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Defensive chemicals of the Spanisch dancer nudibranch *Hexabranchus sanguineus* and its egg ribbons: macrolides derived from a sponge diet

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Abstract: The Spanish dancer nudibranch *Hexabranchus sanguineus* (Rüppell *et* Leuckart), a large brightly colored shell-less sea slug (Gastropoda: Opisthobranchia) common to Indo–Pacific coral reefs, derives a potent chemical defense from a sponge that it eats (*Halichondria* sp.). In turn, the nudibranch passes defensive compounds to its egg ribbons, which are similarly conspicuous and physically defenseless. Slices of the dorsal mantle tissue of the nudibranch were rejected in laboratory feeding assays employing two common sympatric predators: an Indo–Pacific reef fish, *Thalassoma lunare* (Linnaeus), and a reef hermit crab, *Dardanus megistos* (Herbst). The defensive metabolites, a suite of unusual oxazole-containing macrolides, were isolated from the sponge, the nudibranch, and the nudibranch egg masses at 0.14–0.38, 0.14–0.62, and 2.65% of dry weight, respectively, and were effective inhibitors of feeding by *T. lunare* at minimum concentrations of 0.01–0.02% dry weight of food pellet. The macrolides were concentrated in the dorsal mantle of the nudibranch, which is most vulnerable to predatory attack, and in the combined digestive gland/gonad, site of both sponge digestion and egg production. The most abundant macrolide in the sponge tissue was not present in the nudibranch or its egg masses, suggesting that chemical modification of this compound takes place upon digestion. In a reef environment dominated by visually oriented predators, the striking color pattern and behavioral responses of *Hexabranchus* may have arisen with a concomitant elaboration of dietarily derived chemical defenses.

Key words: Chemical defense; *Hexabranchus sanguineus*; Natural product; Nudibranch; Poriferan; Warning coloration

Introduction

The majority of opisthobranch molluscs lack a hard external shell common to other marine snails. The evolution of shell loss appears to have been associated with the development of defense mechanisms of dietary origin (Faulkner & Ghiselin, 1983), including stinging cells procured from the cnidarian prey of eolid nudibranchs (see review in Thompson, 1976), and secondary metabolites from the algal diet of

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ascoglossans and aplysiids (Lewin, 1970; Stallard & Faulkner, 1974; Kinnell *et al.*, 1979). A defensive rôle has been advanced as the suspected function of the unusual secondary metabolites isolated from several dorid nudibranchs, primarily on the basis of the activity of these compounds in various antimicrobial, pharmacological, and toxicity assays (Fuhrman *et al.*, 1981; Walker & Faulkner, 1981; Schulte & Scheuer, 1982; Gunthorpe & Cameron, 1987; review in Karuso, 1987). Only a few studies have addressed the chemical defense of dorid nudibranchs employing ecologically relevant assay methods (Cimino *et al.*, 1982; Thompson *et al.*, 1982).

One of the largest and most active of nudibranch molluscs, *Hexabranchus sanguineus*, is a common and conspicuous inhabitant of coral reefs throughout the Indo–Pacific region (Gohar & Soliman, 1963; Edmunds, 1968; Thompson, 1971; Francis, 1980). It is renowned for its spectacular swimming response, wherein the slug throws its body into sweeping dorsoventral flexions, sending synchronous undulations through the broad and vividly patterned red and white margins of its mantle (Edmunds, 1968; Thompson, 1972). This display has earned it the common name "Spanish dancer". Conspicuous also are the egg masses of these slugs: the bright pink to red egg ribbons are indiscriminately deposited as coiled rosettes on rocks and coral rubble (Gohar & Soliman, 1963). *Hexabranchus* does not appear to have any physical defense mechanism, nor does it protect itself with acidic secretions described for other opisthobranch molluscs (Thompson, 1972; 1976). Instead, we have found that *Hexabranchus* and its egg ribbons are chemically defended from potential predators by macrolides derived from specific sponges upon which the nudibranch feeds.

Methods

We recently collected two large ($\approx$25 cm in length) specimens of *Hexabranchus sanguineus* on a south-facing reef off Kwajalein Atoll, Marshall Islands (September 1986 collection). On the same reef, we harvested large quantities of a small black sponge of uncertain taxonomy, *Halichondria* sp. A, which had been obtained from the same site on a previous expedition. The sponge contained unusual and previously undescribed trisoxazole macrolides, which were highly effective inhibitors of fungal growth (Kernan & Faulkner, 1987; Kernan, 1988; Table I, Fig. 1). A crude organic extract of the mucoid exudate of the frozen nudibranchs was analysed by NMR spectroscopy for the presence of macrolides or other unusual secondary metabolites. The crude extract was sequentially partitioned into hexane, dichloromethane, ethyl acetate, methanol, and water and each extract subjected to the fish feeding assay.

Laboratory feeding assays were performed on two common Indo–Pacific predators, the reef fish (wrasse) *Thalassoma lunare* and the hermit crab *Dardanus megistos*. Selection of these assay organisms took into account two considerations: (1) likelihood that the predatory species would encounter *Hexabranchus* in nature, and (2) ease of collection and maintenance. Wrasses are highly active opportunistic carnivores;

T. lunare in particular is a common reef species distributed throughout the tropical Indo–Pacific (Randall, 1983), a range coincidental with that of *H. sanguineus* (Thompson, 1972). *Thalassoma lunare* is diurnally active, and would be exposed to the egg ribbons of *Hexabranchus* and to the nudibranchs themselves, the latter being active both day and night (Gohar & Soliman, 1963; Francis, 1980; T. F. Molinski, pers. obs.). The reef hermit crab *Dardanus megistos* is found throughout the tropical Indo–Pacific west of Hawaii (Tinker, 1965). One of the largest of its family (reaching 30 cm in length), it is an opportunistic omnivore that would also be expected to encounter both *Hexabranchus* and its egg masses.

Fish and crabs were obtained through Marine Fish Enterprises, Gardena, California. Wrasses were collected in Samoa, Hawaii, and Mexico; crabs were collected in Hawaii. Specimens were shipped to Scripps Institution of Oceanography in healthy condition and commenced feeding within a few days of arrival. Thirteen fish (8–18 cm standard length) were placed into separate 5–10-l aquarium divisions. Ten hermit crabs (in gastropod shells with 4–7-cm apertures) were placed in separate $\approx$ 1-l aquarium divisions.

Assays were first performed using the dorsal mantle tissue of frozen specimens of *Hexabranchus* (September 1986 collection). For these experiments, pieces of squid mantle tissue were used as alternative food items (Calamari, Fiesta del Mar, San Pedro, California). *Hexabranchus* mantle that has been stripped of its red epidermis has the same color and texture as squid mantle. Ten fish were randomly selected and offered both a piece ($\approx 10 \times 4 \times 4$ mm) of stripped *Hexabranchus* dorsal mantle tissue and a similarly sized piece of squid mantle tissue, the order determined by coin toss. Fish rejected the offering if they spat it out three times or more, or abandoned it after spitting it out. Ten crabs were given both a piece ($\approx 2 \times 2 \times 0.4$ cm) of stripped *Hexabranchus* mantle tissue and a similarly sized piece of squid mantle tissue, one immediately after the other, the order determined by coin toss. After 10 min, the tissue remaining in the crabs' feeding appendages was noted.

Feeding assays of extracts and purified compounds were performed with *T. lunare* employing methods described in Pawlik *et al.* (1986, 1987). Organic extracts were dissolved in their respective solvents and applied to freeze-dried krill (*Euphausia* sp.) so as to comprise 5% of the dry weight of the food pellet after evaporation of the solvent. Extract treated pellets and control pellets, treated with solvent alone, were randomly offered to *Thalassoma lunare* as previously described.

Macrolides were purified from the combined hexane, dichloromethane and ethyl acetate extracts of *Hexabranchus* exudate by flash chromatography on silica (E. Merck, 230–400 mesh) employing a 0–10% methanol in dichloromethane gradient followed by HPLC on a Dynamax C-18 column (Rainin Instrument) with 80% methanol in water as the eluant. The identity of all compounds was confirmed or determined by employing standard spectrometric techniques (Kernan & Faulkner, 1987; Kernan, 1988). The purified macrolides, along with pigment and sterol fractions that were similarly isolated, were assayed as previously described.

The two frozen nudibranchs (September 1986 collection) were thawed and four body parts separated from each: the remaining dorsal mantle (a small portion of which had previously been used in feeding assays), the foot, the combined digestive gland/gonad, and the accessory reproductive organs (coiled penis, ampulla and associated glands). Each portion was freeze-dried, extracted, and the macrolides present in the tissue isolated and identified. We subsequently obtained an additional collection of *Hexabranchus* from Kwajalein (November 1986), and two egg ribbons laid by the nudibranchs while in captivity prior to shipment. In this instance, the two nudibranchs were preserved together in methanol, as were the egg masses, so the individual specimens could not be separated for analysis. Both the nudibranchs and the egg ribbons were fully extracted and the macrolides isolated and identified as before.

Given the close proximity of the sponge to the nudibranch at the collection site, and the similarity of their chemistries, we sought evidence to support the dietary origin of the macrolides in the nudibranch tissues. *Hexabranchus* is known to prey predominantly on sponges; remains of other invertebrate and algal species reported from gut content analyses are thought to have resulted from incidental consumption during sponge grazing (see review in Francis, 1980). The gut contents of *Hexabranchus* from the September 1986 collection were examined for sponge spicules. Further, we obtained five living specimens of *Hexabranchus* collected in the Philippines. Three of these were extracted as before upon arrival and the two remaining live nudibranchs were maintained in an aquarium on a diet of thawed pieces of *Halichondria* sp. A. After 2 months, these nudibranchs were extracted as before and macrolides isolated and identified.

Results

In assays of *Hexabranchus* mantle tissue employing the wrasse *Thalassoma lunare*, all 10 fish readily ate pieces of squid mantle, but rejected pieces of *Hexabranchus* mantle. For assays employing the hermit crab, *Dardanus megistos*, in nine of 10 cases, crabs abandoned the *Hexabranchus* tissue within 10 min and commenced feeding on squid mantle. One crab continued to hold the *Hexabranchus* tissue with one claw while it fed on squid mantle with the other.

Spectrometric analyses of crude organic extracts of a mucoid exudate of *Hexabranchus* revealed the presence of macrolides similar to those found in the cooccurring sponge *Halichondria* sp. A. Subsequent purification led to the isolation of three macrolides, two of which were previously undescribed: dihydro- and tetrahydrohalichodramide (Table I; Fig. 1; Kernan, 1988). The same macrolides were isolated from the nudibranchs that had produced the exudate. Two *Hexabranchus* from a subsequent collection from the same location contained approximately the same proportion of macrolides found in the September 1986 collection, but at lower concentrations (Table I). The egg ribbons produced by these nudibranchs, however, contained a

TABLE I

Concentration of macrolides in tissues of sponges *Halichondria* spp., nudibranch *Hexabranchus*, and in mucoid exudate and egg masses of *Hexabranchus*. Structures of macrolides are shown in Fig. 1.

Sample	Collection		Sample	% Dry weight					
	Site	Date		1 Halichond.	2 Dihydro.	3 Tetra.	4 K.B	5 K.C	Total
Halichondria sp. A	Kwajalein	9/86		0.350	0.030	–	–	–	0.380
Halichondria sp. B	Palau	1/85		–	–	–	0.080	0.055	0.135
Hexabranchus sanguineus	Kwajalein	9/86	1	–	0.210	0.048	–	0.009	0.267
			2		0.320	0.072	–	0.018	0.410
Hexabranchus sanguineus	Kwajalein	11/86		–	0.100	0.020	0.010	0.010	0.140
Hexabranchus sanguineus	Philippines	4/87	1, 2, 3	–	–	–	–	–	[b]
Hexabranchus sanguineus[a]	Philippines	6/87	1	–	0.052	0.220	–	–	0.272
			2	–	0.253	0.370	–	–	0.623
Mucoid exudate of *Hexabranchus sanguineus*	Kwajalein	9/86		–	0.104	0.015	–	0.008	0.127
Egg mass of *Hexabranchus sanguineus*	Kwajalein	11/86		–	1.710	0.160	0.100	0.680	2.650

[a] Fed frozen *Halichondria* sp. A from Kwajalein 9/86 collection for 2 months prior to analysis. [b] Two undescribed macrolides isolated in small quantities from one of three specimens. –, not detected.

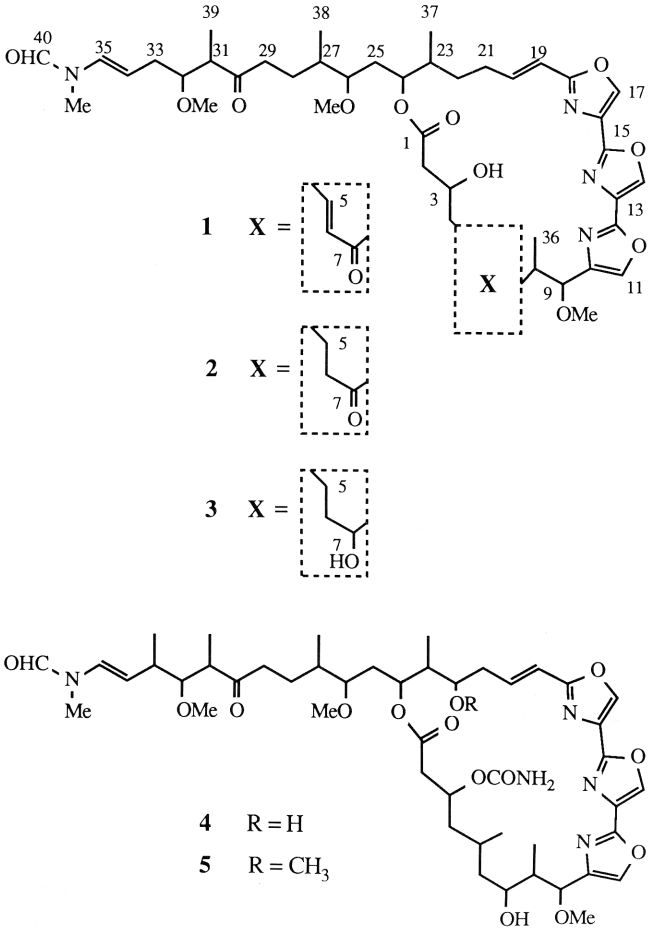

Fig. 1. Macrolides isolated from sponges *Halichondria* sp. A (**1**, **2**) and *Halichondria* sp. B (**4**, **5**) and from nudibranch *Hexabranchus sanguineus* and its egg ribbons (**2–5**). Halichondramide, **1**; dihydrohalichondramide, **2**; tetrahydrohalichondramide, **3**; kabiramide B, **4**; kabiramide C, **5**. Structural elucidation of **2** and **3** will be presented elsewhere. Structures **1**, **4**, and **5** have been described (Matsunaga *et al.*, 1986; Kernan & Faulkner, 1987; N. Fusetani, pers. comm.).

$\geqslant$ 10-fold enrichment in each of the macrolides as compared to the concentration in the tissues of the nudibranchs that laid them. Another undescribed macrolide, kabiramide B, was isolated from both the egg masses and the nudibranchs (Table I, Fig. 1).

HPLC analysis of extracts of dissected portions of two *Hexabranchus* revealed that macrolides were concentrated in the dorsal mantle and in the digestive gland/gonad of the nudibranchs (Table II). Only small amounts of macrolides were present in the foot tissue and none were detected in the accessory reproductive organs.

The gut contents of *Hexabranchus* from the September 1986 collection contained

TABLE II

Concentration of macrolides in four body parts of *Hexabranchus* (Kwajalein 9/86 collection). Macrolide concentration was determined for four parts of each of two nudibranchs.

Sample	Sample	% Dry weight					Total
		1 Halichond.	2 Dihydro.	3 Tetra.	4 K.B	5 K.C	
Dorsal mantle	1	–	0.700	0.033	0.007	0.016	0.756
	2	–	0.490	0.120	–	0.032	0.642
Foot	1	–	0.026	0.008	–	–	0.034
	2	–	0.006	0.003	–	–	0.009
Digestive gland	1	–	0.470	0.110	–	0.068	0.648
and gonad	2	–	0.190	0.032	–	0.005	0.227
Accessory	1	–	–	–	–	–	–
reproductive organs	2	–	–	–	–	–	–

–, not detected.

agglutinated masses of spicules: oxea megascleres, ≈ 545 μm in length. These spicules were identical to those found in the tissues of *Halichondria* sp. A collected at the same site.

Living *Hexabranchus* were maintained for 2 months on thawed pieces of *Halichondria* sp. A. Each nudibranch ate ≈ 1 cm^3 of sponge tissue every 2 days. There was no apparent growth of the nudibranchs during this time. The nudibranchs would not feed on five other unidentified thawed sponges from Kwajalein, or on living pieces of *Spheciospongia confoederata* deLaubenfels or *Aplysina fistularis* (Pallas) from San Diego, California. Extraction and isolation of macrolides from these nudibranchs revealed high concentrations of dihydro- and tetrahydrohalichondramide (Table I).

Only the hexane, dichloromethane and ethyl acetate extracts of the mucoid exudate of *Hexabranchus* inhibited feeding of the wrasse *T. lunare* in laboratory assays. These three extracts were similarly composed of a mixture of pigments, sterols, and macrolides. Following isolation by HPLC, the pigment and sterol fractions were found to be palatable to fish, but each of the macrolides inhibited feeding at a minimum concentration of 0.01–0.02% dry weight of food pellet (Table III). Purified samples of halichondramide and kabiramide B, from *Halichondria* sp. A and the egg ribbons of *Hexabranchus*, respectively, were similarly unpalatable to fish (Table III).

DISCUSSION

The macrolides isolated from *Hexabranchus*, its egg masses, and the sponge *Halichondria* are the most potent inhibitors of fish feeding reported to date (cf. Cimino *et al.*, 1982; Thompson *et al.*, 1982; Pawlik *et al.*, 1986; 1987). Deployment of these compounds is specific: macrolides were concentrated in the dorsal mantle of the

TABLE III

Effects of macrolides applied to food pellets on feeding of reef fish *Thalassoma lunare*. Five pairs of treated and control food pellets were offered randomly, one pair to each of five fish, in assays of tetrahydrohalichrondramide and kabiramide B. Ten replicates were performed for each of other three macrolides.

Concentration % dry weight of pellet	Treated pellets eaten/control pellets eaten				
	1 Halichond.	2 Dihydro.	3 Tetra.	4 K.B	5 K.C
1.0	1/9*	1/10*	–	–	0/10*
0.5	1/10*	0/10*	–	–	0/10*
0.1	0/9*	0/10*	–	–	0/9*
0.02	3/10*	1/8*	0/5*	0/5*	2/9*
0.01	6/7	5/10*	4/5	2/5	4/9*
0.005	–	7/10	–	–	9/10

* $P < 0.05$, Fisher exact test; –, not assayed.

nudibranch and in the combined digestive gland/gonad, while little or none were present in foot or accessory reproductive tissues. The dorsal mantle of the nudibranch is most vulnerable to attack by predators; localization of the macrolides in the mantle further supports their putative defensive function. Although the mucoid exudate of the nudibranchs contained high concentrations of macrolides, it is unclear to what extent this was a result of their having been frozen. It seems likely, however, that macrolides are deployed into the mucus on the dorsal surface of *Hexabranchus* under natural conditions. The high concentration of macrolides in the digestive gland/gonad is not surprising, as this is the site of sponge digestion, and likely, of macrolide uptake. Furthermore, the gonad is the site of egg production, and it is probable that the macrolides are transferred to, and stored in, the eggs prior to their deposition.

The absence of halichondramide, the dominant sponge macrolide, in the tissues of the sponge-fed nudibranchs suggests that this macrolide is being converted by the nudibranch to dihydro- or tetrahydrohalichondramide. An alternative hypothesis is that halichondramide is specifically eliminated by the nudibranchs. Given the paucity of dihydrohalichondramide relative to halichondramide in the sponge tissue (Table I), the former seems the most likely explanation. The absence of either kabiramide B or C in the tissues of these nudibranchs suggests that these macrolides, present in the Kwajalein nudibranchs, were derived from another sponge source. Kabiramides B and C were previously isolated by our group from a very similar but morphologically distinct sponge, *Halichondria* sp. B, from Palau (Table I).

There is evidence to suggest that the chemical defense of *Hexabranchus* and its egg ribbons by macrolides is not restricted to Kwajalein Atoll, and, therefore, that macrolide-containing sponges are similarly distributed. Trisoxazole macrolides have recently been isolated from *Hexabranchus* and its egg masses from Hawaii (Roesener & Scheuer, 1986), and from an unidentified egg ribbon (most likely from *Hexabranchus*)

collected in the Ryukyus Islands (Matsunaga *et al.*, 1986). As previously stated, we isolated small quantities of macrolides from one of three specimens of *Hexabranchus* collected in the Philippines. The lack of defensive metabolites isolated from the other two specimens may be attributable to starvation and rough treatment during captivity, which would result in exudation of metabolites that could not be replaced. Alternatively, these animals may never have procured defensive compounds at all. If the foraging pattern of *Hexabranchus* is relatively indiscriminate and includes several species of sponges that do not contain the defensive compounds or precursors, these metabolites may never be elaborated by the nudibranch or may be subsequently lost through exudation or chemical decomposition. Individual *Hexabranchus* might therefore be expected to contain highly variable concentrations of metabolites, depending on the chemistry of the sponges they have most recently eaten. There is evidence to suggest that spongivory by *Hexabranchus* is nonselective (Francis, 1980), and this may explain the variable concentrations of macrolides isolated from the nudibranchs in the present study.

The macrolides involved in this nudibranch-sponge association are most closely related to the scytophycins, a group of cytotoxic and antifungal macrolides recently isolated from cultures of terrestrial blue-green algae (cyanophytes; Ishibashi *et al.*, 1986). A more distantly related group of macrolides has been isolated from Indo–Pacific sponges of the genus *Theonella*, which are known to contain symbiotic unicellular cyanophytes (Carmely & Kashman, 1985; Sakai *et al.*, 1986). Extracts of *Halichondria* sp. A were free of both Chl *a* and *b*, ruling out a symbiotic cyanobacterial origin for these complex metabolites. The presence of heterotrophic bacterial symbionts in the tissues of *Halichondria*, and their involvement in the production of macrolides, remains a possibility that has yet to be explored.

Although the potent antifungal properties of the macrolides sequestered by *Hexabranchus* may be important for its survival, particularly for the protection of its egg masses from microbial attack, defense against predation seems the most likely primary function. *Hexabranchus* is particularly conspicuous when swimming or crawling on the reef, and its egg masses are prominently deposited. When resting, the nudibranch folds its mantle margins against its body, presenting a nondistinctive mottled aspect. If disturbed while in this state, however, the mantle margins are quickly unfurled in a display strikingly similar to the startle response of some insects (Edmunds, 1968; Wickler, 1968). In an environment where visual cues dominate, especially among coral reef fishes (Lowe-McConnell, 1987), the brilliant red and white contrasting pattern on the mantle of *Hexabranchus* may serve as a warning to potential predators. If some members of a population of *Hexabranchus* lack defensive chemicals due to the absence of suitable sponges in their diet, a form of automimicry may have arisen. Among butterflies, automimicry describes a type of intraspecific mimicry in which larvae of the same species reared on both poisonous and nonpoisonous plants produce identical brightly colored adults that are unpalatable (model) and palatable (mimic), respectively, to potential predators (Brower, 1969). Avian predators of butterflies learn to avoid both

model and mimic after attempting to eat an unpalatable model. In a similar vein, the evolution of the distinctive color pattern and associated behavior of *Hexabranchus* is likely linked to its acquisition and elaboration of dietarily derived defensive metabolites.

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