Tetrahedron Vol. 38, No. 13, pp. 1865 to 1873, 1982
Printed in Great Britain.

0040-4020/82/131865-09$03.00/0
Pergamon Press Ltd.

A CHEMICAL DEFENSE MECHANISM FOR THE NUDIBRANCH *CADLINA LUTEOMARGINATA*

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(*Received in U.S.A.* 13 *July* 1981)

Abstract—The nudibranch *Cadlina luteomarginata* from San Diego, California, concentrates selected metabolites from the sponges that constitute its diet. Gut analyses revealed that *C. luteomarginata* consumes at least ten sponges although *Axinella* sp. and *Myxilla incrustans* are most frequently eaten. Field observations and analysis of metabolites suggest that keratose sponges *Leiosella idia* (= *Spongia idia*) and *Dysidea amblia* are also consumed. Three novel compounds, the furan **20**, the isonitrile **23** and the isothiocyanate **24** were identified by analysis of spectral data. The secondary metabolites of *C. luteomarginata* were found only in the dorsum, which is exposed to potential predators. Five metabolites of *C. luteomarginata* were screened for antifeedant activity against fish and all showed some activity at 10–100 μg/mg in food pellets.

INTRODUCTION

There are over 3000 described species of opisthobranchs, a subclass of the gastropod molluscs.[1] Most opisthobranchs lack the protective shell possessed by other gastropods and appear relatively defenseless against potential predators.[2] Despite the apparent lack of protection, these animals have few known predators and those predators are generally other carnivorous opisthobranchs.[3] Laboratory experiments support the hypothesis that unidentified materials localized on or in the tissues of opisthobranchs protect the animals from predation; these tissues are rapidly rejected as a food source by carnivorous fish.[4] In this paper we will present evidence to support the hypothesis that noxious chemicals of dietary origin protect adult *Cadlina luteomarginata* MacFarland, 1966 from some potential predators.

The nudibranchs, of which *C. luteomarginata* is an example, form the largest order in the subclass Opisthobranchia. They have been described as the "butterflies of the ocean" as they are often brightly colored and highly visible. Since these animals lack any obvious protection against fish, mollusc, asteroid and crab predators, there have been many studies of their defense mechanisms. It has been pointed out that the bright coloration of some nudibranchs, such as *Rostanga* species, may render them camouflaged against the background of a similarly colored food source.[5] Some members of the suborder Aeolidaceae have developed a remarkable defense mechanism using undischarged nematocysts acquired from their coelenterate diet.[6] The spicules that fortify the integument of many members of the suborder Doridaceae may act as a deterrent to predation[7] but they are probably not responsible for the rapid rejection of nudibranchs by carnivorous fish—noxious secretions seem more important in most cases. Two types of chemical defense mechanisms have evolved: some dorids have glands that secrete strong acid (pH ~ 1)[8] while others are thought to secrete toxic or noxious substances[9] that can often be traced to their diet of sponges. *Cadlina luteomarginata* falls into the latter group of dorid nudibranchs.

The hypothesis that some opistobranch molluscs use a chemical defense mechanism is supported by few experiments and much circumstantial evidence. To support the hypothesis that a particular dorid nudibranch employs a chemical defense mechanism using chemicals obtained from their diet of sponges, it is essential to show that the nudibranch stores specific chemicals from sponges that these chemicals have antifeedant properties. The nudibranch may eat a variety of sponges but should store only selected compounds from which they derive benefit. The possibility that the metabolites extracted from nudibranchs could all be derived from sponge material passing through the digestive tract at the time of death must also be excluded. Finally, the antifeedant bioassay should be performed using predators that might reasonably be expected to eat nudibranchs if they had no chemical defense mechanism. Even so, it is impossible to establish categorically that a chemical exhibiting antifeedant properties is solely responsible for an observed lack of expected predation.

Many unusual and *potentially* noxious chemicals have been isolated from opisthobranch molluscs. Chemical studies began with the larger aplysiomorphs (sea hares) and have gradually progressed to the smaller saccoglossans and nudibranchs. The compounds isolated from opisthobranchs have often been traced to dietary sources but few of these compounds have been tested for toxicity or antifeedant activity.

The first marine natural product research on opisthobranchs was performed in the early 1960's by Yamamura and Hirata[10] who isolated brominated terpenes from *Aplysia kurodai*. The sea hares were ideal for marine natural product studies since they were large, easily collected and contained large quantities of metabolites that often contained halogens. Rather than review all of the metabolites from sea hares, we wish to emphasize the dietary sources of these metabolites. The metabolites of *Aplysia californica* were shown to be identical to or closely related to metabolites of red algae of the genera *Laurencia* and *Plocamium*.[11] The metabolites were found predominantly in the digestive gland and the skin

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of the sea hare and the residence time of an average metabolite was ~90 days. Comparison of the compounds obtained from sea hares with algal metabolites showed that *Aplysia angasi*,[12] *A. brasiliana*,[13] *A. dactylomela*,[14] and *A. kurodai*[10,15] all contained *Laurencia* metabolites, *A. limicina*[16] contained *Plocamium* metabolites while *A. depilans*[17] and *A. vaccaria*[18] contained metabolites from brown algae of the genus *Dictyota*. *Dolabella californica*[19] and *D. auricularia*[20] probably obtained their metabolites from *Dictyota* species or related brown algae but no direct feeding observations have been made. The observation that *Laurencia* and *Dictyota* species were avoided by herbivorous fish[21] supported the hypothesis that the algal metabolites were involved in the sea hares' defense mechanisms. Of all the metabolites isolated from sea hares, only brasilenyne (**1**) and *cis*-dihydrorhodophytin (**2**), both assumed to be *Laurencia* metabolites, have demonstrated fish antifeedant activity.[13]

Aplysiatoxin (**3**) and debromoaplysiatoxin (**4**) are toxins isolated from the Hawaiian sea hare *Stylocheilus longicauda*.[22] They are also obtained from the blue-green algae that cause the irritation called "swimmer's itch".[23] Although these compounds have not been reported as antifeedants, their irritant properties should make them useful chemical deterrents.

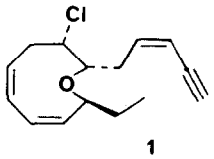
The saccoglossans feed on marine algae by piercing the cell wall with their radulae and sucking out the cellular material. The three saccoglossans that have been studied all assimilate functional chloroplasts from siphonous algae and are able to employ the photosynthetic ability of the chloroplasts to convert bicarbonate into complex metabolites. *Tridachiella diomedea*,[24] *Tridachia crispata*[25] and *Placobranchus ocellatus*[26] all contain "propionate"-derived metabolites, such as tridachione (**5**), that result from *de novo* biosynthesis rather than dietary sources.

The onchid, *Onchidella binneyi*, provided one of the clearest examples of a chemical defense mechanism. When molested, *O. binneyi* expelled a defense secretion that contained the highly reactive molecule onchidal (**6**).[27] Onchidal (**6**) is the enol acetate of ancistrodial, a component of the defensive secretion of the termite *Ancistrotermes carvithorax*.[28]

One of the few animals that preys on opisthobranchs is the cephalaspideal opisthobranch *Navanax inermis*. In order to deter cannibalism, *N. inermis* is able to dissuade other members of the species from following its slime trail by releasing a yellow secretion. The active trail-breaking substances are navenone-A (**7**) and two related compounds.[29]

Until recently, studies of the metabolites of nudibranchs were rare. The small size of most nudibranchs and the correspondingly small quantities of metabolites isolated made structural elucidation difficult. While studying the chemical constituents of the defensive secretion from *Phyllidia varicosa*, Hagadone *et al.*[30] found that the ichthyotoxic constituents were present in a sponge *Hymeniacidon* sp., on which the nudibranch was observed to feed. By using the sponge as a source of the ichthyotoxic substance, sufficient material was obtained to enable the structural elucidation of the isonitriles **8** and **9**. Using the same strategy, Castiello *et al.* have identified the metabolites of the nudibranch *Peltodoris atromaculata* as a mixture of acetylenes **10** from the sponge *Petrosia ficiformis*.[31] Schulte *et al.* showed that one specimen of *Hypselodoris godeffroyana* and three specimens of *Chromodoris maridadilus*, all collected while feeding on *Dysidea fragilis*, contained the same furanosesquiterpenes found in the sponge.[32]

Advances in instrumental analysis, particularly the increased availability of FT NMR instruments, has made it possible to identify some metabolites from nudibranchs



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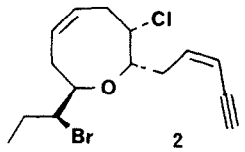

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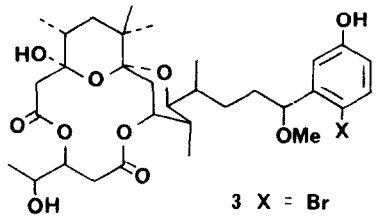

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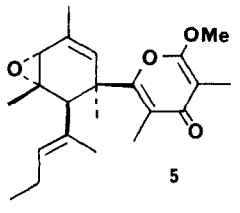
4 X = H



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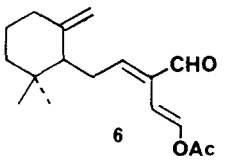

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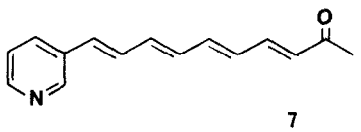

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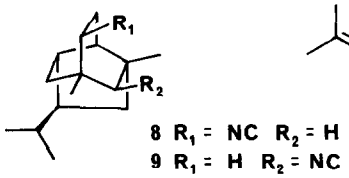
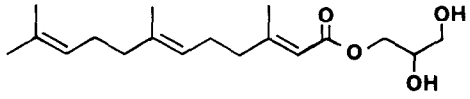
using only the material isolated from the nudibranch. Andersen and Sum[33] have reported the isolation of 2,3-dihydroxypropyl farnesoate (**11**) and two mono-acetyl derivatives from the nudibranch *Archidoris odhneri*. We have recently identified marislin (**12**), the major metabolite of *Chromodoris marislae*, and shown that it can be converted into pleraplysillin-2 (**13**) a metabolite of the sponge *Pleraplysilla spinifera*.[34] We have also described a series of chlorinated acetylenes (e.g. **14**) from the nudibranch *Diaulula sandiegensis*.[35]



8 R_1 = NC R_2 = H
9 R_1 = H R_2 = NC



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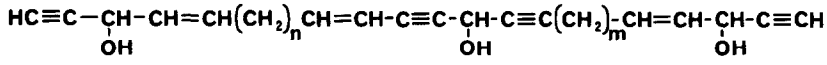

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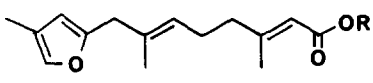
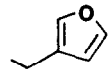
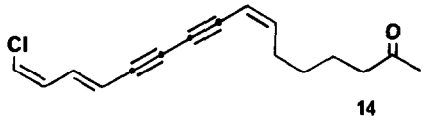


12 R =

13 R =





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Studies of Cadlina luteomarginata

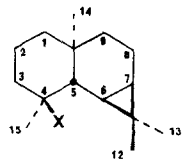
Samples of *Cadlina luteomarginata* were collected at Scripps Canyon, La Jolla, California during January, July and October 1977 and at Point Loma, San Diego, California during October 1978 and July to September 1980. The January 1977 collection of 25 animals was homogenized in ethanol and the chloroform soluble material from the ethanol extract chromatographed on silica gel. Examination of the ^{1}H NMR spectra of various fractions indicated that the least polar fractions contained a mixture of terpenoid furans. The mixture was separated on silver nitrate impregnated silica gel to obtain dendrolasin (**15**, 0.12% dry weight),[36] pallescensin-A (**16**, 0.16% dry weight),[37] pleraplysillin-1 (**17**, 0.14% dry weight)[38] and furodysinin (**18**, 0.2% dry weight).[39] Each of these compounds was identified by comparison of spectral data with published data.[40] The ^{1}H NMR spectra of more polar fractions indicated that most fractions contained either very complex mixtures of minor metabolites, sterols or fats. The one exception was a fraction eluted with 50% ether in hexane that contained predominantly one compound, later identified as idiadione (**19**, 5 mg, 0.1% dry weight).[41] It is significant that no isonitriles or related compounds were observed in this sample.

The July 1977 collection of approximately 100 animals (22 g dry weight) was extracted and the extract chromatographed on Florisil. Examination of the ^{1}H NMR spectra immediately indicated that several different compounds were present. The non-polar furanoid fraction contained only two compounds, pallescensin-A (**16**, 7 mg, 0.03% dry weight) and an undescribed furan **20** (6 mg, 0.03% dry weight). The structure of the furan **20** was suggested from the mass spectrum and the ^{1}H NMR data but has not been confirmed by synthesis or interconversion with a known compound. The ^{1}H NMR spectrum contained signals at δ 7.20 (br s, 1 H), 7.10 (br s, 1 H) and 6.14 (br s, 1 H), assigned to a β-substituted furan, at 4.77 (br s, 1 H) and 4.55 (br s, 1 H) due to the exocyclic methylene protons and at 0.92 (s, 3 H) and 0.85 (s, 3 H) due to the gem-dimethyl group. The structure proposed for the furan **20** is based on the terpenoid skeleton of pallescensins 1–3[42] and corresponds to a dihydro derivative of pallescensin-2 (**21**).

The material eluted from the Florisil column with 1% dichloromethane in hexane contained predominantly one compound, subsequently identified as the isothiocyanate **24** (~20 mg, 0.09% dry weight). The major secondary metabolites were eluted as two fractions with 3% dichloromethane in hexane. Rechromatography of the first fraction on silica gel gave the isonitrile **22** (15 mg, 0.07% dry weight) and the isonitrile **23** (65 mg, 0.3% dry weight). The second minor fraction also contained a mixture of isonitriles that has subsequently been separated using reverse phase chromatography. Only the isonitrile **23** and the related isothiocyanate **24** were identified at this time.

The isonitrile **23**, $[\alpha]_D$ +36° (*c* 0.02, $CHCl_3$), had the molecular formula $C_{16}H_{25}N$. The IR spectrum contained

Table 1. ^{1}H NMR data for isonitrile **23** and isothiocyanate **24**



23 X = NC
24 X = NCS

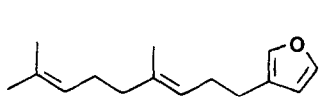
	23	mult.	J (Hz)	NOEDS	24
α	1.37	m	13, 3, 3, 2		1.33
β	1.05	m	*		1.02
α	∿1.59	m	*		∿1.58
β	∿1.59	m	*		∿1.58
α	2.04	m	13, 3, 3, 2		1.96
β	∿1.80	m	*		∿1.75
	1.23	d	6.5		1.25
	0.56	dd	9, 6.5		0.56
	0.67	dd	9,8		0.64
α	1.81	m	15, 13, 8, 8		1.77
β	∿1.59	dd	15, 7		∿1.58
α	1.15	dd	13, 8		1.13
β	0.82	td	13, 3, 7		0.81
2	1.00			n.O.e. to 5	1.01
3	1.11			n.O.e. to 6, 7	1.12
4	0.88			n.O.e. to 15	0.89
5	1.45	1:1:1	2, 2	n.O.e. to 14, 6	1.42

*2nd order coupling

an isonitrile band at 2125 cm^{-1}. The ^{1}H NMR spectrum (Table 1) was particularly detailed and allowed assignment of the structure. The isonitrile group was attached to a fully substituted carbon atom bearing a methyl group that gave rise to a diagnostic signal at δ 1.45 (1:1:1 J = 2 Hz). The spectrum also contained three methyl singlets at δ 0.88 (s, 3 H), 0.99 (s, 3 H) and 1.11 (s, 3 H) and two cyclopropyl proton signals at δ 0.56 (dd, 1 H, J = 9, 6.5 Hz, H-6) and 0.67 (dd, 1 H, J = 9, 8 Hz, H-7). The signal at δ 0.56 was coupled to a signal at δ 1.23 (d, 1 H, J = 6.5 Hz, H-5) assigned to the bridgehead proton. The signal at δ 0.67 was coupled to a signal at 1.81 (m, 1 H, J = 15, 13, 8, 8 Hz, H-8α) that was in turn coupled geminally to a signal at ~1.59 (H-8β) and vicinally to signals at 0.82 (td, 1 H, J = 13, 13, 7 Hz, H-9β) and 1.15 (dd, 1 H, J = 13, 8 Hz, H-9α). A molecular model of the isonitrile indicated that the dihedral angle between H-8β and both H-7 and H-9α was ~90° and thus no coupling should be expected. The 9β proton lies within the shielding cone of the cyclopropyl ring[43] and is therefore at unusually high field. The 1α and 3α proton signals were clearly visible at δ 1.37 (m, 1 H, J = 13, 3, 3, 2 Hz) and δ 2.04 (m, 1 H, J = 13, 3, 3, 2 Hz) respectively and showed a 2 Hz W-coupling. The 1β and 3β proton signals were at δ 1.05 and ~1.80 respectively but both signals were complex since they exhibited second order coupling to two methylene protons (H-2α, H-2β) at ~1.59. We observed nuclear Overhauser effects (NOEDS[44]) in accord with the stereochemistry shown (Table 1). The ^{13}C NMR spectrum is consistent with the structure shown. The isonitrile **23** was hydrolyzed in aqueous acetic acid to obtain the formamide **25**. A single crystal X-ray diffraction analysis on the formamide **25** confirmed the structure and stereochemistry of the isonitrile **23**.[45]

The isothiocyanate **24** had the molecular formula $C_{16}H_{25}NS$. The IR spectrum contained a strong isothiocyanate band at 2100 cm^{-1}. The ^{1}H NMR spectrum (Table 1) was almost identical with that of isonitrile **23** with the exception that the C-15 methyl signal at δ 1.42 (s, 3 H) was now a sharp singlet. The minor isonitrile **22** could not be identified from spectral data and could not be converted into a suitable crystalline derivative.

The October 1977 collection from Scripps Canyon was used exclusively for gut content analysis (*vide infra*). Specimens of *C. luteomarginata* collected in 1978 from Point Loma were extracted and chromatographed in the usual way in order to obtain a new sample of the isonitrile **22** but only the isonitrile **23** was isolated. The



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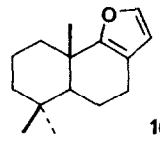

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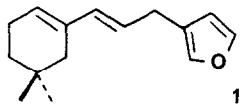

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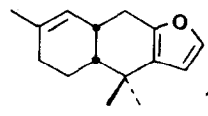

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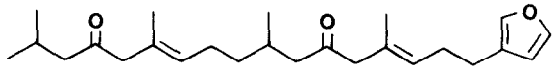

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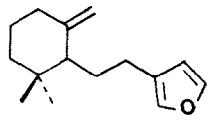

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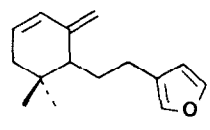

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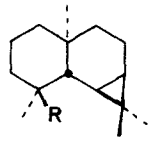

23 R = -NC
24 R = -NCS
25 R = -NHCHO

Table 2. Metabolites isolated from collections of *Cadlina luteomarginata*

	Scripps Canyon				Pt. Loma	
	January 1977		July 1977		Summer 1980	
	% dry wt.	mg/animal	% dry wt.	mg/animal	% dry wt.	mg/animal
dendrolasin (15)	0.13	0.24	--	--	--	--
pallescensin-A (16)	0.16	0.32	0.03	0.06	--	--
pleraplysillin-1 (17)	0.14	0.28	--	--	0.007	0.07
furodysinin (18)	0.2	0.4	--	--	0.018	0.17
idiadione (19)	0.1	0.2	--	--	--	--
furan 20	--	--	0.03	0.06	0.006	0.06
isonitrile 23	--	--	0.3	0.6	0.089	0.87
unknown isonitrile 22	--	--	0.07	0.15	0.033	0.32
unknown isonitrile 26	--	--	--	--	0.06	0.59
isothiocyanate 24	--	--	0.09	0.18	0.014	0.14
unknown isothiocyanate 27	--	--	--	--	0.006	0.055
unknown isothiocyanate 28	--	--	--	--	0.006	0.055
Total	0.73	1.44	0.52	1.05	0.239	2.33

animals were not dried after extraction but were used for analysis of gut contents.

Samples of *C. luteomarginata* collected at Point Loma in summer 1980 were carefully analyzed using a combination of chemical and biological techniques. The animals were soaked in methanol and the dichloromethane-soluble material from the extract was chromatographed by LC, first on Partisil using dichloromethane as eluant then on Partisil or Partisil-ODS for final purification. The major metabolite was the isonitrile **23** (0.089% dry weight) followed by a new unknown isonitrile **26** (0.06% dry weight) and the unknown isonitrile **22** (0.033% dry weight). The remaining compounds isolated are listed in Table 2.[57] The unknown isothiocyanate **27** corresponds to isonitrile **26** and isothiocyanate **28** to isonitrile **22**. The tertiary isonitriles **22** and **26** and the corresponding isothiocyanates **27** and **28** were unusually unreactive with respect to reactions at the isonitrile and isothiocyanate groups. The ^{1}H NMR spectral data did not give clearly defined signals suitable for analysis so that we have been unable to elucidate these structures using material isolated from the nudibranchs. However, we expect to obtain further data on these compounds since gut content analysis has enabled us to locate the sponge source of these compounds.[56]

Dorid nudibranchs feed predominantly, if not exclusively, on sponges. We were able to identify ten sponges that had been eaten by *C. luteomarginata* by examining the siliceous spicules found in their guts (Table 3). All but one individual (n = 45) contained spicules from only one sponge species. The most frequently encountered spicule types were identified as belonging to an undescribed *Axinella* sp. that had been neither encountered in the sponge-dominated reef habitat being

Table 3. Spicule-containing sponges consumed by *Cadlina luteomarginata*. Numbers refer to the number of individuals containing the diagnostic suite of spicules for each sponge species listed

Sponge	October* 1977	October** 1978	Summer** 1980	Total
Axinella sp.	1	7	14	22
Myxilla incrustans (Esper, 1805-1814)		7	4	11
Higginsia higginissima Dickinson, 1945		1	3	4
Zygherpe hyaloderma de Laubenfels, 1932			1	1
Desmacella sp.		1		1
Forcepia sp. A		1		1
Forcepia sp. B		1		1
Hymedesmia sp.		1		1
Hymenamphiastra sp.		1		1
Leptolabis sp.		1		1
Axinella sp. + M. incrustans		1		1
No spicules present	5	31	18	54
Total	6	53	40	99

*Collected at Scripps Canyon

**Collected at Pt. Loma

studies at Point Loma[46] nor previously recorded for Southern California.[47] Of the remaining nine sponges identified, only *Myxilla incrustans* was frequently encountered in the nudibranch. The sponges identified at the genus level are all undescribed species. Only *M. incrustans* and *Zygherpe haloderma* have been previously reported for Southern California[47] although *Higginsia higginissima* has been reported for the Gulf of California.[49] A comparison of data for collections of *C. luteomarginata* from San Diego and Vancouver, Canada, revealed that only *M. incrustans* and *Z. haloderma* were found in nudibranchs from both locations.[48]

The gut contents did not contain any recognizable fragments of spongin that would allow identification of keratose sponges. However, the presence of sand grains in the guts of some individuals may signify that the nudibranch had eaten a local keratose sponge, such as *Dysidea amblia*, that is characterized by sand cored spongin fibers. *C. luteomarginata* has been observed feeding on a keratose sponge, *Leiosella idia* (= *Spongia idia*). It is apparent, both from analysis of gut contents and from direct feeding observations, that *C. luteomarginata* is highly selective in its feeding habits.

Examination of the sponge *Axinella* sp. revealed that the proportions of the isonitriles **22**, **23** and **26** found in the sponge were almost identical with those found in the nudibranch. The isothiocyanates **24**, **27** and **28** were not found in the same ratios in both organisms although the same order of abundance was observed. The nudibranch contained less of the isothiocyanates than would have been expected from analysis of the sponge. The absence of isonitriles and isothiocyanates from the January 1977 collection of *C. luteomarginata* implies that the diet may vary with time, possibly due to seasonal availability of preferred food items.

In order to locate the storage site for the metabolites, specimens of *C. luteomarginata* were dissected into three portions: the dorsum, the guts and the foot and head. Each portion was extracted in the usual manner and the extracts analyzed by LC on Partisil using dichloromethane as eluant. The results of this experiment, shown in Fig. 1, clearly demonstrate that the metabolites

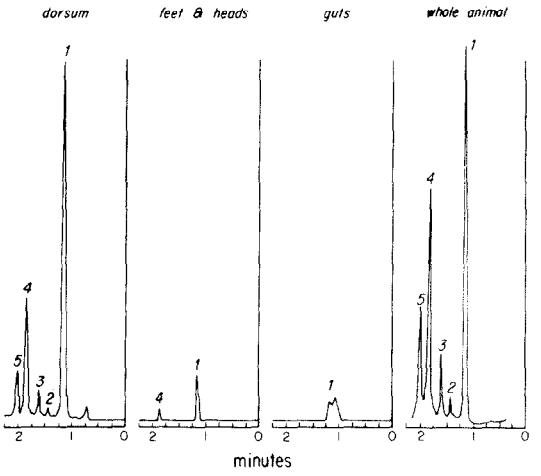

Fig. 1. Liquid chromatography traces of extracts of the dorsum, feet and heads and guts of *Cadlina luteomarginata* compared with a LC trace of an extract of a whole animal [Partisil, dichloromethane]. LC peaks are numbered to indicate peaks of identical retention time. ^{1}H NMR analysis showed that the peaks 1–5 were identical in the dorsum and the whole animal, that peak 1 in the guts contained no terpenoids and that peaks 1 and 4 in the feet and heads contained traces of terpenoids and isonitrile **23** respectively.

are stored in the dorsum, presumably in skin glands similar to those described by Thompson.[4]

Toxicity and antifeedant assays were performed to determine whether the metabolites found in *C. luteomarginata* were effective deterrents at concentrations that might be encountered by predators. Since the assays generally required more compound than was available from the nudibranchs we have only screened compounds available from sponge sources and did not separate the natural mixture of isonitriles **22**, **23** and **26** or isothiocyanates **24**, **27** and **28**. All compounds screened (Table 4) were toxic to the commercial goldfish (*Carassius auratus*) at 100 μg/mL. The isonitrile mixture was effective as an antifeedant against goldfish at 10 μg/mg in a food pellet, while all other compounds,

Table 4. Toxicity and antifeedant activity of selected metabolites

Assay	Toxicity		Antifeedant Activity			
Organism	Goldfish[a]		Goldfish			Sculpin[b]
Dose / Compound	10μg/mL	100μg/mL	1μg/mg	10μg/mg	100μg/mg	10μg/mg
Isonitriles 22, 23, 26	-[c]	+	-	+	+	±
Furodysinin (18)	-	+	-	±	+	+
Isothiocyanate 24, 27, 28	-	+	-	±	+	±
Idiadione (19)	-	+	-	±	±	+
Pallescensin-A (16)	-	+	-	-	±	+
"Sterols"[d]	NT	NT	NT	-	-	NT

a) Carassius auratus

b) Clinocottus analis

c) + active
± partially active
- inactive
NT not tested

d) The sterol fraction from C. luteomarginata was expected to be inactive and served as a negative control.

except pallescensin-A (**16**), caused partial inhibition of feeding. Using the wooly sculpin (*Clinocottus analis*) as assay organism, pallescensin-A (**16**) and idiadione (**19**) were the most effective antifeedants at 10 μg/mg. Since the soft tissues of the dorsum contained ~5.4 μg/mg of isonitrile mixture, these antifeedant data suggest that the dorsum contains enough of the metabolites to constitute an effective chemical defense mechanism, particularly if the compounds are actively secreted into the mouth of the predator.

DISCUSSION

From the results of this and other investigations, there can now be no doubt that the secondary metabolites isolated from dorid nudibranchs are predominantly of dietary origin. The similarities and differences in the metabolites isolated from *C. luteomarginata* samples of different geographical origin[48] reflect variations in the sponge fauna at the respective collection sites. We have shown, both by feeding observations and by examination of the gut contents, that the isonitriles isolated from *C. luteomarginata* were obtained from the relatively uncommon sponge *Axinella* sp. The keratose sponge *Leiosella idia* is the dietary source of idiadione (**19**) and we have observed *C. luteomarginata* feeding on this sponge many times. It is significant that only idiadione (**19**) of the three major sesterterpene metabolites of *Leiosella idia* could be detected in *C. luteomarginata*. Furodysinin (**18**), pallescensin-A (**16**) and possibly other sesquiterpene furans are metabolites of *Dysidea amblia*. However, none of the diterpenes found in *D. amblia* have been detected in *C. luteomarginata*. We have not observed *C. luteomarginata* feeding on *D. amblia* but the presence of sand grains among the gut contents suggests that the nudibranchs had consumed *D. amblia* or another sponge characterized by detritus cored fibers. Since keratose sponges cannot be identified from well-digested gut contents, it is possible, indeed probable, that the nudibranchs had consumed other keratose sponges, for we have not found sources for dendrolasin (**15**), pleraplysillin-1 (**17**) or the furan **20**. Since *C. luteomarginata* contains only a few metabolites of *L. idia* and *D. amblia* from a range of available compounds with similar lipophilic properties, we suggest that these compounds are selectively sequestered to provide an effective defensive secretion.

While describing defensive adaptations in opisthobranchs, Thompson[4] observed that "skin glands have been found whose position and function can only be explained satisfactorily as defensive. They are always present in addition to the usual mucous glands associated with ciliated epithelia". In dorid nudibranchs, Thompson found non-mucous skin glands to be abundant in the dorsum. Since the secondary metabolites of *C. luteomarginata* were found exclusively in the dorsum and can be extracted by soaking the nudibranchs in an appropriate solvent for a few hours, it seems reasonable to propose that the metabolites are sequestered in the non-mucous skin glands and that they are secreted when the nudibranch is molested.

The antifeedant properties of the metabolites from *C. luteomarginata* were measured in an assay that required a fish to detect the compounds in a food particle. We have not been able to mimic the natural situation where the metabolites are released directly into the mouth of an inquisitive predator but we suspect that the compounds might be more effective as antifeedants when delivered directly. Thompson's observation[4] that dead nudibranchs were eaten by fish supports the argument that the nudibranchs actively secrete distasteful metabolites when molested.

In summary, we have shown that *Cadlina luteomarginata* stores selected metabolites from a diet of many sponge species into its dorsum. We propose that these metabolites are sequestered in the non-mucous skin glands described by Thompson and are secreted when the nudibranch is molested. We have demonstrated that the major metabolites have antifeedant properties. These observations clearly establish a chemical defense mechanism for *Cadlina luteomarginata*.

EXPERIMENTAL

Collection, extraction and chromatography of Cadlina luteomarginata

(a) *January* 1977 *collection.* 25 individuals were collected by hand using SCUBA (−20 to −30 m) at Scripps Canyon, La Jolla. The animals were homogenized in EtOH (200 mL), allowed to stand for 16 hr and filtered to obtain the non-soluble material (5 g) and an EtOH extract. The EtOH was evaporated and the resulting aqueous suspension washed with $CHCl_3$ (3 × 5 mL). The combined organic extracts were dried over Na_2SO_4 and the solvent evaporated to obtain a brown oil (360 mg, 7.2% dry weight). The brown oil was preadsorbed on silica gel (1 g) that was applied to the top of a column (30 × 1 cm dia.) of silica gel (15 g). The column was eluted with solvents of increasing polarity from hexane through ether to chloroform. Material eluted with hexane was rechromatographed on a silica gel plate impregnated with silica gel to obtain pallescensin-A (**16**, 8 mg, 0.16% dry weight), furodysinin (**18**, 10 mg, 0.2% dry weight) and pleraplysillin-1 (**17**, 7 mg, 0.14% dry weight), having spectral data identical with literature values. Fractions eluted with 5% ether in hexane contained predominantly dendrolasin (**15**, 6.5 mg, 0.13% dry weight), as judged by spectral data and coinjection on gas chromatography. A fraction eluted with 50% ether in hexane contained predominantly idiadione (**19**, 5 mg, 0.1% dry weight).[41]

(b) *July* 1977 *collection.* 100 individuals (22 g dry weight) from Scripps Canyon were homogenized in EtOH (500 mL) and filtered to obtain an ethanolic extract. The EtOH was evaporated and the resulting aqueous suspension extracted with ether (3 × 100 mL). The ether extract was dried over anhyd. $MgSO_4$ and the solvent evaporated to obtain a brown oil (1.40 g, 6.4% dry weight). The oil was chromatographed on a column of Florisil (25 g) using solvents of increasing polarity from hexane through CH_2Cl_2 to EtOAc. The material eluted with hexane consisted of a mixture of furans (~50 mg) that was rechromatographed on a $AgNO_3$-impregnated silica gel plate to obtain pallescensin-A (**16**, 7 mg, 0.03% dry weight) and **20** (6 mg, 0.03% dry weight). Material eluted with 1% CH_2Cl_2 in hexane was combined to give a fraction (27 mg, 0.12% dry weight) that contained >75% of **22**. Material (330 mg) eluted with 3% CH_2Cl_2 in hexane was rechromatographed on a silica gel column using 2% CH_2Cl_2 in ether as eluant to obtain **23** (65 mg, 0.3% dry weight) and **22** (15 mg, 0.07% dry weight).

(c) *Summer* 1980 *collection.* 109 individuals were collected (−13 to −18 m) at Point Loma, San Diego between July and September 1980. The animals were stored in MeOH (1 L) at −5° for 6 months then the solvent was decanted and the animals soaked in fresh MeOH (1 L) for 2 days. The combined MeOH extracts were evaporated to obtain an aqueous suspension that was partitioned between water (100 mL) and hexane (5 × 200 mL) then CH_2Cl_2 (3 × 200 mL). The combined organic extracts were evaporated to obtain a gum that was dissolved in ether and passed through a plug (5 × 2 cm dia.) of silica gel using ether as eluant. Evaporation of the ether gave a pale yellow oil (1.08 g, 10 mg/animal). The animals (105 g dry weight) were dried and weighed. The oil was separated into six fractions by LC on a preparative Partisil column using CH_2Cl_2 as eluant. The first fraction (136 mg) was rechromatographed by LC on Partisil using hexane as eluant to obtain 16 fractions, many of which contained 1–2 mg of material. The major fractions contained furodysinin (**18**, 19 mg, 0.018% dry weight, 0.175 mg/animal), pleraplysillin-1

(**17**, 7.5 mg, 0.007% dry weight, 0.07 mg/animal), **20** (6.4 mg, 0.006% dry weight, 0.06 mg/animal), **24** (15 mg, 0.014% dry weight, 0.13 mg/animal), **27** (6 mg, 0.006% dry weight, 0.055 mg/animal) and **28** (6 mg, 0.006% dry weight, 0.055 mg/animal). The second fraction (8 mg) was a complex mixture and was not investigated further. Fraction 3 (35 mg) was rechromatographed by LC on Partisil using 1:1 hexane/CH_2Cl_2 as eluant to obtain **22** (35 mg, 0.03% dry weight, 0.32 mg/animal). Fraction 4 (154 mg) was rechromatographed by LC on Partisil ODS using 7:3 MeOH/water as eluant to obtain **23** (95 mg, 0.09% dry weight, 0.87 mg/animal) and **26** (64 mg, 0.06% dry weight, 0.59 mg/animal). Fractions 5 (99 mg) and 6 (350 mg) contained predominantly fats and sterols but appeared to contain traces of the formamides corresponding to **22**, **23** and **26**.

Pallescensin-A (**16**)[37] and furodysinin (**18**)[39] were identical in all respects with authentic samples while pleraplysillin-1 (**17**) was identified by comparison of spectral data with literature values.[38]

The furan **20** (*dihydropallescensin-2*). ^{1}H NMR (CCl_4) δ 0.85 (s, 3 H), 0.92 (s, 3 H), 2.17 (m, 4 H), 4.55 (br s, 1 H), 4.77 (br s, 1 H), 6.14 (br s, 1 H), 7.10 (br s, 1 H), 7.20 (br s, 1 H); mass spectrum, *m/z* (rel. int., %) 218 (8), 203 (1), 147 (3), 109 (42), 81 (100), 69 (50); high resolution mass measurement, obsd. *m/z* 218.166, $C_{15}H_{22}O$ requires 218.167.

Isothiocyanate **24**. $[\alpha]_D - 12°$ (*c* 1.1, $CHCl_3$); IR (film) 2110, 2085 cm^{-1}; ^{1}H NMR (CCl_4) see Table 1; mass spectrum, *m/z*, 263, 248, 230, 205, 189, 161, 149, 123.

Isonitrile **23**. $[\alpha]_D + 36°$ (*c* 0.2, $CHCl_3$); IR ($CHCl_3$) 2950, 2143, 1470, 1400 cm^{-1}; ^{1}H NMR (CCl_4) see Table 1; ^{13}C NMR ($CDCl_3$) δ 154.9 (t, J = 3.8 Hz), 60.65 (t, J = 4.8 Hz), 48.8 (d), 41.9 (t), 39.0 (t), 32.7 (s), 29.0 (q), 22.2 (q), 21.0 (d), 19.6 (d), 19.0 (q), 18.7 (t), 18.0 (s), 15.8 (t), 15.5 (q); mass spectrum, *m/z* (rel. int., %) 231 (2), 216 (2), 204 (3), 189 (6), 163 (9), 161 (10), 81 (100); high resolution mass measurement, obsd. *m/z*, 231.198, $C_{16}H_{25}N$ requires 231.199.

Hydrolysis of isonitrile **23**. **23** (10 mg, 0.04 mmol) was dissolved in glacial AcOH (2 mL) containing water (2 drops) and the mixture was allowed to stand at 25° overnight. The solvents were evaporated and the residue recrystallized from hexane to obtain **25** (5 mg, 46% theoretical): m.p. 151–2°; $[\alpha]_D + 27°$ (*c* 0.75, $CHCl_3$); IR ($CHCl_3$) 1675 cm^{-1}; ^{1}H NMR (CCl_4) δ 0.43 (dd, 1 H, J = 9, 6.5 Hz), 0.62 and 0.64 (m, 1 H), 0.93 (s, 3 H), 0.93 and 0.95 (2 × s, 3 H), 1.04 and 1.05 (2 × s, 3 H), 1.36 (br s, 3 H), 7.14 and 7.16 (2 × br s, 1 H), 8.07 and 8.10 (2 × s, 1 H) [2 geometrical isomers of formamide are observed].

Field feeding observations. The majority of individuals were collected off a sand-swept botton (− 13 to − 18 m) within the Point Loma kelp bed during October 1980. There we observed *Cadlina luteomarginata* feeding on one of the few sponges which grows in this habitat, *Axinella* sp. Only a few individuals were collected from the undersides of reef overhangs where sponges dominate space; there we observed seven individuals feeding on the kerotose sponge *Leiosella idia*.

Gut content analysis. The gut contents of two MeOH-extracted collections (October 1977, n = 6; October 1978, n = 53) were examined at 400X. The majority (n = 36) of the nudibranch guts contained no recognizable sponge skeletal parts (spongin or spicules) probably due in large part to evacuation after collection and upon solvent immersion. Many of the guts, however, were packed with well-digested chunks of sponges possessing siliceous skeletons in accordance with the findings of Bloom.[51] All gut contents were sufficiently digested to preclude any observation of spongin. In three cases, however, sufficient sand grains of an appropriate size suggest a keratose sponge, *Dysidea amblia*, characterized by detritus-filled fibers, might have been consumed.

To identify the sponge species characterized by siliceous spicules, these gut samples, plus an additional 40 individuals from our 1980 collection were digested in conc. HNO_3 and examined to determine the spicule types present and thus the sponge species consumed. Species were identified according to standard references.[52]

Localization of metabolites 6 individuals were collected at Point Loma (−13 m) in June 1981 and maintained in running seawater for 2 days. During this time the gut contents were evacuated. Dissection was performed by a dorsoventral incision through the entire animal. The guts were removed and a second incision made to separate the dorsum from the remaining tissue comprising the foot and head. The parts of the animals were combined into 3 portions and extracted with MeOH at 5° for 2 days. The extracts were filtered and the solvent evaporated to obtain crude extracts. The guts (742 mg dry wt) gave 62 mg of crude extract (8.3% dry wt). The foot and head portions (306 mg dry wt) gave 11 mg of extract (3.6% dry wt). The dorsum portions (1.78 g dry wt) gave 30 mg of extract (1.7% dry wt). A 10 mg portion of each extract was analyzed by LC on Partisil using CH_2Cl_2 as eluant. Each peak was collected and analyzed by ^{1}H NMR spectroscopy. The fractions from the dorsum gave identical ^{1}H NMR spectra to the corresponding fractions in an extract of the whole animals. ^{1}H NMR analysis of the gut extract revealed no signals due to terpenoids while fractions labeled 1 and 4 in the LC trace of feet and heads contained traces of terpenoid materials and **23** respectively.

In an attempt to quantify the amount of metabolites in the soft tissue of the dorsum, the dorsum portions were ashed at 500° for 4 hr to obtain an ash-free dry weight (954 mg). The inorganic ash consisted predominantly of siliceous spicules characteristic of *C. luteomarginata*. The soft tissue of the dorsum is 33.7% of the dry weight of the animal. Using data for the quantities of **22**, **23** and **26** found in the summer 1980 collection (Table 2), we calculated that the soft tissue of the dorsum contained ~ 5.4 μg/mg tissue of isonitriles.

Bioassays

Two bioassays were developed after Sun and Fenical,[53] Paul *et al.*[54] and Bakus.[55] Toxicity was measured against feeder goldfish (*Carassius auratus*) by treating replicate fish (n = 4) with pure and mixed metabolites at 10 μg/mL and 100 μg/mL. Compounds were added to deionized water (20 mL) in distilled acetone; controls (n = 6) employed solvent only. Goldfish were randomly placed within individual treatment and control regimes and observed for 1 hr. Toxicity was defined as death or loss of equilibrium after 1 hr. There was little variation within treatment groups. Solvent controls were non-toxic in all cases.

Feeding inhibition was assessed by observing the feeding response of feeder goldfish, *Carassius auratus*, and wooly sculpin, *Clinocottus analis*, toward food pellets (Wardley's[c] Shrimp Pellets) treated with 1–100 μg/mg of these compounds. The sculpin was collected intertidally at La Jolla, California. The sculpin is capable of eating dorid nudibranchs; in aquarium situations, we have observed it to prey on other species of dorids. The test compounds were applied to the treatment pellets (n = 3 to 6 depending on compound availability) with distilled acetone and evaporated at room temp. Control pellets (n = 6) were treated with solvent only. Fish were randomly isolated in groups of 10 in 1 gal glass jars filled with fresh or seawater (2 L), as required, at room temp. Single pellets were randomly added to the jars and the fish were observed for 1 hr in the case of goldfish and 3 hr in the case of the sculpin. The feeding response was scored as not consumed (+), partially consumed (+/−), or completely consumed (−). Indigestible hard parts in the pellets were ignored. The feeding ability of the fish was verified by feeding them untreated pellets before and after treatment and control pellets. In all cases control pellets were consumed; very little variation in response within treatment groups was observed.

Acknowledgements—This research was supported in part by the National Science Foundation (Grants OCE-8008338 and PCM-7714946). We wish to thank William H. Hagey for laboratory assistance.

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