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DITERPENOID FEEDING DETERRENTS FROM THE PACIFIC GREEN ALGA *PSEUDOCHLORODESMIS FURCELLATA*

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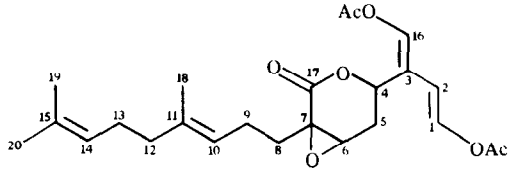
Key Word Index—*Pseudochlorodesmis furcellata*, Udoteaceae, Chlorophyta, herbivory, chemical defense, marine natural products

Abstract—Our continuing research with tropical green algae of the family Udoteaceae has led to the isolation of two new diterpenoid metabolites from the uncalcified Pacific alga *Pseudochlorodesmis furcellata*. The isolation and structural determination of these secondary metabolites is presented here along with data on the susceptibility of this alga to herbivorous fishes on Guam In addition, data on the feeding deterrent effects of the extract and major metabolite toward natural populations of herbivorous fishes and toward the herbivorous surgeonfish, *Zebrasoma flavescens*, and the rabbitfish, *Siganus spinus*, are presented. Results from these ecological assays support the proposal that chemical defenses effectively reduce grazing on *P furcellata* by herbivorous fishes

INTRODUCTION

In connection with our interest in the chemical adaptations of tropical marine algae, we have investigated the natural products chemistry and *in situ* palatability of the Pacific green alga *Pseudochlorodesmis furcellata* (Zanard) Boerg (Udoteaceae) Our results have shown that *P furcellata* contains two new diterpenoids (**1** and **2**) which are related to other compounds found in this family of algae. Like several of the related diacetoxybutadienes from this group, the major compound (1) possesses significant feeding deterrent properties in field assays [1]

The importance of plant secondary metabolites in reducing herbivory in terrestrial communities is well documented and generally accepted as one of the most effective means of herbivore defense [2, 3]. Coral reefs have been reported to have some of the highest grazing intensities of any terrestrial or marine habitats [4] and therefore many secondary metabolites from tropical algae have been hypothesized to function in chemical defense against herbivores However, little experimental evidence exists to support these proposals [1, 5, 6].



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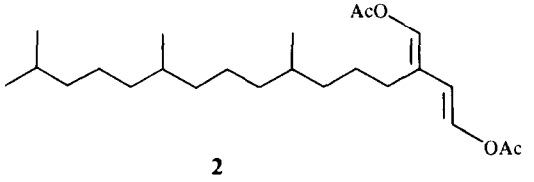

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We have previously isolated numerous terpenoid metabolites from tropical green algae of the families Udoteaceae and Caulerpaceae [7–9], and have shown that these compounds are toxic and feeding deterrent in laboratory assays [10] However, recent evidence indicates that these laboratory assays must be interpreted with caution in the way in which they relate to the behaviour of herbivores under natural conditions [1, 6] It is important to use herbivores that co-occur with the algae and may naturally feed on the plants to test the chemical defensive role of algal secondary metabolites Although useful information about the bioactivity of algal secondary metabolites can be gained from laboratory assays, ecologically relevant field and aquarium assays should be used to test hypotheses regarding the chemical ecology of marine algae

In this study, we first examined the susceptibility of *Pseudochlorodesmis furcellata* to grazing by herbivorous fishes in a reef habitat on Guam, Mariana Islands. The secondary metabolites were isolated, and the major metabolite, epoxylactone (**1**) was tested as a feeding deterrent toward natural populations of herbivorous fishes on Guam The deterrent effects of the crude extract were tested toward the herbivorous surgeonfish, *Zebrasoma flavescens*, in aquarium assays The crude extract and compound **1** were also tested as feeding deterrents toward the rabbitfish, *Siganus spinus*, an important herbivore in the tropical western Pacific. By utilizing these biological assays, we felt we could more accurately determine the role of the *Pseudochlorodesmis* terpenoids in chemical defense

RESULTS AND DISCUSSION

Pseudochlorodesmis furcellata was collected in reef habitats in Cetti Bay and Sella Bay on Guam *P. furcellata* is a soft, uncalcified green alga which is related to *Chlorodesmis*, a genus of green algae which is known to contain similar diterpenoids [9, 11] Fresh *P. furcellata*

was extracted repeatedly with dichloromethane–methanol (2 1), and the solvents were removed under vacuum to yield a dark green gum (0 9 g from *ca* 80 g wet algae, 12 g dry algae) Flash chromatography of the extract on silica gel, using ethyl acetate–isooctane mixtures, yielded two fractions rich in diterpenoids **1** and **2** Final purification of one fraction (EtOAc–isooctane, 1 1) by silica HPLC (EtOAc–isooctane, 35 65) yielded the major diterpenoid **1** (250 mg, 28% extract) Silica HPLC purification (EtOAc–isooctane, 1 9) of the more nonpolar fraction yielded the minor metabolite **2** (50 mg, 5% extract)

The epoxylactone **1** showed $[\alpha]_D^{25}$ + 1 6° ($CHCl_3$, *c* 0 7), and analysed for $C_{24}H_{32}O_7$ by HRMS (M^+ *m/z* = 432 2139, calc 432 2149). The presence of an *E,E*-1,4-diacetoxybutadiene functionality, commonly found in metabolites from this algal group, was readily apparent from the characteristic UV absorption at 248 nm (ε = 14000) ^{1}H NMR bands for the *trans*-olefinic protons at C-1 and C-2, and the lone olefin proton at C-16 were observed at δ 7 38 (*d*, *J* = 12 5 Hz), 5 86 (*d*, *J* = 12 5 Hz) and 7 25 (*s*) (Table 1) Corresponding acetate ester carbonyl bands and appropriate enol acetate olefin carbons were also observed in the ^{13}C NMR spectrum of **1** (Table 1)

The IR spectrum of **1** showed broad absorption at 1760 cm^{-1} This absorption, considered with a carbonyl band in the ^{13}C NMR spectrum at δ 168 5 indicated the presence of a δ-lactone functionality Also present in lactone **1** were two typical trisubstituted olefinic bonds, one of which was geminally dimethylated, and a trisubstituted epoxide (Table 1) Two ^{13}C NMR shifts above 18 ppm proved the C-10/C-11 olefin geometry to be *E* The mass spectrum of **1** showed $C_5H_9^+$ and $C_{10}H_{17}^+$ fragments which indicated cleavage of the C-8/C-9 bond and hence the positioning of all oxygenation before C-8

Final assignment of the structure of epoxylactone **1** was achieved by interpretation of heteronuclear correlation (HETCOR) long range coupling (J_{C-H} = 4–9 Hz, Table 1) Particularly diagnostic were the long range couplings between the lactone proton (C-4) and the carbon signals for C-2, C-16, and C-6 These data positioned the δ-lactone as originating from C-17 (carbonyl) and as possessing the α,β-epoxy constellation Analysis of the proton coupling constants of the 6-membered lactone ring failed to yield reliable stereochemical information Lactone **1** is similar in structure to the α,β-epoxy-γ-lactone we recently reported from *Udotea argentea* [9]

A second metabolite of *P furcellata* was the saturated bis-enol acetate **2** This compound showed $[\alpha]_D^{25}$ + 1 6° ($CHCl_3$, *c* 0 68) and analysed for $C_{24}H_{42}O_2$ by HRMS (found 394 3072, calc 394 3085) Structural assignment of **2** as the bisdihydro derivative of trifarin [12] was straight

Table 1 ^{1}H NMR and ^{13}C NMR spectra of epoxylactone **1**

	^{1}H NMR*		^{13}C NMR†		
C	^{1}H shift	*J*(Hz)	^{13}C shift		Observed $^{2,3}J_{CH}$ to H at C #
1	7 38 *d*	12 5	136 5	CH	2
2	5 86 *d*	12 5	108 6	CH	1 16
3			118 4	C	1, 16
4	5 37 *t*	6	73 2	CH	2, 6, 16
5	2 45 *ddd*	14 6, 2	27 7	CH_2	6
	2 29 *ddd*	14, 6, 2			
6	3 52 *bs*(*t*)	2	58 0	CH	
7			54 8	C	
8	2 25 *m*		30 6	CH_2	6
	1 58 *m*				
9	2 20 *m*		23 0	CH_2	
10	5 12 *t*	7	122 6	CH	18-Me
11			136 5	C	18-Me
12	2 02 *m*		39 6	CH_2	18-Me
13	2 06 *m*		26 6	CH_2	14
14	5 07 *t*	7	124 0	CH	19-Me, 20-Me
15			131 5	C	19-Me, 20-Me
16	7 25 *s*		132 4	CH	2
17			168 5	C=O	
18	1 63 *s*		16 0	Me	10, 12
19	1 60 *s*		17 5	Me	20-Me, 14
20	1 68 *s*		25 5	Me	19-Me & 20-Me
OAc	2 17 *s*		20 5	Me	
	2 14 *s*		20 5	Me	
			166 4	C=O	Me
			167 5	C=O	Me

*^{1}H NMR spectra recorded at 360 MHz in $CDCl_3$ solution

†^{13}C NMR spectra recorded at 50 MHz in $CDCl_3$ Attached protons were determined by DEPT sequence analyses

forward on the basis of its simplified spectral characteristics No attempt was made to determine the absolute or relative stereochemistries of the methyl-bearing carbons at C-7 and C-11.

Because *P. furcellata* occurs in reef habitats on Guam and lacks morphological defenses such as calcification, we hypothesized that chemical defenses were important for this alga. The alga is also taxonomically related to *Chlorodesmis fastigiata* which is known to produce diterpenoid feeding deterrents [1, 10]. The susceptibility of the whole alga to grazing by herbivorous fishes was hence tested in Pago Bay on Guam. Arrays ($N=12$) of *P. furcellata*, *Chlorodesmis fastigiata*, *Cladophoropsis membranacea*, and *Udotea geppii* attached to polypropylene lines were placed on the reef slope for six hours. During this time, 50% of the *Cladophoropsis membranacea* individuals were eaten, 42% of the *Pseudochlorodesmis* individuals were eaten, 8% of the *Chlorodesmis* was eaten, and virtually no *Udotea* was eaten. We ranked *P. furcellata* as a medium preference alga based on these and similar studies conducted on the Pago Bay reef slope. Many species of preferred algae were rapidly consumed in this herbivore-intense habitat (60–80% of individuals consumed in 2–3 hr), yet *P furcellata* was not avoided like *Chlorodesmis* and the calcified alga, *Udotea*.

The crude extract of *P. furcellata* was tested in aquarium assays toward the herbivorous surgeonfish, *Zebrasoma flavescens*, which is common in some reef habitats on Guam. We coated the highly preferred alga, *Enteromorpha clathrata*, with the extract at a concentration of 5% of the dry mass which approximates the natural concentrations of 7.5% of the dry mass in *Pseudochlorodesmis*. The coated *Enteromorpha* was offered to 10 individual *Z. flavescens* along with solvent controls Results of the assay are shown in Fig. 1. The extract reduced grazing by 80.6% relative to solvent controls and was a significant feeding deterrent ($N=10$, $p<0.005$). The extract was also tested toward *Z flavescens* at a lower concentration of 3% of the *Enteromorpha* dry mass. The extract reduced grazing by 25% relative to solvent controls but was not a significant deterrent ($N=9$, $p>0.06$). Thus, the *Pseudochlorodesmis* extract is a potent feeding deterrent toward *Z. flavescens*, especially at naturally occurring concentrations.

The crude extract of *P. furcellata* and the major epoxylactone (**1**) were also tested in aquarium assays toward the herbivorous rabbitfish, *Siganus spinus*, an abundant herbivore in the tropical western Pacific Again, we coated *Enteromorpha clathrata* with the extract and the epoxylactone (**1**) at naturally-occurring concentrations and offered these to a school of 25 rabbitfish along with solvent controls. The crude extract reduced grazing by 50% relative to solvent controls at 8% of the dry mass of *Enteromorpha*. In three trials, 18 pieces of control *Enteromorpha* and nine pieces of treated *Enteromorpha* were eaten The epoxylactone **1** also significantly reduced grazing by 30% when coated on *Enteromorpha* at 2% of the algal dry mass ($N=6$, $p=0.016$, Fig 2) The minor metabolite **2** was not tested in these assays because insufficient amounts were isolated (See Experimental for details of the assays)

The feeding deterrent effects of the epoxylactone **1** toward natural populations of herbivorous fishes on Guam were reported in an earlier paper [1] The compound was coated on the preferred red alga, *Acanthophora specifera*, at 2% of the algal dry mass and offered to grazing fishes in Cocos Lagoon on Guam for 30–60 min The compound significantly ($p=0.04$) reduced grazing by 20% relative to the solvent controls. The *Acanthophora* was grazed by a wide variety of herbivorous fishes including surgeonfishes, parrotfishes, and rabbitfishes in these assays. The compound thus appeared to be an effective deterrent toward natural populations of these herbivores.

Chemical defense appears to contribute to the survival of *Pseudochlorodesmis furcellata* in some reef habitats on Guam. The alga is soft and lacks calcification, yet it is not highly susceptible to grazing by herbivorous fishes The major metabolite, epoxylactone **1**, is a significant feeding deterrent toward herbivorous fishes in field and aquarium experiments This study utilizes natural herbivore species in assays to test predictions about the feeding deterrent role of the *Pseudochlorodesmis* diterpenoids. Similar studies will yield further information regarding the complex interactions between tropical algae and the numerous and diverse species of herbivores that occur on coral reefs.

EXPERIMENTAL

General The instrumentation and general experimental methods used in this research have been recently described [13] High resolution ^{1}H NMR spectra were obtained on a 360 MHz Nicolet-Oxford Magnetics FT spectrometer, ^{1}H and ^{13}C NMR

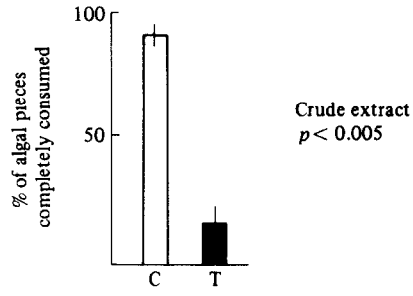

Fig 1 Results of feeding deterrent assays with the *Pseudochlorodesmis* crude extract tested toward the herbivorous surgeonfish, *Zebrasoma flavescens*. Results were analysed by the Wilcoxon Signed-Ranks test for paired comparisons ($N=10$, 90% of control algae eaten, 17 5% of treated algae eaten, $p<0.005$)

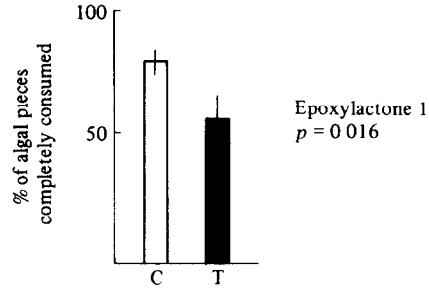

Fig 2 Results of feeding deterrent assays with the epoxylactone 1 toward the herbivorous rabbitfish, *Siganus spinus* Statistical tests are as in Fig 1 Nine trials were performed, three resulted in ties ($N=6$, 83 3% of control algae eaten, 58 3% of treated algae eaten, $p=0.016$)

200 MHz spectrometer (50 MHz carbon) Multiplicities for attached protons in the ^{13}C NMR were determined by DEPT sequence analyses High resolution mass spectra were obtained by the mass spectrometry service laboratory, University of California, Riverside Algae were collected at Cetti Bay and Sella Bay on the southern portion of Guam, Mariana Islands, in the tropical western Pacific (13°N, 143° E) in October 1985 and January 1986

Epoxylactone **1** The diterpenoid showed $[\alpha]_D^{25}$ +1 6° ($CHCl_3$, *c* 0 7), IR 3020, 2960, 1730–1770 broad, 1370, 1220, 1200, 1100, 1085, 930, 800 cm^{-1} ($CHCl_3$), UV (MeOH) λ_{max} 248 nm (ε = 14,000), HRMS M^+ found 432 2139, calc 432 2149 See Table 1 for ^1H NMR and ^{13}C NMR values

Bisdihydro trifarin **2** The diterpenoid showed $[\alpha]_D^{25}$ +1 6° ($CHCl_3$, *c* 0 68), IR ($CHCl_3$) 2910, 1750, 1630, 1370, 1080, 930 cm^{-1}, UV (MeOH) λ_{max} 250 nm (ε = 14,500), HRMS M^+ found *m/z* 394 3072, calc 394 3085, ^1H NMR ($CDCl_3$) δ 7 40 (1H, *d*, *J* = 12 5 Hz), 7 17 (1H, s), 5 92 (1H, *d*, *J* = 12 5 Hz), 2 38 (*t*, *J* = 6 5 Hz), 2 17 (3H, s), 2 15 (3H, s), 0 87 (12H, *d*, *J* = 6 5 Hz), ^{13}C NMR (50 MHz, $CDCl_3$) 167 8 (C=O), 167 4 (C=O), 135 7 (CH), 134 1 (CH), 121 7 (C), 113 4 (CH), 39 4 (CH_2), 37 4 (CH_2), 37 35 (CH_2), 37 3 (CH_2), 36 8 (CH_2), 32 8 (CH), 32 5 (CH), 27 9 (CH), 25 5 (CH_2), 25 4 (CH_2), 24 8 (CH_2), 24 4 (CH_2), 22 6 (Me), 22 5 (Me), 20 6 (Me), 19 7 (Me), 19 6 (Me)

Algal susceptibility assays The susceptibility of different species of algae to grazing by herbivorous fishes was examined by weaving small (3 to 4 cm long) pieces of thalli, at 5 cm intervals, into a 0 5 m length of 3-strand, 7 mm thick, polypropylene line that was then fastened to the reef at Pago Bay on Guam (N = 12 ropes) When an herbivorous fish encountered a rope, all species of algae should have been equally apparent and available Grazing was allowed to continue for 6 hr At the end of the experiment, each species on each rope was recorded as still present or totally consumed Similar methods have been used in other studies of algal susceptibility to grazers [14, 15]

Feeding deterrent assays A school of 25 rabbitfish, *Siganus spinus*, were maintained in a 5000 l, flowing seawater aquarium The crude extract of *Pseudochlorodesmis furcellata* and isolated epoxylactone **1** were dissolved in Et_2O and applied to the palatable green seaweed *Enteromorpha clathrata* at *ca* natural concentrations Et_2O alone was applied to the control *Enteromorpha* Small pieces of *Enteromorpha* (200–300 mg wet mass) were woven into a 0 5 m length of polypropylene line To test the crude extracts, 6 pieces of control and 6 pieces of treated *Enteromorpha* were offered to the rabbitfish for 3 trials (10 min each) The total number of pieces of control and treated algae that were eaten and uneaten were recorded To test the isolated epoxylactone **1** 4 pieces of control and 4 pieces of treated *Enteromorpha* were offered to the rabbitfish for 9 trials The control and treated algae were placed at opposite ends of the polypropylene line and offered as a matched pair The number of pieces of control and treated algae that were eaten were recorded for each trial and results were analysed by the Wilcoxon Signed-ranks Test for paired comparisons Results were significant at p = 0 016 (Fig 1)

Feeding deterrent assays with *Zebrasoma flavescens* were conducted similarly Ten individual fish were maintained in 500 l, flowing seawater tanks that were sectioned into 4–6 individual compartments Four pieces of treated and four pieces of control (Et_2O) *Enteromorpha* were placed at opposite ends of a polypropylene line and one line was offered to each fish Assays were allowed to continue until at least half of the available algae was eaten or up to 2 hr Results were analysed by the Wilcoxon Signed-Ranks Test for paired comparisons

Field feeding deterrent assays Methods and results for this assay have been previously described [1] The experiments were similar to the rabbitfish assays, but were conducted in reef habitats in Cocos Lagoon on Guam instead of in large aquaria Five pieces of the palatable red seaweed, *Acanthophora specifera*, were coated with the epoxylactone **1** and placed on polypropylene lines Five Et_2O controls were also placed on lines and the lines were placed on the reef, 0 25 m apart, as paired samples for 30 min (N = 15 pairs) Results were analysed by the Wilcoxon Signed-Rank Test and showed that the epoxylactone **1** was a significant feeding deterrent (p = 0 04)

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