet. Control foods contained identical amounts of organic solvent to control for any effect of solvent on feeding; however, the bulk of the solvent quickly evaporated from the food mixtures. Pieces of food were attached to 50-cm-long pieces of polypropylene rope (10 pieces/rope, spaced 3 cm apart for multiple comparisons, or 4 pieces/rope, spaced 5 cm apart for paired comparisons). Ropes were attached to the reef in groups of five to eight for multiple comparisons or in pairs (an extract versus a control). Ropes within a group or pair were spaced ~30 cm apart; different groups or pairs were spaced >2 m apart. All ropes in a group or pair were retrieved after fishes had removed

TABLE 1. FEEDING EXPERIMENT PROTOCOLS<sup>a</sup>

Assay	Design	Consumers	Diet	Treatments
November 1989 collection	MC	reef fish	squid	S, BW, DG, E, C, UC
October 1990 collection	MC	reef fish	squid	dorsal S, dorsal BW, foot S, foot BW, DG, E, I, C
Single diets	MC	reef fish	squid	S+BW, DG ( <i>Dicotyota</i> spp. diet), S+BW, DG ( <i>Padina tenuis</i> diet), C, UC
Single diets DG	MC	reef fish	squid	DG ( <i>Dicotyota</i> spp. diet), DG ( <i>Padina tenuis</i> diet), DG (field diet), C, UC
DG fractions	MC	reef fish	squid	C, 5 fractions
S fractions (June 1990)	MC	reef fish	squid	C, 5 fractions
S fractions (October 1990)	P	reef fish	pellet food	5 fractions versus paired C
S HPLC fractions	P	reef fish	pellet food	2 HPLC fractions versus paired C
Cholesterol	P	pufferfish	Prime Reef	Cholesterol versus paired C
(-)-7-Dehydrocholesterol	P	pufferfish	Prime Reef	(-)-7-Dehydrocholesterol versus paired C
I	P	reef fish	squid	I versus paired C
I (two assays)	P	pufferfish	Prime Reef	I versus paired C
I MeOH-soluble fraction	P	pufferfish	Prime Reef	fraction versus paired C
I C18 fractions	P	pufferfish	Prime Reef	fraction versus paired C
E	P	reef fish	squid	E versus paired C
E fractions	MC	reef fish	squid	C, 5 fractions
E HPLC fractions	P	reef fish	pellet food	5 HPLC fractions versus paired C

<sup>a</sup>MC = multiple choice, P = paired choice, S = skin, BW = body wall, DG = digestive gland, E = eggs, I = ink, C = control, UC = unexposed control. All multiple choice assays had  $N = 12$ ; sample sizes for paired assays differ for each comparison and are given in figures.

approximately half the total food pieces from that group or pair (1–10 min). For all multiple comparisons, cubes were weighed before and after the experiment to measure the proportion of food eaten. Unexposed controls included in several assays were handled identically to other ropes except that they were kept in mesh bags so that fish could not eat them. Results from unexposed controls indicated that changes in mass due to handling were minor relative to effects of feeding by fish. Proportional data were arcsine–square-root transformed before analysis. Results of multiple comparisons were analyzed by calculating all possible paired *t* tests and setting the critical *P* value equal to 0.05 divided by the number of tests. For paired comparisons, the number of food cubes completely eaten from each rope was scored, the data were analyzed with Wilcoxon's signed rank test, and the results reported as two-tailed *P* values.

Additional feeding assays (Table 1) were conducted in the laboratory by using the spotted sharpnose pufferfish *Canthigaster solandri* (Richardson 1844). *Canthigaster solandri* is an omnivore that feeds on a variety of filamentous algae and benthic invertebrates (Myers, 1989). We collected pufferfish from Pago Bay, Guam, by dipnet and held them individually in 4-liter flow-through containers submerged in water tables supplied with fresh running seawater. Laboratory experiments used a diet composed of carrageenan (2.5 g), frozen Prime Reef mix (35 g, Ocean Nutrition), and tap water (50 ml) (the Prime Reef diet). Individual pufferfish were offered one piece of food containing an extract or secondary metabolite and one piece containing only solvent. Paired trials without fish were conducted to estimate changes in the mass of food in the absence of a consumer. Individual replicates with fish and paired no-fish replicates were terminated after approximately half the total food was eaten (1–48 hr). Food pieces were weighed before the experiment, then blotted dry and weighed again after the experiment. Data were analyzed following Peterson and Renaud (1989): the variable “(change in mass of treated food) – (change in mass of control food)” was compared for replicates with and without fish by using a paired *t* test.

#### *Distribution of Unpalatable Compounds*

*November 1989 Collection.* Five *D. auricularia* and two egg masses were collected. The animals were dissected into three parts (skin, body wall, and digestive gland—other internal organs were discarded). Body parts and egg masses were extracted. Extract yields were typical of other collections except that the body wall had a relatively low yield (Table 2). Organic extracts were tested at natural concentrations (Table 1).

*October 1990 Collection.* Five *D. auricularia* and five egg masses were collected, and the *D. auricularia* were stimulated to release ink. The animals were dissected into five parts (dorsal skin, dorsal body wall, foot skin, foot body wall, and digestive gland—other internal organs were discarded). Body parts,

TABLE 2. EXTRACT YIELDS (% DRY MASS) FROM VARIOUS COLLECTIONS OF *Dolabella auricularia*<sup>a</sup>

	Field 11/89 ( <i>na</i> = 5)	Field 6/90 ( <i>na</i> = 12; <i>ne</i> = 19)	Field 10/90 ( <i>na</i> = 5; <i>ne</i> = 5)	<i>Dictyota</i> diet ( <i>na</i> = 4)	<i>Padina</i> diet ( <i>na</i> = 3)
Digestive gland	24.4 ± 12.1	14.4 ± 1.6	31.7 ± 7.3	28.4 ± 12.1	29.0 ± 20.8
Skin	4.8 ± 1.2	6.4 ± 0.9			
Dorsal skin			9.9 ± 6.5		
Foot skin			6.1 ± 2.6		
Body wall	0.6 ± 0.1	3.5 ± 0.4			
Dorsal body wall			5.0 ± 2.0		
Foot body wall			2.3 ± 1.5		
Skin + body wall				1.7 ± 0.7	2.4 ± 0.3
Eggs		11.3 ± 1.0	28.4 ± 6.4		

<sup>a</sup>Data are means ± 1SD. *na* = number of animals extracted, *ne* = number of egg masses extracted.

eggs, and ink were extracted. Yields were typical of other collections (Table 2). Most organic extracts were tested at natural concentrations (Table 1). Because we had limited material, digestive gland and egg extracts were tested at concentrations of 25%, although their yields were slightly higher. Ink extract was tested at an arbitrary concentration of 25%.

*Animals from Single Diets.* Seven *D. auricularia* were collected and maintained individually in 40-liter aquaria with running seawater. Four individuals were fed a diet of *Dictyota* spp. (mostly *D. bartayresii*) and three a diet of *Padina tenuis* for 11 weeks, after which they were dissected into two parts (skin + body wall, and digestive gland—other internal organs were discarded) and extracted. Yields were within the range of other collections (Table 2). Extracts were tested at natural concentrations (Table 1).

To explore further if the diet that a *D. auricularia* had been eating affected the palatability of the digestive-gland extracts, we compared digestive-gland extracts from animals fed *Dictyota* spp. and *Padina tenuis* with extracts from animals eating a field diet (November 1989 collection), at a standard concentration of 10% (Table 1).

*Sea Urchin Cleavage.* We examined the toxic effect of different extracts from wild-caught animals and from animals fed single diets in a standard pharmacological assay. Extracts were incubated at 16 µg/ml with zygotes of the sea urchin, *Strongylocentrotus purpuratus*, in filtered seawater from the time of fertilization through the completion of the first cleavage in control zygotes (approx. 2.5 hr); controls were exposed to identical amounts of solvent. Results were recorded as percent change in division relative to controls.

### *Fractionation of Extracts*

In order to attempt to isolate deterrent compounds from extracts of *D. auricularia*, a collection of 12 animals and ca. 40 egg masses was made in June 1990. *D. auricularia* were stimulated to release ink and dissected into three parts (skin, body wall, and digestive gland—other internal organs were discarded). Body parts, eggs, and ink were extracted. Extract yields (Table 2) were similar to other collections except that digestive glands and eggs had relatively low yields, perhaps because of seasonal variation or because extraction was not complete. Because the body-wall extract was highly palatable in previous assays, it was not tested further. Extracts of the digestive gland, skin, and eggs were separated into fractions of increasing polarity by using silica gel vacuum flash column chromatography [mobile phases: 100% hexanes, 25% ethyl acetate (EtOAc)—75% hexanes, 50% EtOAc—50% hexanes, 75% EtOAc—25% hexanes, 100% EtOAc].

*Digestive Gland Fractions.* Flash-column fractions from digestive glands were tested at a total concentration of 10% (Table 1). This concentration was lower than natural, but was used to encourage fish to feed because the crude extract of the digestive gland was extremely unpalatable at natural concentrations (see Results).

*Skin Fractions.* Flash-column fractions from skin were tested twice (Table 1). In the first assay, fractions from the June 1990 collection were tested at an overall concentration of 6% (slightly lower than the extract yield; Table 2). Based on the results of this assay, we fractionated the October 1990 dorsal-skin extract by using slightly different mobile phases (10% EtOAc—90% hexanes, 20% EtOAc—80% hexanes, 100% EtOAc, 50% EtOAc—50% methanol (MeOH), 100% MeOH). These five fractions were tested at an overall concentration of 9.9% (based on the extract yield; Table 2).

These two assays consistently identified the 20–25% EtOAc fraction as the only one deterring feeding (see Results). We isolated and tested (Table 1) two major HPLC fractions from this flash-column fraction by silica-gel HPLC (15% EtOAc—hexanes). Results indicated that a common sterol, (–)-7-dehydrocholesterol, was responsible for the unpalatability of the skin extracts. We purchased cholesterol (Sigma) and (–)-7-dehydrocholesterol (Aldrich) and tested these compounds at several concentrations (Table 1). Because (–)-7-dehydrocholesterol decomposes in the presence of oxygen and sunlight to give a variety of breakdown products, including vitamin Ds and sterol endoperoxides that are known to deter feeding (Bobzin and Faulkner, 1992), we cannot unambiguously assign the results of these assays to one particular compound. Based on TLC analysis, an unknown but large proportion of the (–)-7-dehydrocholesterol in our assays had decomposed by the time we returned uneaten food to the laboratory and reextracted it.

*Ink Fractions.* To determine if ink extract deterred feeding at lower con-

centrations than tested above (distribution of unpalatable compounds), we tested it in three assays at concentrations of 6.3, 5, and 5% (Table 1). Reasoning that ink might contain a water-soluble feeding deterrent, we partitioned ink between aqueous MeOH and dichloromethane, lypophilized the MeOH-soluble fraction, and tested it at a concentration of 32% (Table 1). Since ink organic extract deterred feeding in some assays, we obtained fractions using a reverse-phase C<sub>18</sub> column eluted with MeOH, 50% H<sub>2</sub>O–50% MeOH, 20% H<sub>2</sub>O–80% MeOH, MeOH, 50% dichloromethane–50% MeOH, 100% dichloromethane, and 100% MeOH. The first two fractions were tested individually, while fractions 3–7 were pooled; all were tested at 4% (Table 1).

*Egg Fractions.* To confirm that extracts of eggs deterred feeding, we tested them at a concentration of 6% (Table 1). Flash-column fractions of eggs were tested at an overall concentration of 10% (lower than the extract yield, Table 2). Because only the 25% EtOAc–75% hexanes fraction deterred feeding, we further separated this fraction using silica-gel HPLC (5% EtOAc–95% hexanes mobile phase) into five HPLC fractions that we tested at an overall concentration of 6.3% (Table 1).

## RESULTS

### *Distribution of Unpalatable Compounds*

*November 1989 Collection.* Reef fish discriminated strongly among diets containing different extracts (Figure 1, top). Those with digestive glands and eggs were consumed significantly less than the control. Digestive-gland extract diet was so unpalatable that it did not lose any more mass than did the unexposed control. There was a nonsignificant trend towards the skin extract deterring feeding.

*October 1990 Collection.* Reef fish discriminated strongly among diets containing different extracts (Figure 1, bottom). Those containing digestive glands and ink were consumed significantly less than the control. There were nonsignificant trends towards the egg and skin extracts deterring feeding. Although body-wall extract was tested at a much higher concentration than in the previous assay, it still had no significant effect.

*Animals from Single Diets.* Extracts of digestive glands from animals fed either *Dictyota* spp. or *Padina tenuis* strongly deterred feeding by reef fishes (Figure 2, top). Body wall + skin extracts did not significantly deter feeding relative to controls. When extracts of digestive-glands from animals fed *Dictyota* spp., *Padina tenuis*, or a field diet were compared at a standardized concentration of 10%, all three extracts deterred feeding, but reef fish did not discriminate between extracts (Figure 2, bottom).

*Sea Urchin Cleavage.* Digestive gland extracts strongly inhibited cleavage

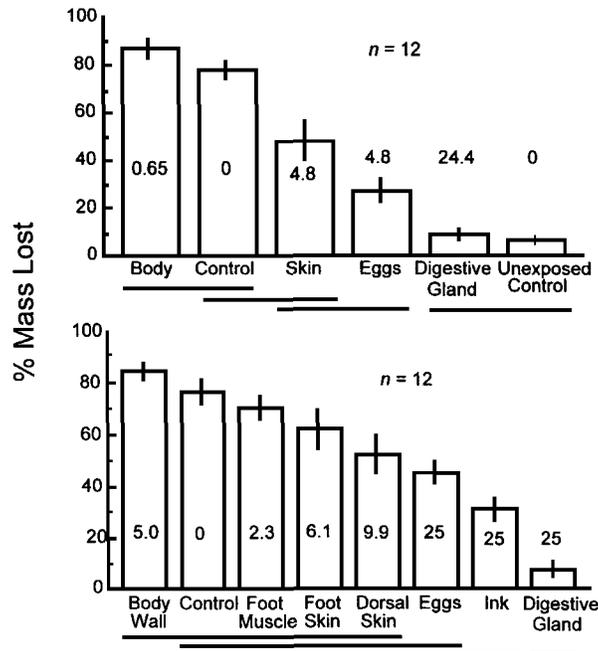


FIG. 1. Effect of extracts of *D. auricularia* on feeding by reef fishes. Data are means  $\pm$  1 SE; extract concentrations (% dry mass) are shown in or above bars. Horizontal bars connect means that are not significantly different. Top: November 1989 collection. Bottom: October 1990 collection.

of sea urchin zygotes, but extracts of skin, eggs, body wall, and ink had no effect (Table 3).

#### *Digestive Gland Fractions*

All digestive gland fractions deterred feeding, even at the relatively low total concentration of 10% (Figure 3). Fish did not significantly discriminate among digestive gland fractions. Examination by TLC indicated that all contained numerous compounds. Since all fractions deterred feeding and were chemically complex, we did not attempt a systematic study of which metabolites deterred feeding. However, we did isolate two secondary metabolites, prepaicifenol epoxide and its derivative johnstonol, by HPLC. We targeted these because they were easy to purify from the complex extracts. They were also isolated from the digestive glands of animals that were fed *Dictyota* spp. in the laboratory (see Methods and Materials: Animals from Single Diets). Presumably,

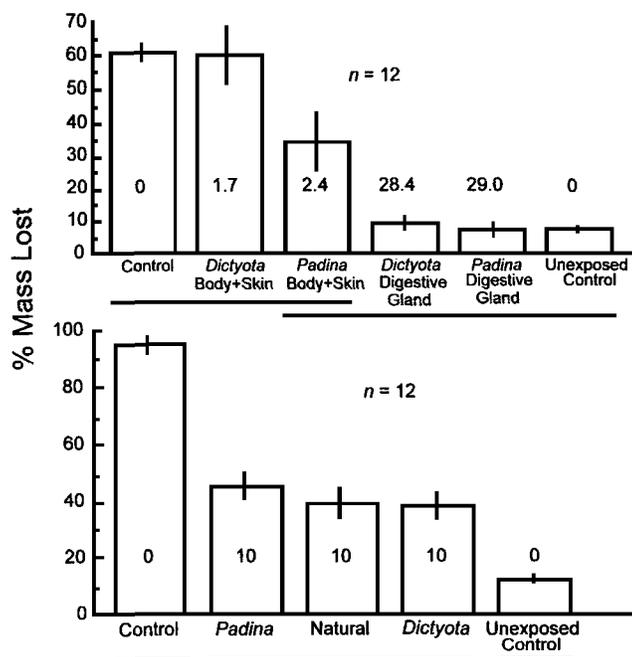


FIG. 2. Effect of extracts of *D. auricularia* on feeding by reef fishes. Data are means  $\pm$  1 SE; extract concentrations (% dry mass) are shown in or above bars. Horizontal bars connect means that are not significantly different. Top: animals fed single diets; natural concentrations. Bottom: digestive gland extracts from animals fed single diets and from November 1989 collection; identical concentrations.

TABLE 3. EFFECT OF EXTRACTS FROM *Dolabella auricularia* (16  $\mu$ g/ml) ON ZYGOTE CLEAVAGE OF SEA URCHIN, *Strongylocentrotus purpuratus*

	Effect	Change (%)
Digestive gland (field diet)	inhibition	-67
Digestive gland ( <i>Padina tenuis</i> diet)	inhibition	-73
Digestive gland ( <i>Dictyota</i> spp. diet)	inhibition	-100
Skin	no effect	-7
Eggs	no effect	0
Body wall	no effect	0
Ink MeOH extract	no effect	0
Ink organic extract	no effect	0

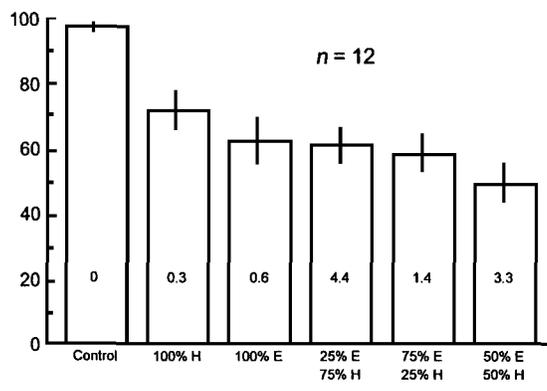


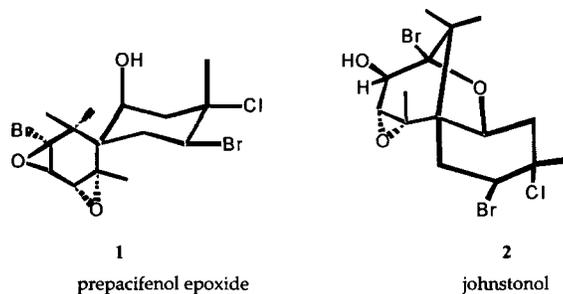
FIG. 3. Effect of digestive gland fractions (10% dry mass total concentration) of *D. auricularia* on feeding by reef fishes. Data are means  $\pm$  1 SE, extract concentrations (% dry mass) are shown in bars. Horizontal bars connect means that are not significantly different. H, hexanes; E, ethyl acetate.

they were eaten in the field before the experiment began and were slow to be excreted.

Final purification of prepacifenol epoxide and johnstonol was accomplished with HPLC by using a  $C_{18}$  column (5- $\mu$ m Phenomenex Ultracarb  $10 \times 250$  mm) and a gradient from  $H_2O$  to MeCN. Prepacifenol (**1**) rearranged to form johnstonol (**2**) over the course of time required to accumulate 2D NMR data (24 hr) in  $CDCl_3$  (Scheme 1). The structure of johnstonol was supported by  $^{13}C$  NMR and 2D NMR including COSY, HMQC, and HMBC experiments. Prepacifenol epoxide, physical data:  $^1H$  NMR  $\Delta \pm 0.05$  ppm from published values (300 MHz,  $CDCl_3$ ) (Faulkner et al., 1974). Mass spectrum EI  $m/z$  (fragment, %): 442 ( $M^+$ , 2) (calcd. for  $C_{15}H_{21}O_3ClBr_2$ : 442); HREI  $m/z$  (fragment, %): 345.0242 ( $M^+ - H_2OBr$ , 20) (calcd. for  $C_{15}H_{19}O_2ClBr$ : 345.0256,  $\Delta$  1.4 mmu). The physical data for johnstonol were comparable to published values. We also partially purified another digestive gland compound that appeared to be a dictyol diacetate.

#### Skin Fractions

The most deterrent fraction in each of the two sets of skin fractions was located in a region of similar polarity. In the first set, the 25% EtOAc–75% hexanes fraction was the least palatable (Figure 4, top). In the second, only the 20% EtOAc–80% hexanes fraction significantly deterred feeding (Figure 4, bottom). This fraction yielded two major mixtures after HPLC purification. Examination of these by NMR revealed that one was composed primarily of sterols (mostly



SCHEME 1.

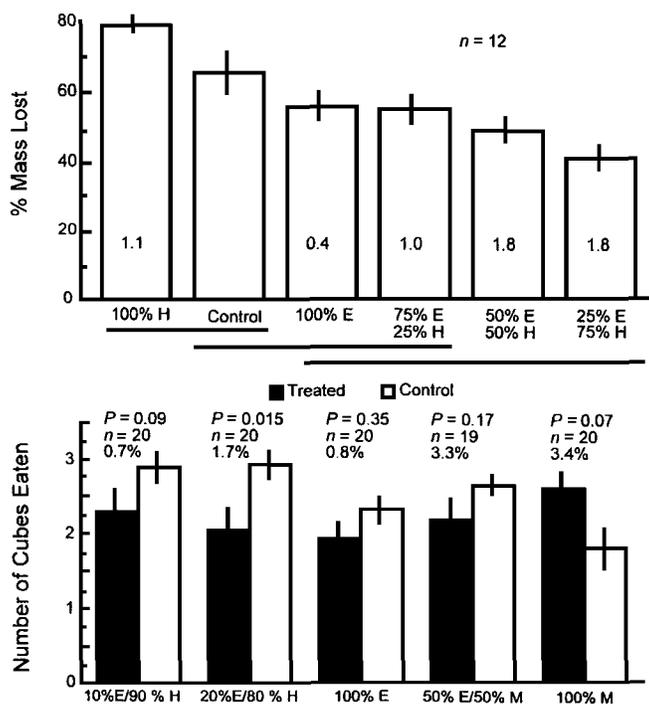
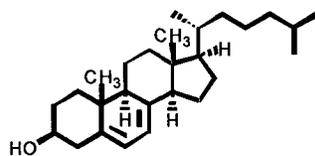


FIG. 4. Effect of two sets of fractions of extract of skin from *D. auricularia* on feeding by reef fishes. Data are means  $\pm$  1 SE. H, hexanes; E, ethyl acetate; M, methanol. Top: First set of extracts (6.1% dry mass total concentration); extract concentrations (% dry mass) are shown in bars. Horizontal bars connect means that are not significantly different. Bottom: Second set of extracts (9.9% dry mass total concentration); data are number of cubes out of four eaten. *P* values, sample sizes, and extract concentrations are given above bars.



3

7-dehydrocholesterol

SCHEME 2.

cholesterol) and the second of (-)-7-dehydrocholesterol. We compared the latter with an authentic sample of (-)-7-dehydrocholesterol (**3**) (Scheme 2) obtained from Aldrich (D300-1):  $[\alpha]_D^{100^\circ}$  ( $c = 0.0028$  g/mL, MeOH),  $^1\text{H NMR } \Delta \pm 0.05$  ppm from standard material (300 MHz,  $\text{CDCl}_3$ );  $^{13}\text{C NMR } \Delta \pm 0.15$  ppm from standard material (75 MHz,  $\text{CDCl}_3$ ). Trace amounts of the corresponding 5,8 endoperoxide were detected in the  $^1\text{H NMR}$  spectrum, likely due to the oxidation of (-)-7-dehydrocholesterol.

The sterol fraction significantly stimulated and the (-)-7-dehydrocholesterol fraction significantly deterred feeding by reef fish (Figure 5). Cholesterol had no effect on feeding by pufferfish at a concentration of 1% (Figure 6). (-)-7-

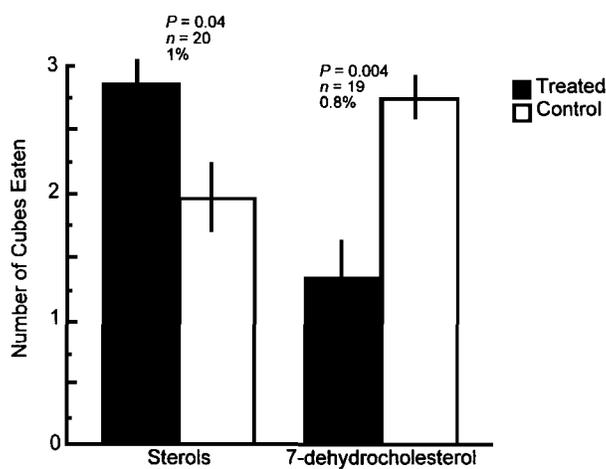


FIG. 5. Effect of two skin HPLC fractions [sterols and almost pure (-)-7-dehydrocholesterol] from *D. auricularia* on feeding by reef fishes. Data (means  $\pm$  1 SE) are number of cubes out of four eaten. *P* values, sample sizes, and extract concentrations are given above bars.

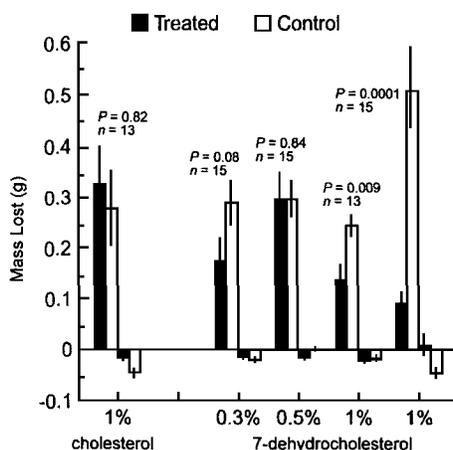


FIG. 6. Effect of pure metabolites cholesterol and (-)-7-dehydrocholesterol on feeding by pufferfish in the laboratory. Data (means  $\pm$  1 SE) are mass lost from diets exposed to (left two bars of each group) and not exposed to (right two bars of each group) pufferfish. *P* values and sample sizes are given above bars.

Dehydrocholesterol deterred feeding at a concentration of 1% in two different assays, but had no effect at 0.5 or 0.3% (Figure 6).

#### *Ink Fractions*

Although earlier tests with ink organic extract at a concentration of 25% indicated that it deterred feeding by reef fishes (Figure 1, bottom), effects at lower concentrations were inconsistent. Reef fish were not deterred by ink extract at 6.3% ( $N = 17$ ,  $P = 0.09$ , trend towards stimulating feeding, data not shown). In one assay, ink extract at 5% had no effect on pufferfish feeding ( $N = 8$ ,  $P = 0.75$ , data not shown); in a second assay it deterred feeding ( $N = 7$ ,  $P = 0.0016$ , data not shown). The MeOH-soluble ink extract had no effect on feeding at 32% ( $N = 11$ ,  $P = 0.28$ , data not shown). Of the three  $C_{18}$  fractions from the organic extract, only fraction 1 (MeOH) significantly deterred feeding by pufferfish (Figure 7). This fraction was purple; fractions 2 and 3–7 were off-white in color.

#### *Egg Fractions*

Egg extracts deterred feeding by reef fish when tested in a paired design at a concentration of 6% ( $N = 18$ ,  $0.67 \pm 0.20$  cubes with extract eaten versus  $3.78 \pm 0.13$  control cubes eaten,  $P < 0.001$ ). When the flash-column fractions were compared, only the 25% EtOAc–75% hexanes fraction, which comprised 64% of the total extract, deterred feeding (Figure 8, top). Of five HPLC fractions from

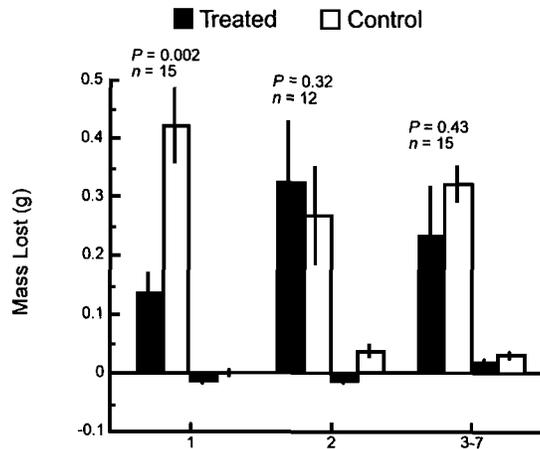


FIG. 7. Effect of fractions of extract of ink from *D. auricularia* on feeding by pufferfish in the laboratory. Data (means  $\pm$  1 SE) are mass lost from diets exposed to (left two bars of each group) and not exposed to (right two bars of each group) pufferfish. *P* values and sample sizes are given above bars.

this flash-column fraction, only one deterred feeding (Figure 8, bottom). This fraction had an orange-red color. A  $^1\text{H}$  NMR spectrum of this active fraction suggested it was a triglyceride with unsaturated fatty acids.

#### DISCUSSION

Our results indicate striking differences in the secondary metabolite composition of various tissues, ink, and eggs from *D. auricularia*, and in their palatability. Extracts of digestive glands contained a variety of algal-derived secondary metabolites and were very unpalatable. Extracts of ink, eggs, and skin were unpalatable; however, the unpalatability did not appear to originate from algal compounds. Extracts of body-wall were palatable. Extracts of digestive glands inhibited cleavage of sea urchin zygotes, but extracts of ink, skin, body wall, and eggs did not. These results suggest that *D. auricularia* is chemically defended, but that these defenses involve more than just the utilization of dietary metabolites.

Digestive glands of *D. auricularia* contained numerous secondary metabolites, consistent with previous findings that *D. auricularia* is a generalized feeder (Pennings and Paul, 1992; Pennings et al., 1993). Although we did not identify all these compounds, we did isolate prepacifenol epoxide, known from the red algal genus *Laurencia* and the sea hare *Aplysia californica*, and its derivative

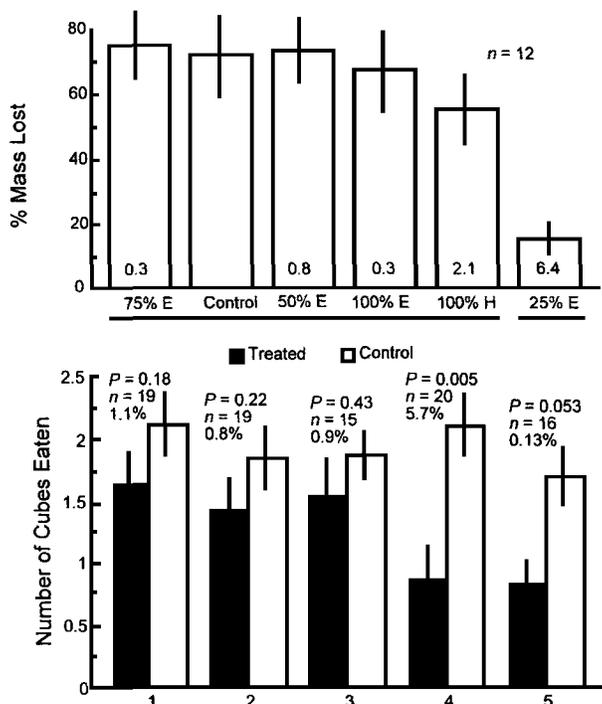


FIG. 8. Effect of fractions of extract of eggs from *D. auricularia* on feeding by reef fishes. Data are means  $\pm$  1 SE. H, hexanes; E, ethyl acetate. Top: Flash column fractions (10% total concentration); extract concentrations (% dry mass) are shown in bars. Horizontal bars connect means that are not significantly different. Bottom: HPLC fractions; data are number of cubes out of four eaten by reef fishes from paired treated and control ropes. *P* values, sample sizes, and extract concentrations are given above bars.

johnstonol (Sims et al., 1972; Faulkner et al., 1974; Ireland et al., 1976) and another compound that was probably a dictyol diacetate similar to a group of compounds known from the brown algal genus *Dictyota* (Faulkner, 1984). Other workers have isolated brown algal metabolites from *D. auricularia* (Faulkner, 1984, 1988). *D. auricularia* has also been a source of the promising anti-cancer compounds, dolastatins (Pettit, 1996), some of which probably originate from cyanophytes (Pettit et al., 1989).

Extracts of digestive glands were extremely unpalatable to reef fishes in our assays and strongly reduced cleavage of sea urchin zygotes. The digestive gland is the primary location of dietary-derived secondary metabolites in a variety of sea hares (Winkler, 1969; Faulkner, 1992; Pennings and Paul, 1993; de Nys et al., 1996). The simplest explanation for the accumulation of algal metabolites

in the digestive gland is that it is a way of detoxifying a chemically rich diet (much as the human liver removes a variety of toxic materials). An accumulation of unpalatable metabolites in an internal organ could not provide any selective advantage to *D. auricularia* through deterring predators since an individual *D. auricularia* would have to be disemboweled before the unpalatable metabolites could be detected. Consequently, even though an individual that sequestered secondary metabolites might be found distasteful by predators, it would not disproportionately survive to pass on its genes. However, if dietary-derived secondary metabolites were transferred to the skin, ink, or eggs, they might serve as a chemical defense. We evaluate this possibility below.

Although we found that extracts of skin were moderately unpalatable, our bioassay-guided fractionation did not lead us to an algal metabolite. Instead, we identified (-)-7-dehydrocholesterol as being responsible. This was surprising and may be an artifact since (-)-7-dehydrocholesterol decomposes to form vitamin Ds and sterol endoperoxides that could have been the source of the unpalatability. We do not know if this transformation would take place *in vivo*, nevertheless, no other skin fractions deterred feeding, suggesting either that the skin of *D. auricularia* is not unpalatable at all (i.e., all the skin results are artifacts), or that (-)-7-dehydrocholesterol is responsible for the unpalatability. (-)-7-Dehydrocholesterol is related to similar sterols isolated from sponges, tunicates, corals, and *Aplysia* spp. (Gunatilaka et al., 1981; Bobzin and Faulkner, 1992; Ortega et al., 1997; Slattery et al., 1997). Some of these have been shown to deter potential consumers (Slattery et al., 1997), indicating that similar compounds may be widespread in marine invertebrates, possibly playing an unappreciated role in defense against predators. Dietary-derived secondary metabolites are found in low concentrations in the skin of species of *Dolabella*, *Stylocheilus*, and *Aplysia* (Winkler, 1969; Faulkner, 1992; Pennings and Paul, 1993). Our results suggest that these compounds occur in *D. auricularia* at concentrations too low to deter feeding; however, they may be concentrated enough in some other sea hares to be a defense (Faulkner, 1992; de Nys et al., 1996).

The function of sea hare ink has been a long-standing mystery (Chapman and Fox, 1969; Nolen et al., 1995; Carefoot et al., 1999). The hypothesis that sea hare ink is unpalatable has been tested for *Stylocheilus longicauda* (Paul and Pennings, 1991), *D. auricularia* (Pennings, 1994), and a number of *Aplysia* spp. (Ambrose and Givens, 1979; DiMatteo, 1981, 1982; Pennings, 1994; Nolen et al., 1995) with ambiguous results. The results of our experiments are also ambiguous. Although organic extract of ink deterred feeding at a high concentration, it did so in only one of three assays when tested at lower concentrations. Since ink is rapidly diluted once released, our results suggest that ink could deter predators only at close range (e.g., if released directly into the mouth of a fish). Alternatively, it might be that ink contains unpalatable compounds that rapidly decompose and, hence, were absent in our experiments. Finally, ink might serve

a purpose other than antipredator defense or might deter predators by a mechanism other than bad taste (Carefoot et al., 1999).

The observation that the active ink fraction was purple and the two nonactive fractions were off-white is consistent with the hypothesis that the unpalatable material is the known ink constituent aplysiocyanin, a monomethyl ester of the red algal pigment phycoerythrobilin (Faulkner, 1992). Thus, the possibility remains that sea hares incorporate dietary-derived compounds into their ink to deter predators. However, both the defensive nature of the ink and the identity of the unpalatable compound(s) remain to be established.

Our results indicate that eggs of *D. auricularia* contain an unpalatable, lipid-soluble compound or compounds. We did not completely purify this substance, but NMR analysis following preliminary purification suggested that it was more likely a triglyceride than a diet-derived algal secondary metabolite. Sea hare eggs are generally ignored by predators (Carefoot, 1987; Pennings, 1994), but efforts to isolate algal secondary metabolites from them have thus far failed (Faulkner, 1992). Work in our laboratory on eggs of *Aplysia juliana* also indicate that they are unpalatable and that the responsible compound is triglyceride-like in nature (Paul and Pennings, unpublished). Antimicrobial peptides and dietary-derived, mycosporine-like amino acids have been isolated from sea hare eggs (Kamiya et al., 1984; Carefoot et al., 1998), but it is unlikely that peptides or amino acids would have been extracted in the organic solvents we used.

If dietary-derived metabolites are important in defending sea hares from predators, we would expect that animals fed different diets would differ in palatability. Pennings (1990) raised small *Aplysia californica* on different diets and found that they differed in palatability to one species of predator, but not to others. By using a similar experimental design, Nolen et al. (1995) concluded that sea hare ink was an effective defense against predation by sea anemones but that secondary metabolites sequestered in the body were not. Moreover, there is little evidence that species of sea hares that eat chemically rich diets are less palatable than species that eat chemically poor diets (Pennings, 1994). In our experiments, the palatability of extracts from the digestive gland and skin of animals fed *Dictyota* spp. and *Padina tenuis* for 11 weeks did not differ from each other or from field animals. These results suggest that diet does not make a difference to the palatability of *D. auricularia*, at least on this time scale. Since diet-derived compounds may be retained in sea hare digestive glands for months (Stallard and Faulkner, 1974; Pennings and Paul, 1993), it is possible that maintaining animals on diets for a longer time period might alter their palatability.

Two important caveats apply to our results. First, it is possible that the process of dissecting animals and extracting the tissues might have introduced artifacts. Such artifacts could occur, for example, if bound chemicals were released, or if chemicals were not stable under the extraction conditions. If such artifacts occurred differentially in different extracts, our tests of relative palatability

could have been compromised. For example, ink might have contained a highly unpalatable but unstable compound that we could not detect with our methods. However, since our techniques were similar to those that have been used to isolate an endless array of algal metabolites from the digestive glands of various sea hares, it is unlikely that this potential artifact could cause typical algal secondary metabolites such as terpenes to be absent from the skin, eggs, or ink. Second, it is possible that our results might have differed had we used different species of consumers. This concern is difficult to address for organisms such as *D. auricularia* that have no known important predators since it is not obvious what the "correct" consumer to test would be. Most of our assays were conducted in the field with a diverse assemblage of fishes and therefore are unlikely to have been affected by the idiosyncratic behavior of any one consumer species. Nevertheless, it is possible that, for example, there could be compounds in the skin of *D. auricularia* that are highly unpalatable to crustaceans, but palatable to fishes.

Given these caveats, our results provide little support for the hypothesis that *D. auricularia* utilizes dietary-derived compounds to protect itself from predators. Dietary-derived compounds may make the ink of *D. auricularia* moderately unpalatable, but the role of ink in defense and the compounds involved have yet to be rigorously established. The skin and eggs of *D. auricularia* may be chemically defended, but the responsible compounds do not appear to be typical algal metabolites. *D. auricularia* does accumulate extremely high concentrations of algal secondary metabolites in the digestive gland, but this may serve to detoxify a chemically rich diet rather than to provide chemical defense.

*Acknowledgments*—Funding was provided by NIH (GM38624 and GM44796) and NSF (OCE-9116307). HPLC instrumentation was funded by grants from the Research Foundation and NSF (BSR-8605299). We thank J. Dobbert, K. Meyer, and K. Sonoda for field and laboratory help and T. Carefoot, the editor J. Romeo, and two anonymous reviewers for comments on the manuscript. This is contribution #410 from the Marine Laboratory of the University of Guam.

#### REFERENCES

- AMBROSE, H. W., III, and GIVENS, R. P. 1979. Distastefulness as a defense mechanism in *Aplysia brasiliana* (Mollusca: Gastropoda). *Mar. Behav. Physiol.* 6:57–64.
- AVILA, C. 1995. Natural products of opisthobranch molluscs: A biological review. *Oceanogr. Mar. Biol. Annu. Rev.* 33:487–559.
- BOBZIN, S. C., and FAULKNER, J. D. 1992. Chemistry and chemical ecology of the Bahamian sponge *Aplysilla glacialis*. *J. Chem. Ecol.* 18:309–332.
- CAREFOOT, T. H. 1987. *Aplysia*: Its biology and ecology. *Oceanogr. Mar. Biol. Annu. Rev.* 25:167–284.
- CAREFOOT, T. H., HARRIS, M., TAYLOR, B. E., DONOVAN, D., and KARENTZ, D. 1998. Mycosporine-like amino acids: Possible UV protection in eggs of the sea hare *Aplysia dactylomela*. *Mar. Biol.* 130:389–396.

- CAREFOOT, T. H., PENNINGS, S. C., and DANKO, J. P. 1999. A test of novel function(s) for the ink of sea hares. *J. Exp. Mar. Biol. Ecol.* 234:185–197.
- CHAPMAN, D. J., and FOX, D. L., 1969. Bile pigment metabolism in the sea-hare *Aplysia*. *J. Exp. Mar. Biol. Ecol.* 4:71–78.
- DE NYS, R., STEINBERG, P. D., ROGERS, C. N., CHARLTON, T. S., and DUNCAN, M. W. 1996. Quantitative variation of secondary metabolites in the sea hare *Aplysia parvula* and its host plant, *Delisea pulchra*. *Mar. Ecol. Prog. Ser.* 130:135–146.
- DIMATTEO, T. 1981. The inking behavior of *Aplysia dactylomela* (Gastropoda: Opisthobranchia): Evidence for distatefulness. *Mar. Behav. Physiol.* 7:285–290.
- DIMATTEO, T. 1982. The ink of *Aplysia dactylomela* (Rang, 1828) (Gastropoda: Opisthobranchia) and its role as a defense mechanism. *J. Exp. Mar. Biol. Ecol.* 57:169–180.
- FAULKNER, D. J. 1984. Marine natural products: Metabolites of marine algae and herbivorous marine molluscs. *Nat. Prod. Rep.* 1:251–280.
- FAULKNER, D. J. 1988. Feeding deterrents in molluscs, pp. 29–36, in D. G. Fautin (ed.). *Biomedical Importance of Marine Organisms*. California Academy of Sciences, San Francisco.
- FAULKNER, D. J. 1992. Chemical defenses of marine molluscs, pp. 119–163 in V. J. Paul (ed.). *Ecological Roles of Marine Natural Products*. Comstock Publishing Associates, Ithaca, New York.
- FAULKNER, D. J. 1997. Marine natural products. *Nat. Prod. Rep.* 14:259–302.
- FAULKNER, D. J., STALLARD, M. O., and IRELAND, C. 1974. Prepacifenol epoxide: a halogenated sesquiterpene diepoxide. *Tetrahedron Lett.* 40:3571–3574.
- FLURY, F. 1915. Uber das Apsysiengift. *Arch. Exp. Pathol. Pharmacol.* 79:250–263.
- GUNATILAKA, A. A. L., GOPICHAND, Y., SCHMITZ, F. J., and DJERASSI, C. 1981. Minor and trace sterols in marine invertebrates. 26. Isolation and structure elucidation of nine new 5 $\alpha$ ,8 $\alpha$ -epidioxysterols from four marine organisms. *J. Org. Chem.* 46:3860–3866.
- IRELAND, C., STALLARD, M. O., FAULKNER, D. J., FINER, J., and CLARDY, J. 1976. Some chemical constituents of the digestive gland of the sea hare *Aplysia californica*. *J. Org. Chem.* 41:2461–2465.
- KAMIYA, H., MURAMOTO, K., and OGATA, K. 1984. Antibacterial activity in the egg mass of a sea hare. *Experientia* 40:947–949.
- MYERS, R. F. 1989. *Micronesian Reef Fishes*. Coral Graphics, Guam.
- NOLEN, T. G., JOHNSON, P. M., KICKLIGHTER, C. E., and CAPO, T. 1995. Ink secretion by the marine snail *Aplysia californica* enhances its ability to escape from a natural predator. *J. Comp. Physiol. A* 176:239–254.
- ORTEGA, M. J., ZUBÍA, E., and SALVÁ, J. 1997. 3-*epi*-Aplykurodinone B, a new degraded sterol from *Aplysia fasciata*. *J. Nat. Prod.* 60:488–489.
- PAUL, V. J., and PENNINGS, S. C. 1991. Diet-derived chemical defenses in the sea hare *Stylocheilus longicauda* (Quoy et Gaimard, 1824). *J. Exp. Mar. Biol. Ecol.* 151:227–243.
- PENNINGS, S. C. 1990. Multiple factors promoting narrow host range in the sea hare, *Aplysia californica*. *Oecologia* 82:192–200.
- PENNINGS, S. C. 1994. Interspecific variation in chemical defenses in the sea hares (Opisthobranchia: Anaspidea). *J. Exp. Mar. Biol. Ecol.* 180:203–219.
- PENNINGS, S. C., and PAUL, V. J. 1992. Effect of plant toughness, calcification, and chemistry on herbivory by *Dolabella auricularia*. *Ecology* 73:1606–1619.
- PENNINGS, S. C., and PAUL, V. J. 1993. Sequestration of dietary secondary metabolites by three species of sea hares: Location, specificity and dynamics. *Mar. Biol.* 117:535–546.
- PENNINGS, S. C., NADEAU, M. T., and PAUL, V. J. 1993. Selectivity and growth of the generalist herbivore *Dolabella auricularia* feeding upon complementary resources. *Ecology* 74:879–890.
- PETERSON, C. H., and RENAUD, P. E. 1989. Analysis of feeding preference experiments. *Oecologia* 80:82–86.

- PETTIT, G. R. 1996. Progress in the discovery of biosynthetic anticancer drugs. *J. Nat. Prod.* 59:812–821.
- PETTIT, G. R., KAMANO, Y., KIZU, H., DUFRESNE, C., HERALD, C. L., BONTEMS, R. J., SCHMIDT, J. M., BOETTNER, F. E., and NIEMAN, R. A. 1989. Isolation and structure of the cell growth inhibitory depsipeptides dolastains 11 and 12. *Heterocycles* 28:553–558.
- SIMS, J. J., FENICAL, W., WING, R. M., and RADLICK, P. 1972. Marine Natural Products III. Johnstonol; an unusual halogenated epoxide from *Laurencia johnstonii*. *Tetrahedron Lett.* 3:195–198.
- SLATTERY, M., HAMANN, M. T., MCCLINTOCK, J. B., PERRY, T. L., PUGLISI, M. P., and YOSHIDA, W. Y. 1997. Ecological roles for water-borne metabolites from Antarctic soft corals. *Mar. Ecol. Prog. Ser.* 161:133–144.
- STALLARD, M. O., and FAULKNER, D. J. 1974. Chemical constituents of the digestive gland of the sea hare *Aplysia californica*—I. Importance of diet. *Comp. Biochem. Physiol.* 49B:25–35.
- SWITZER-DUNLAP, M., and HADFIELD, M. G. 1977. Observations on development, larval growth and metamorphosis of four species of aplysiidae (Gastropoda: Opisthobranchia) in laboratory culture. *J. Exp. Mar. Biol. Ecol.* 29:245–261.
- SWITZER-DUNLAP, M., and HADFIELD, M. G. 1979. Reproductive patterns of Hawaiian aplysiid gastropods, pp. 199–210 in S. E. Stancyk (ed.). *Reproductive Ecology of Marine Invertebrates*, University of South Carolina Press, Columbia.
- WINKLER, L. R. 1969. Distribution of organic bromide compounds in *Aplysia californica* Cooper, 1863. *Veliger* 11:268–271.
- YAMADA, K., and KIGOSHI, H. 1997. Bioactive compounds from the sea hares of two genera: *Aplysia* and *Dolabella*. *Bull. Chem. Soc. Jpn.* 70:1479–1489.