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Chemical defense in tropical green algae, order Caulerpales

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ABSTRACT: Forty marine algae of the order Caulerpales were chemically investigated. Virtually all produce toxic secondary metabolites of a unique and unprecedented class. These metabolites are generally linear terpenoids, but unusual structural features such as aldehydes and bis-enol acetate functional groups make these compounds unique. The compounds are toxic or deterrent toward microorganisms, sea urchin larvae, and herbivous fishes, and when incorporated into diets at naturally occurring concentrations cause mortality in juvenile conch. Concentrations of bioactive metabolites were found to show little variation in different plant parts such as blades, stipes and holdfasts. Young growing tips and reproductive structures contained higher concentrations than mature plant tissues on a dry weight and ash-free dry weight basis. Chemical variation, both qualitative and quantitative, was observed in different populations of the same species. In the cases examined, algae growing in areas known to have the highest herbivory produced the greatest concentrations and varieties of secondary metabolites. Based upon these observations and prior feeding preference data, we conclude that chemical defense is a major factor in the survival of marine algae within this order.

INTRODUCTION

Herbivory in both marine and terrestrial communities can be very intense, reducing the growth and survival of individual plants and influencing interspecific competition and community structure (Rockwood 1973, Vadas 1977, Lubchenco 1978, 1980, Morrow & LaMarch 1978, Ogden & Lobel 1978, Rausher & Feeny 1980, Hay 1981b,c, Lubchenco & Gaines 1981, Gaines & Lubchenco 1982, Coley 1983, Hay et al. 1983). The importance of plant defensive mechanisms, especially the role of secondary metabolites as chemical defenses against herbivores, has been recognized and studied extensively in terrestrial communities (Fraenkel 1959, Sondheimer & Simeone 1970, Whittaker & Feeny 1971, Levin 1976, Harborne 1977, 1978, Rosenthal & Janzen 1979). Over 12000 different natural products of varied biogenetic origins including alkaloids, terpenoids, acetogenins and aromatic compounds are produced by terrestrial plants (Devon & Scott 1972). The diversity and ubiquity of these secondary metabolites has generated considerable debate regarding their costs and benefits and the selective forces influencing their biosynthesis. Although approximately 500 secondary metabolites have been described from seaweeds, few studies have assessed the ecological roles of these compounds (Scheuer 1978–1983, Faulkner 1984).

An extensive literature discussing the theory of plant-herbivore interactions and biochemical evolution has developed from studies of bioactive metabolites from terrestrial plants (Feeny 1975, 1976, Rhoades & Cates 1976, Rhoades 1979, 1985, Fox 1981). These studies suggest that the evolution of plant defense mechanisms is responsive to the plant's risk of discovery by herbivores, the cost of defense, and the value to the plant of various plant parts (Rhoades 1979). Although there is currently general acceptance of the defensive roles of these compounds, there is still considerable speculation regarding how herbivores and the physical environment interact to affect plant chemistry (Coley 1983, Louda 1984, Rhoades 1985, Coley et al. 1985).

In contrast, much less is known about plant-herbivore interactions in the marine environment and especially the role of algal secondary metabolites as adaptations against herbivores. Compounds from

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marine algae have been hypothesized to play important defensive roles in marine algae (Ogden & Lobel 1978, Norris & Fenical 1982, Hay 1984b), but experimental bioassays with these secondary metabolites have rarely been conducted (Geiselman & McConnell 1981, McConnell et al. 1982, Paul & Fenical 1983, Steinberg 1984, 1985).

Marine algae of the order Caulerpales (Chlorophyta) are perhaps the most abundant and widely distributed algae in tropical oceans (Taylor 1960, Hillis-Colinvaux 1980, Norris & Fenical 1982). They are important primary producers, and in many reef systems are conspicuously abundant in habitats where herbivores are common. This algal group is also a major contributor to coral reef sediments and reef structure (Ginsburg 1956, Stockman et al. 1967, Hillis-Colinvaux 1980), since most species have the ability to deposit $CaCO_3$ on their thalli and become highly calcified (50 to 90 % dry weight) (Böhm 1973, Littler 1976, Borowitzka 1977, Bold & Wynne 1978, Hillis-Colinvaux 1980, Drew 1983).

Algae within the order Caulerpales possess a siphonous or coenocytic construction consisting of multinucleate tubular filaments lacking cross walls, except to delineate reproductive structures. They are further characterized by specialized pigments and cell-wall components. The order is subdivided into the families Caulerpaceae, containing the single large genus *Caulerpa,* and Udoteaceae containing the genera *Chlorodesmis, Halimeda, Avrainvillea, Penicillus, Tydemani, Udotea* and others (Round 1973, Bold & Wynne 1978).

The major goal of this investigation was to study the physiological effects of a wide range of caulerpalean algal metabolites and to examine the hypothesis that chemical defense is a significant factor in the survival of these algae.

We asked the following questions: (1) Have marine plants evolved the synthesis of secondary metabolites that function as adaptations against herbivory? If so, what are their chemical structures and are they similar to those of terrestrial organisms? (2) Do the metabolites in question show toxicity and feeding deterrent effects in laboratory bioassays designed to examine some potential defensive roles? (3) Do the concentrations and structures of secondary metabolites vary in different plant parts? (4) Does chemical variation occur, perhaps in response to increased levels of predation, in different populations of the same species?

METHODS

Collection and study sites. Algae for these studies were collected in the tropical Atlantic and Pacific Oceans. During 4 consecutive years, algae were collected extensively throughout the Bahama Islands in both reef and seagrass habitats. Collections were also made on 2 occasions in the Florida Keys, in Puerto Rico, and in St. Croix, U.S.V.I. Pacific collections were made from reef and lagoonal habitats on Guam, on nearby Saipan and in Palau. Over 200 collections of 40 species of green algae of the order Caulerpales were chemically analyzed during this 4 yr period.

General terpenoid extraction and purification techniques. After collection, algae were immediately ground and extracted with dichloromethane. Extracts were chromatographed over Florisil (to remove chlorophyll pigments) and the semi-purified fractions were frozen and stored for further analysis. Silica gel column chromatography, followed by high performance liquid chromatography (HPLC), was utilized to purify all metabolites. We found that it was important to immediately extract and chromatograph many of these unstable and chemically reactive metabolites, since many compounds were found to decompose when algae were stored frozen or preserved in alcohol.

Details of the final purifications and structure elucidations of these green algal metabolites can be found in Paul & Fenical (1984a,b).

Antimicrobial assays. A standard agar plate-assay disc method was used. Petri plates containing typical marine media were inoculated with both known and undefined marine bacteria and fungi. Known marine bacteria were provided by Drs. A. Carlucci and K. Nealson of this institution, and marine fungi were provided by Dr. J. Kohlmeyer, University of North Carolina. Undescribed microorganisms (both bacteria and fungi given VJP numbers) were also isolated for these tests from the surfaces of numerous brown algae collected in the Bahamas in September 1981. Each compound (0.10 mg) was applied with diethyl ether to standard test disks. The disks were applied to a freshly inoculated plate and incubated at 20°C for several days. Inhibition of growth was assessed by measuring the 'clear zones' around the assay disks.

Toxicity assays. *Sea urchin fertilized egg cytotoxicity.* Sea urchin egg development is frequently used as a pharmacological screen for compounds that inhibit cell division. The details of this assay have been reported by Cornman (1950) and Jacobs et al. (1981). This assay was used as a general cytotoxicity screen to assess the potential biological activity of the metabolites. Both quantitative and qualitative effects upon cell division in the southern California sea urchin *Lytechinus pictus* Verrill were assessed.

Compounds were dissolved in 25 µl of EtOH and added to 6 ml seawater at known concentrations, and several hundred fertilized eggs were added. Solvent and seawater controls were run simultaneously. Serial dilutions were tested until the lowest ED_{100} (lowest

concentration for 100 % inhibition of cell division) was determined. After 90 min, inhibition of cell division was measured relative to controls. Active compounds completely blocked or caused abnormal cell division. The assay was performed in triplicate at the lowest effective concentrations for each compound.

Many of the metabolites were also tested in this assay using eggs of the tropical Pacific urchin *Echinometra mathei* (de Blainville).

Sea urchin sperm assay. This assay was utilized as a general toxicity screen using sea urchin sperm obtained from the sea urchin *Lytechinus pictus*. Concentrations of sperm per ml seawater could be approximated spectrophotometrically (Vacquier & Payne 1973). Generally, approximately 3×10^7 sperm ml^{-1} were used. Sperm were placed in contact with known concentrations of compounds dissolved in seawater for 30 min. Assays were performed in duplicate at the lowest effective dosages, and solvent (EtOH) and seawater controls were run simultaneously. Toxicity was assessed microscopically as the complete loss of flagellar motility.

Sea urchin larval toxicity assay. Toxicity was measured against pluteus larvae (36 h after fertilization) of the urchin *Lytechinus pictus*. Approximately 20 to 30 larvae were treated with the compounds at known concentrations for 1 h (acute toxicity) and for 24 h. Seawater and solvent (EtOH) controls were also assayed. Toxicity was defined as 100 % inhibition of larval swimming and ciliary motion when viewed microscopically. The assay was repeated 2 or 3 times for each metabolite. Most of the metabolites were also tested against larvae of *Echinometra mathaei* for acute toxicity.

Fish toxicity assay. Tropical damselfishes *Pomacentrus coeruleus* (Atlantic) and *Dascyllus aruanus* (Pacific) (Pomacentridae) were used for toxicity of the algal metabolites toward potential fish predators. These fish were locally available from pet stores. Compounds were stirred into seawater at known concentrations using a small amount of EtOH (100 µl EtOH in 200 ml seawater). A damselfish was placed into the seawater and observed for 1 h. Solvent controls were run simultaneously. Toxicity was defined as death of the fish within 1 h. Each compound was tested in triplicate at the minimum effective concentrations.

Feeding deterrence assay. Natural concentrations of each compound on a dry weight basis (1000 to 5000 ppm) were prepared in diethyl ether and applied volumetrically to 20 mg pieces of fish food (Longlife pelleted food). Control pellets were treated with the same volume of ether. Ether was evaporated at room temperature. Results of the assay were measured by counting the number of bites taken of treated and control pellets by individual fish in a school of 6 to 8 fish. Damselfish *Pomacentrus coeruleus* and *Eupomacentrus leucostictus* were used in separate experiments and most of the compounds were tested against both groups of fish. At least 10 treated and 10 control pellets were added randomly to the tank and scored in this way. The number of bites taken of control and treated pellets were compared statistically using the Mann-Whitney Test. The feeding deterrence assays were also conducted in 2 other ways for several of the metabolites. First, control and treated pellets were added simultaneously to a tank of 6 to 8 fish. After 30 min pellets were scored as eaten or uneaten. This experiment was repeated at least 6 times. Results were analyzed using Fishers Exact Test (Sokal & Rohlf 1969). Finally, individual fish were placed in separate smaller containers and given both control and treated pellets. After 1 h pellets were scored as eaten or not eaten and the results were analyzed using Fishers Exact Test.

Gastropod feeding assay. Juvenile conch *Strombus costatus* (20 to 30 mm shell length) from Puerto Rico were fed 4 separate diets and their survival recorded for 14 d. Nine juveniles were placed in each of four 1000 ml beakers and the beakers were covered with plastic screen. All beakers were placed into a 68 l, well-aerated aquarium at 24 °C. The green alga *Enteromorpha* sp., a preferred food for juvenile conchs (D. Ballantine pers. comm.), was used as a food source (collected at the Scripps Institution seawall). The *Enteromorpha* was treated with the compounds udoteal (U-3), caulerpenyne (C-10) and halimedatrial (H-7) (Fig. 1). Untreated algae were used as a control diet (ether only). Compounds were dissolved in diethyl ether at known natural concentrations (1 % dry weight of *Enteromorpha*) and added to the algae volumetrically. The solvent was evaporated for 10 min. McConnell et al. (1982) showed that nonpolar compounds such as these terpenoids do not dissolve into surrounding seawater and will coat the algal surface for 3 h or more with almost no loss. Food was changed and the beakers were cleaned every other day to minimize decomposition of the compounds and to keep the glass surfaces free of diatom-bacterial films which the *S. costatus* preferred to graze. Conchs were examined several times per day to assess toxic effects of the diets.

Chemical variation in plant parts. The chemical composition of the growing tips, whole blades, stipes, rhizoidal holdfasts, and reproductive portions of several algae were compared using thin layer chromatographic (TLC) analyses. Different secondary metabolites show very characteristic patterns by TLC, hence presence or absence of secondary metabolites can readily be assessed, and compounds can be compared between plant parts using this technique. Proton nuc-

lear magnetic resonance spectrometry (NMR) and high performance silica gel liquid chromatography (HPLC) were used for quantitative analyses of the concentrations of metabolites in various plant parts.

Chemical variation in algal populations. TLC and HPLC were again used to assess qualitative and quantitative chemical variation in different populations of algae. Proton nuclear magnetic resonance spectrometry was used to identify all metabolites produced by different populations of the algae.

RESULTS

Natural products chemistry of Caulerpales

Over 40 algal species within the order Caulerpales were found to produce unusual secondary metabolites (>95 % of those studied). The results of prior extensive chemical investigations of the algae showed that the bioactive secondary metabolites were present in the lipid extracts, not the aqueous extracts (Paul 1985). The majority of these secondary metabolites are sesquiterpenoids (C_{15}) or diterpenoids (C_{20}). Table 1 summarizes information on the metabolites, including their algal sources, collecting sites and concentrations within the algae. Chemical structures of the metabolites from Table 1 are shown in Fig. 1. Since all of the metabolites from caulerpalean algae are chemically closely related and have nearly identical physiological effects, we focused this study on several substances which are major metabolites and characterize the respective genera involved.

Fig. 1 demonstrates the variety of different metabolites which have been isolated. Letters preceding the compound numbers indicate the genera of algae which produce these metabolites (Table 1). Although diversity is great, some structural features are common to these metabolites. Many of these terpenoids are in the linear or acyclic form. The conjugated bis-enol acetate functional group (encircled in structure U,P,R-1) is a unifying feature of many of these metabolites. Until its discovery in these algae, this unique chemical group was unknown in natural products. This functionality represents a 'masked' or acetylated dialdehyde constellation to which high biological activity is generally attributed.

Table 1. Metabolites present in algae of the order Caulerpales

Compound designation	Common name	Algal sources	Type of compound	Approx. natural conc. (% of dry weight)	Collecting site	References
U, P, R-1	Dihydrorhipocephalin	*Udotea cyathiformis, Penicillus capitatus, Rhipocephalus phoenix*	Sesquiterpenoid triacetate	1.0 %	Bahamas, Florida Keys	Paul & Fenical 1984a
U, P, R-2	Aldehyde 2	*U. cyathiformis, P. capitatus, R. phoenix*	Sesquiterpenoid aldehyde	0.3 %	Bahamas, Florida Keys	Paul & Fenical 1984a
U-3	Udoteal	*U. flabellum, U. argentea, U. petiolata*	Diterpenoid diacetate aldehyde	0.5–1.5 %	Belize, Bahamas, Guam, Mediterranean	Paul et al. 1982, Fattorusso et al. 1983, Paul & Fenical 1984a, 1985
U-4	Petiodial	*U. flabellum, U. petiolata*	Diterpenoid dialdehyde acetate	0.5 %	Bahamas, Mediterranean	Fattorusso et al. 1983, Paul & Fenical 1984a
P-5	Dihydroudoteal	*P. dumetosus, P. pyriformis*	Diterpenoid diacetate, aldehyde	0.8 %	Bahamas	Paul & Fenical 1984a
R-6	Rhipocephalin	*Rhipocephalus phoenix*	Sesquiterpenoid	1.0 %	Belize, Bahamas	Sun & Fenical 1979
H-7	Halimedatrial	*Halimeda* spp.	Diterpenoid cyclopropane trialdehyde	0.1–0.5 %	Bahamas, Puerto Rico, Florida Keys, Guam	Paul & Fenical 1983, Paul & Fenical 1984b
H-8	Halimeda tetraacetate (4,9-diacetoxy-udoteal)	*Halimeda* spp.	Diterpenoid tetraacetate	0.5–1.5 %	Bahamas, Puerto Rico, Florida Keys, Guam, Hawaii	Paul & Fenical 1984b
CH-9	Chlorodesmin	*Chlorodesmis fastigiata*	Diterpenoid tetraacetate	1.0 %	Australia, Guam	Wells & Barrow 1979
C-10	Caulerpenyne	*Caulerpa* spp.	Sesquiterpenoid triacetate	0.2–1.5 %	Bahamas, Puerto Rico, Florida Keys, Guam, Mediterranean	Amico et al. 1978

Antimicrobial assays

All of these metabolites were extensively assayed for antimicrobial activity against many different marine bacteria and fungi (approximately 20 strains). All of the metabolites were inhibitory toward at least 75 % of the bacteria and fungi they were tested against (Tables 2 & 3).

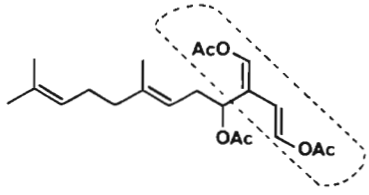
Toxicity assays

Sea urchin fertilized egg development assay

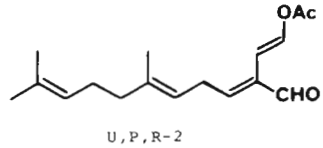
Table 4 shows the 100 % effective dosages (ED_{100}) for inhibition of development of fertilized eggs of the urchin *Lytechinus pictus*. All metabolites were tested at several concentrations (16, 8, 4, 2, 1 µg ml^{-1}) and only the lowest ED_{100} concentrations are reported. Several compounds were active at very low concentrations (1 µg $ml^{-1} \sim 10^{-6}$ M). Compounds 1, 3, 5, 7, 8, and 10 were also assayed against the eggs of the tropical sea urchin *Echinometra mathaei* and found to be active at similar concentrations. Solvent controls (EtOH) did not show inhibitory effects on egg development.

Sea urchin sperm assay

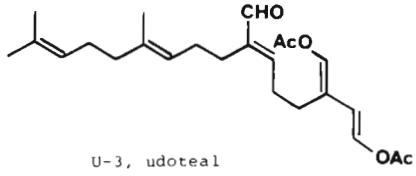
The results of sea urchin sperm assays are presented in Table 4. The majority of the compounds were toxic to sperm in 30 min. Several compounds were toxic at very low concentrations (Compounds 4, 7). EtOH solvent controls showed no toxic effects.



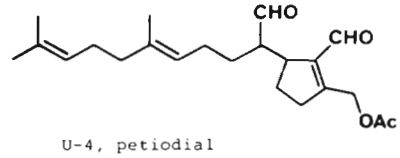
U,P,R-1, dihydrorhipocephalin



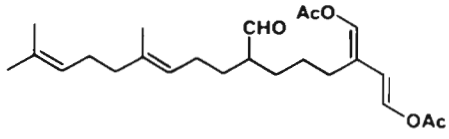
U,P,R-2



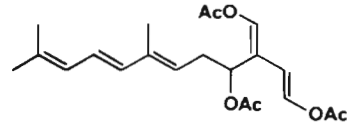
U-3, udoteal



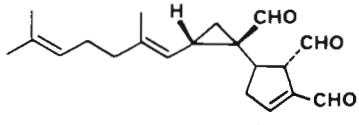
U-4, petiodial



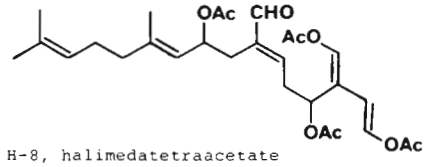
P-5, dihydroudoteal



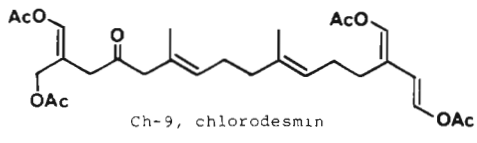
R-6, rhipocephalin



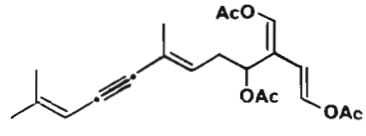
H-7, halimedatrial



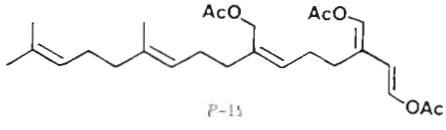
H-8, halimedatetraacetate



Ch-9, chlorodesmin



C-10, caulerpenyne



P-11

Fig. 1. Major terpenoid metabolites produced by Caulerpalean algae. Letters preceding compound numbers indicate genera which produce the compound (see Table 1)

Table 2. Results of antibacterial assays with green algal metabolites

Bacteria	Compounds tested									
	U,P,R-1	U,P,R-2	U-3	U-4	P-5	R-6	H-7	H-8	Ch-9	C-10
Serratia marinorubra	+	+		+	–		+			
Vibrio splendida	+	+		+	+		+			
V. harveyi	+	+	+	+	+	+	+	+	+	
V. leiognathi	+	+	+	+	+	–	+	–	–	+
Vibrio sp.		+			+		+	+	+	
VJP Cal 8101	+	+		–	–		+	+		+
VJP Cal 8102	+	+	–	+	+	+	+	+	–	+
VJP Cal 8103		+			+		+	+		+

+: inhibition of bacterial growth in excess of 2 mm
–: no inhibition, normal growth

Table 3. Results of antifungal assays with green algal metabolites

Fungi	Compounds tested									
	U,P,R-1	U,P,R-2	U-3	U-4	P-5	R-6	H-7	H-8	Ch-9	C-10
Leptosphaeria sp.	+	+	–	–	–	+	–	–	–	+
Lulworthia sp.	+	+	–	–	+	–	+	+	–	+
Alternaria sp.	+	+	+	+	–	+	+	+	–	+
Dreschleria haloides	–	–	–		+	–	+	–		
Lindra thallasiae	+		–	+		–	+	–	–	+
VJP Cal 8104	+	+	–	–	–	+	+	+	+	
VJP Cal 8105	+	+	–	–	+	+	+	–	–	+

+: inhibition of fungal growth in excess of 2 mm
–: no inhibition, normal growth

Sea urchin larval toxicity assay

The results of this assay are presented in Table 4. All compounds tested were toxic to larvae within 24 h and several compounds were active at very low concentrations ($\sim 10^{-7}$ M). Solvent controls showed no toxic effects toward larvae, even in 24 h experiments.

Fish toxicity

Table 5 shows fish toxicity results. The majority of the metabolites were toxic within 1 h and several in concentrations as low as 5 µg ml^{-1} (10^{-5} to 10^{-6} M). The decision to test compounds at concentrations only as high as 20 µg ml^{-1} and for only 1 h was subjective; we were assaying for acute toxicity. Many of the compounds that were not toxic still showed detrimental effects and were most likely toxic at greater concentrations or exposure times. Most fish showed sedated behavior, discoloration and increased respiratory activity. Fish exposed to solvent only (controls) showed no detrimental effects.

Fish feeding deterrence assay

Results of fish feeding deterrence assays comparing number of bites taken of control and treated pellets are summarized in Table 6. All of the metabolites tested induced significant feeding avoidance in these damselfishes. The results of contingency table analyses of eaten and uneaten pellets for groups of fish are shown in Table 7. Table 8 shows the results of feeding deterrence measured with individual fish for 2 metabolites. The results of all tests were significant at $p < 0.01$.

Strombus costatus feeding assay

Fig. 2 shows the survival rates of juvenile *Strombus costatus* fed on the 4 diets for 14 d. Control conchs showed 100 % survivorship and continued to feed on *Enteromorpha* sp. for another 6 wk with no mortality. Most striking were the results of conchs feeding on the halimedatrial (H-7) diet. All 9 specimens died within the first 6 d on the diet. On both the caulerpenyne and udoteal diets ca 50 % of the specimens died. The

Table 4. *Lytechinus pictus*. Results of bioassays with sea urchin fertilized eggs, sperm and larvae. ED_{100}: minimum effective dose for 100 % toxicity

Bioassay	Compounds tested (μg ml^{-1})									
	U,P,R-1	U,P,R-2	U-3	U-4	P-5	R-6	H-7	H-8	Ch-9	C-10
Fertilized egg cytotoxicities ED_{100}	2	2	8	1	16	8	1	8	8	8
Sperm toxicity (30 min) ED_{100}	8	2	8	1	8	8	1	8	8	8
Larval toxicity (1 h) ED_{100}	2	2	8	0.5	4	4	1	8	4	8
Larval toxicity (24 h) ED_{100}	2	1	8	0.2	2	4	0.2	4	2	2

Table 5. *Pomacentrus coruleus* and *Dascyllus aruanus*. Results of fish toxicity assays. Fish toxicity ED_{100} concentrations in μg ml^{-1}

Bioassay	Compounds tested									
	U,P,R-1	U,P,R-2	U-3	U-4	P-5	R-6	H-7	H-8	Ch-9	C-10
Fish toxicity 1 h (N = 3)	5	5	–	5	–	10	5	–	10	20

–: not toxic at 20 μg ml^{-1} in 1 h

Table 6. *Pomacentrus coerulus* and *Eupomacentrus leucostictus*. Results of fish feeding deterrence assay. Number of bites of control treated pellets (at least 10 of each) was compared by Mann-Whitney test

Bioassay	Compounds tested									
	U,P,R-1	U,P,R-2	U-3	U-4	P-5	R-6	H-7	H-8	Ch-9	C-10
No. of bites	+	+	+	+	nt	+	+	+	nt	+

nt: not tested
+: significant avoidance of treated pellets
–: no significant deterrence measured

experiment was discontinued because those conchs on the caulerpenyne and udoteal diets stopped grazing the treated *Enteromorpha* and grazed almost entirely on the diatoms adhering to the walls of the glass beakers. The control conchs, however, continued to graze the *Enteromorpha* almost exclusively. Results were analyzed using contingency table analysis and the G-test (Sokal & Rohlf 1969). Results were significant at $p < 0.005$.

Chemical variation in plant parts

Little difference in the secondary metabolite composition was detected by HPLC and NMR comparisons of the blades, stipes and holdfasts of several species of algae, *Penicillus dumetosus, P. pyriformis, Rhipocephalus phoenix, Caulerpa paspaloides,* and *Udotea flabellum* (Table 9). TLC comparisons of plant parts from several other species of *Udotea* and *Halimeda* also showed no detectable differences in the amounts or kinds of metabolites present.

Halimeda scabra was also examined for secondary metabolite production in the newly produced, uncal-

Table 7. *Pomacentrus coerulus* and *Eupomacentrus leucostictus*. Feeding deterrence results (groups of fish). Table shows number of pellets eaten or uneaten

	Halimedatrial (H-7), 5000 ppm		Petiodial (U-4, alcohol), 2000 ppm	
	Treated	Control	Treated	Control
Eaten	0	6	0	7
Uneaten	6	0	8	3
Fisher's Exact test	$p = 0.002$			$p = 0.004$

Table 8. *Pomacentrus coerulus* and *Eupomacentrus leucostictus*. Feeding deterrence results (individual fish). Table shows number of fish that had or had not eaten pellets

	Dihydrorhipocephalin (U,P,R-1), 5000 ppm		Petiodial (U-4) 5000 ppm	
	Treated	Control	Treated	Control
Eaten	1	9	2	16
Uneaten	8	0	16	2
Fisher's Exact test	$p = 0.0004$		$p = 0.0000$	

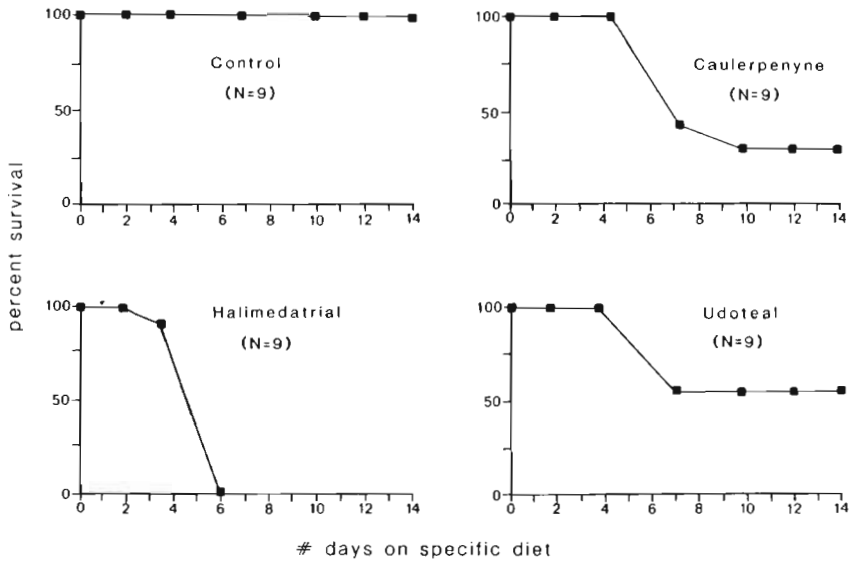


Fig. 2. *Strombus costatus*. Juvenile conch feeding assay

cified growing terminal segments. The same metabolites that were present in the rest of the plant (H-7 and H-8) were found in the growing segments. No $CaCO_3$ was present in these newly produced tissues, and chlorophyll was not detected in the extracts. The reproductive structures (gametangia) in *Halimeda tuna* were separated from the rest of the plant and analysis showed the presence of halimedatetraacetate (H-8) as 2 % of the dry weight of the tissues. This concentration is approximately 4 times greater than in the calcified plant tissues, and when calculated for ash-free dry weights, the growing tips and reproductive structures both have 25 % greater secondary metabolite concentrations than mature plant tissues.

Variation in plant populations

Striking qualitative and quantitative variation was observed in populations of the same species of algae collected from various habitats. Reef collections of *Udotea cyathiformis* and *Rhipocephalus phoenix* showed about 2 times greater concentrations of the major metabolites (U,P.R-1 for *U. cyathiformis*, R-6 for

Table 9. Chemical variation in plant parts

Alga	Metabolites	Concentrations (% dry wt)
Penicillus dumetosus (N = 1)		
Tops	Dihydroudoteal (P-5)	0.45
Rhizoids (holdfasts)	Dihydroudoteal (P-5)	0.45
Penicillus pyriformis (N = 1)		
Tops	Dihydroudoteal (P-5), dialdehyde	0.25, 0.05
Stipes	Dihydroudoteal (P-5), dialdehyde	0.25, 0.05
Rhizoids (holdfasts)	Dihydroudoteal (P-5)	0.25
Rhipocephalus phoenix (N = 1)		
Tops	Rhipocephalin (R-6), minor aldehydes	0.60, 0.05
Stipes	Rhipocephalin (R-6), minor aldehydes	0.40, 0.05
Rhizoids (holdfasts)	Rhipocephalin (R-6), minor aldehydes	0.40, 01.0
Udotea flabellum (N = 1)		
Tops	Udoteal (U-3)	0.30
Stalk	Udoteal (U-3)	0.30
Rhizoids (holdfast)	Udoteal (U-3)	No dry wt. calculations, similar by TLC
Caulpera paspaloides (N = 1)		
Tops	Caulerpenyne (C-10)	0.35
Rhizome and rhizoids	Caulerpenyne (C-10)	0.35

R. phoenix) than grassbed collections of the same species. The diversity of minor metabolites was also much greater for the reef collections including compounds such as U,P,R-1,2 and a variety of unidentified aldehydes. Table 10 summarizes these results.

Reef and grassbed collections of *Penicillus dumetosus* were compared from various habitats in the Florida Keys and Bahama Islands (Table 10). All reef collections of this alga examined contained aldehyde P-5 as the major metabolite. However, all grassbed populations contained a different diterpenoid, P-11 (Fig. 1), as the major metabolite. Compound P-11 does not possess an aldehyde functionality, but like all these compounds does show antimicrobial and toxic effects in laboratory bioassays. Other species of *Penicillus (P. capitatus, P. pyriformis)* also show variations in the amounts and types of terpenoids produced. Shallow reef collections of *Halimeda goreauii* contain greater concentrations of the major metabolite (a diterpenoid tetraacetate related to H-8) than deep reef collections of this alga.

DISCUSSION

Caulerpalean algae have been shown to be of low preference in the diets of most macroherbivores by feeding preference studies (Tsuda & Bryan 1973, Mathieson 1975, Ogden 1976, Hay 1981b, 1984b, Lobel & Ogden 1981, Hay et al. 1983, Littler et al. 1983) and stomach content analyses of herbivorous fishes and sea urchins (Hiatt & Strasburg 1960, Randall 1967, Hobson 1974, Bryan 1975, Lawrence 1975). While the basis for the proliferation and abundance of these algae has not been fully defined, it has been generally accepted that calcification provides a physical defense against herbivores for many species (Ogden 1976, Ogden & Lobel 1978, Hay 1981b, Lobel & Ogden 1981). Our investigations of this group show that these species also produce unique secondary metabolites that possess significant toxicities and produce deterrent effects in feeding assays.

Selection for chemical defenses may be driven by the intense macroherbivory on tropical reefs (Earle 1972, Ogden 1976, Ogden & Lobel 1978, Hay 1981b, 1984b, Littler et al. 1983). However, other factors that may select for chemical defenses include: (1) the need to inhibit (pathogenic) microorganisms such as bacteria and fungi; (2) the need to inhibit fouling organisms such as algal spores and settling larval invertebrates; and (3) interspecific competition for space. The effects of microorganisms (ZoBell & Allen 1935), fouling organisms, micrograzers (Brawley & Adey 1981), and competition for settling space (Sousa 1979) on algal growth and survivorship have not been well documented for most tropical algae. Consideration of these latter possibilities led to the development of the bioassays we used to assess the toxic and deterrent properties of these algal metabolites.

Toxic and deterrent secondary metabolites

The green algal compounds tested were highly biologically active in all the bioassays selected. This

Table 10. Chemical comparisons between algae in different habitats

Algae	Metabolites	Average conc. (% dry wt)	Standard deviation	Standard error
Rhipocephalus phoenix				
Grassbed (N = 4)	Rhipocephalin (R-6)	0.5	0.16	0.08
Reef (N = 3)	Rhipocephalin (R-6)	0.9	0.56	0.32
	Dihydrorhipocephalin (UPR-1)	0.6	0.44	0.25
	Mixture of aldehydes	0.3	0.24	0.14
Udotea cyathiformis				
Shroud Cay Reef (N = 1)	Dihydrorhipocephalin (UPR-1)	0.8	–	
	Aldehyde (UPR-2)	0.6	–	
Shroud Cay grassbed (N = 1)	Dihydrorhipocephalin	0.3	–	
Halimeda goreauii				
Shallow reef (6–8 m, N = 2)	*H. goreauii* tetraacetate	0.09	0.014	0.010
Deep reef (18–24 m, N = 2)	*H. goreauii* tetraacetate	0.05	0.0	0
Penicillus dumetosus				
Grassbed (N = 7)	*Penicillus* triacetate (P-11)	0.7	0.19	0.07
Reef (N = 4)	Dihydroudoteal (P-5)	0.7	0.12	0.06
Penicillus capitatus				
Grassbed and reef (N = 4)	Dihydrorhipocephalin (UPR-1)	0.8	0.37	0.18
Mangroves (N = 3)	Triacetate	0.8	0.47	0.27

high degree of bioactivity supports the hypothesis that these compounds function as defenses against predators and pathogens in tropical waters. Many other compounds isolated from species of caulerpalean algae have been tested and show similar levels of activity (Paul 1985). Of the many diverse metabolites that we and others have isolated from a wider spectrum of marine plants (in particular, many natural products from red and brown algae), few show the potent activities of these green algal metabolites in these bioassays. These compounds inhibit the growth of microorganisms, development of fertilized urchin eggs, and they are toxic to larval and adult stages of potential herbivores.

The compounds possessing one or more aldehyde functional groups (U,P,R-2, U-4, U-7) are the most biologically active in these bioassays. In the terrestrial environment secondary metabolites possessing aldehyde groups are among the most toxic and deterrent compounds that have been isolated. Terpenoid aldehydes such as warburganal, polygodial, isovelleral and the iridoid aldehydes are potent toxins produced by terrestrial plants and insects (Cavill & Hinterberger 1960, Kubo et al. 1976, Camazine et al. 1983). Aldehyde groups, and especially α,β-unsaturated aldehydes, can react with biological molecules in several ways which result in enzyme deactivation and interference with normal metabolic functions. Aldehydes can react with basic amino groups to form 'Schiff Bases' (imines). Biological nucleophiles (e.g. amines, alcohols, sulfhydryl groups) can add to the β-carbon of the unsaturated aldehyde in a 'Michael Addition' reaction (Kupchan et al. 1970). Little is known, however, regarding the interactions of enol-acetate functionalities with biological molecules. Hydrolysis of the enol-acetates should occur *in vivo* to yield the bioactive aldehydes, but addition reactions directly to the unsaturation may also be possible.

The toxic effects of several of these algae have been discussed previously. In particular, the toxic effects of *Caulerpa* spp. have been attributed to the compounds caulerpin and caulerpicin (Doty & Aguilar-Santos 1966, 1970, Aguilar-Santos & Doty 1968). In our assays we have found that the sesquiterpenoid caulerpenyne (C-10), which exists in concentrations of 40 to 50 % of the organic extract (1.0 to 1.5 % dry weight) in many species of *Caulerpa*, shows strong toxic and feeding deterrent properties. We attribute much of the biological activity of *Caulerpa* species to this major metabolite which we have isolated in varying concentrations from *C. taxifolia, C. sertulariodes, C. racemosa, C. mexicana, C. cuppressoides, C. prolifera, C. verticillata, C. paspaloides,* and *C. lanuginosa*. McConnell et al. (1982) showed that caulerpenyne accounted for the feeding inhibition in sea urchins *Lytechinus variegata* observed for *C. prolifera*. Hodgson (1984) showed that caulerpenyne was responsible for the antibiotic and antineoplastic activity of *C. prolifera* extracts. Caulerpin, on the other hand, shows little if any activity in assays testing feeding deterrence (McConnell et al. 1982) or ichthyotoxicity (V. Paul unpubl. results).

Several biological investigations have shown that these algae represent poor diets for many tropical herbivores. Balazs (1982) showed that juvenile green turtles *Chelonia mydas* in the northwestern Hawaiian Islands feed on *Caulerpa racemosa* and other unpreferred algae only when other more desirable algae are absent. This population of turtles also showed lower growth rates than turtles from the southern Hawaiian islands which feed on more desirable algae. Lobel & Ogden (1981) showed that the parrotfish *Sparisoma radians* demonstrated very low survivorship (equivalent to or lower than starvation) when fed on diets of *Caulerpa mexicana, Halimeda incrassata,* and *Penicillus pyriformis*. They attributed this mortality to toxins in *Caulerpa* spp. and calcification in *Halimeda* and *Penicillus* spp. However, we feel that the toxic terpenoids present in all 3 of these species were likely responsible for the high mortality observed. Their results compare with the mortality observed in our experiments with juvenile conch fed diets including the toxic terpenoids caulerpenyne (C-10), udoteal (U-3) and halimedatrial (H-7).

Calcification may be an effective deterrent to some herbivores and should also lower algal nutritive value and digestibility. However, it should be emphasized that many tropical reef herbivores are well adapted for the consumption of calcareous material (Randall 1967, Hiatt & Strasburg 1960, Ogden 1977). The parrotfishes (family Scaridae) for example, are abundant tropical herbivores that consume large quantities of calcareous material. These fishes possess a pharyngeal mill requiring calcareous material to aid in grinding food, and their stomach contents commonly contain between 50 and 90 % $CaCO_3$ (Randall 1967). The lack of stomach acid (measured pH 7 to 8) observed in these fishes further indicates their specialization for dealing with calcareous material (Lobel 1981). We believe multicomponent defenses, involving both physical and chemical methods, are very important for the successful survival of these green algae in herbivore-rich tropical waters. In terrestrial habitats, multiple defenses including spatial and temporal escapes, and chemical and physical defenses are also considered important antiherbivore adaptations (Feeny 1976, Coley 1983).

Our discussion has focused on antiherbivore defenses as a primary function of these terpenoid metabolites in members of the order Caulerpales. The role of herbivory as a selective pressure influencing

algal adaptation in tropical waters has been more extensively investigated than the effects of pathogenic microorganisms or fouling. However, our bioassay results indicate that these compounds may be effective defenses against the growth of pathogenic marine microorganisms and the settling of marine invertebrate larvae. The broad spectrum of physiological activity shown by these compounds suggests that the metabolites may be adaptations against a number of detrimental factors, thereby increasing the adaptive benefit of secondary metabolite production and enhancing the survivorship of these algae in more general ways.

Chemical variation

Results of studies of terrestrial plant-herbivore interactions suggest defenses should be allocated in proportion to the risk of predation to a particular tissue and the value of that tissue to the plant (McKey 1979). Therefore, we might expect to find lower concentrations of the terpenoid metabolites in the holdfasts of green algae since these portions are not available to most macroherbivores. Within these algae, however, we found little variation in the secondary metabolite composition in blades, stipes and holdfasts. Unlike higher plants, the allocation of secondary metabolites to specific tissues may be unfeasible or costly due to the coenocytic constructions of these green algae. Newly growing segments and reproductive structures, which are valuable to the plants, are well defended in these algae. Alternatively, higher secondary metabolite concentrations in these actively growing plant tissues may be a result of enhanced biosynthetic capability.

In terrestrial plants defense often appears to decrease when enemies are absent and increase when plants are attacked (Denno & McClure 1983, Rhoades 1985). Spatial variation in herbivory is well known in tropical habitats (Hay 1981b, 1984b,c, Hay et al. 1983). In particular, reef habitats have been shown to have much greater levels of herbivory than their adjacent seagrass beds. Shallow reef areas have also been found to show higher levels than deep reef areas (Hay & Goertemiller 1983, Hay 1984a). The chemical variation we observed in different populations of these green algae may reflect a differential commitment to chemical defense under varying levels of herbivory. In our preliminary comparisons, the reef populations usually showed higher concentrations and a greater variation of secondary metabolites than corresponding grassbed populations. In some cases, different metabolites were produced by reef and grassbed populations. Greater concentrations of biologically active molecules (aldehydes) were also found in algae collected in reef habitats. More studies are clearly needed to determine if secondary metabolite production in algae is induced under increased levels of herbivory (artificial grazing and transplant experiments are in progress), or if other environmental factors are responsible for this variation.

In summary, the secondary metabolites produced by virtually all members of caulerpalean algae that we tested show toxic and feeding deterrent effects in laboratory assays designed to examine their potential defensive functions in tropical habitats. The results indicate that these metabolites may play a significant role as chemical defenses against herbivores and pathogens in the marine environment. Herbivory appears to be an important factor selecting for algal defense mechanisms in tropical habitats; however, toxic metabolites may also play a role against fouling organisms and pathogens. Our results suggest that multiple benefits against diverse predators are derived from secondary metabolite biosynthesis. Between-habitat variation in secondary metabolite production was observed and may be related to the degree of herbivory the plants experience.

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