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PLANT ANIMAL INTERACTIONS

Michael S. Deal · Mark E. Hay · Dean Wilson · William Fenical

Galactolipids rather than phlorotannins as herbivore deterrents in the brown seaweed *Fucus vesiculosus*

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Abstract The first investigation of seaweed chemical defense against herbivores involved the brown seaweed *Fucus vesiculosus* and suggested defense via phlorotannins. The first demonstration of seaweed induction of secondary metabolites in response to herbivory also involved the genus *Fucus* and assumed a defensive function for phlorotannins. Many other investigations correlate herbivore feeding preference with changing levels of phlorotannins in this genus and others, but few directly test the effects of phlorotannins. No studies have assessed *Fucus* chemical defenses using bioassay-guided separation to investigate the complete complement of compounds deterring herbivores. We investigated the deterrence of *F. vesiculosus* chemical extracts using herbivore bioassays to guide our chemical investigations. Although crude extracts from *F. vesiculosus* strongly deterred feeding by the sea urchin *Arbacia punctulata*, phlorotannins from this extract did not deter feeding at 2× or 4× natural concentration by dry mass. Feeding deterrence was due to: (1) a polar galactolipid in the ethyl acetate-soluble extract, and (2) a non-phenolic compound, or compounds, in the water-soluble extract. Although this is the first evidence of galactolipids deterring herbivores, such defenses could be geographically and taxonomically widespread. The galactolipid we discovered in *Fucus* occurs in marine dinoflagellates, and a related metabolite that deters herbivory has recently been discovered in a tropical green seaweed. We were unable to identify the second deterrent compound, but deterrence occurred in a fraction containing carbohydrates, including sulfated sugars, but no phlorotannins. Given the polarity of these chemical deterrents, they could co-occur with and confound bioassays of phlorotannins if investigators test phlorotannin-containing algal extracts without further purification.

Keywords *Arbacia punctulata* · Glycerolipids · Plant-herbivore interactions · Polyphenolics · Seaweed chemical defenses

M. S. Deal
Institute of Marine Sciences,
University of North Carolina at Chapel Hill,
Morehead City, NC 28557, USA

M. E. Hay (✉)
School of Biology,
Georgia Institute of Technology,
Atlanta, GA 30332-0230, USA
e-mail: mark.hay@biology.gatech.edu
Tel.: +1-404-8948429
Fax: +1-404-8940519

D. Wilson · W. Fenical
Scripps Institution of Oceanography,
University of California — San Diego,
La Jolla, CA 92093-0204, USA

Present address:
M. S. Deal, Biopraxis,
10655 Sorento Valley Road, Suite 200, San Diego, CA 92191, USA

Introduction

Investigations of plant chemical defenses against herbivores have shaped our understanding of the ecology and evolution of prey-consumer interactions, coevolution, and the forces structuring populations and communities (Strong et al. 1984; Hay and Fenical 1988; Rosenthal and Berenbaum 1992; Fritz and Simms 1992; Duffy and Hay 2001; Paul et al. 2001). The role of plant tannins as herbivore deterrents has played a central role in virtually all general theories of plant defenses against herbivores (e.g., Feeny 1976; Rhoades and Cates 1976; Bryant et al. 1983; Coley et al. 1985; Herms and Mattson 1992), even though these large molecules are difficult to purify (Ragan and Glombitza 1986) or to accurately quantify (Appel et al. 2001), and have therefore rarely been tested directly for herbivore deterrent effects following careful purification and separation from other potentially confounding chemical deterrents (but see Steinberg 1988; Clausen et al. 1990; Steinberg and van Altena 1992).

Like tannins from terrestrial plants, phlorotannins from brown seaweeds have received considerable attention

from chemical ecologists (reviewed by Ragan and Glombitza 1986; Steinberg 1992; Van Alstyne et al. 2001) due to some phlorotannins serving as chemical defenses against herbivores (e.g., Steinberg 1988). Numerous ecological investigations have correlated phlorotannin concentrations with patterns of susceptibility to grazers (e.g., Steinberg 1985; Van Alstyne 1988; Yates and Peckol 1993; Martinez 1996; Pavia et al. 1997; Van Alstyne et al. 2001), and there have been several careful chemistry studies of brown algal phlorotannins (e.g., Ragan and Craigie 1976; Glombitza et al. 1976; Koch et al. 1980; McInnes et al. 1985; Ragan 1985). A few investigators have coated phlorotannin-containing crude (i.e., methanolic) extracts onto palatable seaweeds (Van Alstyne and Paul 1990) or incorporated them into, or removed them from, agar-based experimental foods (e.g., Pavia and Toth 2000). Better still, some studies have incorporated purified or partially purified phlorotannins into foods that are then offered to herbivores (Geiselman and McConnell 1981; Steinberg 1988; Steinberg and van Altena 1992; Winter and Estes 1992; Boettcher and Targett 1993). This approach provides the most convincing evidence for the deterrent properties of specific phlorotannins, or phlorotannin mixtures. However, numerous studies of the relationship between phlorotannins and seaweed palatability have reported conflicting or variable results. Phlorotannin concentrations are sometimes predictive of resistance to herbivores (Steinberg 1985), but in other investigations show no correlation with resistance (Steinberg and van Altena 1992; Targett et al. 1995; Pavia et al. 1997; Van Alstyne et al. 2001). More direct tests sometimes show strong effects of phlorotannins on feeding or digestive efficiency (Steinberg 1988; Boettcher and Targett 1993; Steinberg et al. 1995), but at other times show little, if any, effect (Steinberg and van Altena 1992; Steinberg et al. 1995). Some of this variance is due to differential susceptibility of different herbivores to phlorotannins (e.g., Steinberg et al. 1995), or differential effects of different phlorotannins on herbivore physiology (Boettcher and Targett 1993). However, the frequent lack of correlation between phlorotannin levels and seaweed palatability could also suggest that phlorotannins *as a class* do not have a predictable activity against herbivores (Boettcher and Targett 1993; Targett and Arnold 1998), that some phlorotannins have primary roles (Schoenwaelder and Clayton 1999) while others function as defenses, or that phlorotannin-containing seaweeds also produce other deterrent metabolites that have been overlooked and could be confounding studies focused primarily on phlorotannins. Our investigation evaluates this last possibility.

In a previous study (Deal 1997) investigating intraspecific variation in susceptibility of *Fucus vesiculosus* to herbivores, we found phlorotannin concentrations to be an inconsistent predictor of palatability. Aware of the variable effects of seaweed phlorotannins reported in the literature (Steinberg et al. 1991; Steinberg and van Altena 1992; Boettcher and Targett 1993; Targett et al. 1995), we decided to use bioassay-guided separations of *Fucus* extracts to determine if metabolites other than phlorotannins could be functioning as herbivore deterrents.

We asked the following questions:

1. Do extracts from *F. vesiculosus* deter sea urchin feeding?
2. If extracts are deterrent, what specific compounds are responsible?
3. Do phlorotannins from our population of *Fucus* deter sea urchin feeding?
4. If not, will phlorotannins become deterrent if elevated to 2–4 times their natural concentrations?

Materials and methods

The brown intertidal seaweed *F. vesiculosus* and the herbivorous sea urchin *Arbacia punctulata* were both collected from rocky habitats near Beaufort, North Carolina, USA (34°43′N, 76°41′W). The *Fucus* used in chemical investigations and bioassays was collected in January of 1996. This population of *Fucus* is near the southern limit of the distribution for this species (Schneider and Searles 1991), but has persisted at this location for more than 20 years (M. E. Hay, personal observation). We also obtained *F. vesciculosus* from a protected western shore of Appledore Island, Maine (42°59′N, 70°37′W) in June 1996, and compared the chemistry and deterrence of extracts from this northern population with the population we investigated in North Carolina.

General approach

We used assays of sea urchin feeding preferences (following methods of Hay et al. 1994, 1998) to guide our chromatographic fractionation of *Fucus* extract along polarity and molecular size gradients (Fig. 1). This allowed for the possibility of any fraction being active and did not focus on phlorotannins alone. However, because phlorotannins, or phlorotannin-containing extracts, from *Fucus*, or related genera, have sometimes been shown to be deterrent (Geiselman and McConnell 1981; Steinberg 1988; Steinberg and van Altena 1992; Pavia and Toth 2000), we also explicitly assayed the phlorotannins from this population. Initial solvent partitions and bioassays of extracts were followed by further separation and purification of deterrent fractions using Sephadex LH20 chromatography (to separate extracts by compound size and shape) or silica gel flash chromatography (to separate extracts by compound polarity). Final purification and quantification of deterrent metabolites was performed by high performance liquid chromatography (HPLC). Structures were assigned by proton and carbon NMR in combination with other spectral methods.

Although the sea urchin *A. punctulata* co-occurs with *Fucus* in the lowest portions of the intertidal, *Arbacia* has less access to *Fucus* than do some snails and amphipods, which are more tolerant of desiccation than is *Arbacia*. We chose *Arbacia* as our bioassay organism because: (1) it is a generalist herbivore unlikely to be specifically adapted to dealing with *Fucus* metabolites, (2) it fed rapidly (our assays ran for <2 h), allowing us to test polar metabolites that might leach from our test food if assays ran for long periods (Steinberg 1988; Pavia and Toth 2000), and (3) its feeding preferences and responses to seaweed chemical defenses are similar to those of other generalist consumers in the region (Hay et al. 1987; 1988; Duffy and Hay 1994; Bolser and Hay 1996; Cronin and Hay 1996). *Arbacia* also feeds reluctantly on intact *Fucus*, dried and finely ground *Fucus* imbedded in agar (Deal 1997), and on other Fucalean seaweeds in the region (Hay et al.

1986; 1987), all suggesting that the alga many be chemically distasteful.

Previous feeding investigations conducted with snails necessitated assay durations of 6 h to 8 days in order to observe sufficient feeding (e.g., Steinberg 1988; Pavia and Toth 2000). In preliminary assays performed for 24 h, but checked frequently (i.e., after 1 h, 2 h, 4 h, etc.) to assess consumption, we commonly found that some extracts were deterrent when assessed after 2–4 h, but lost deterrence if assessed after longer periods. This suggests that deterrent compounds were leaching from the foods or degrading over this period, so we constrained our bioassays to <2 h in duration and conducted assays only at night when urchins fed most rapidly. We also set up 35 replicate urchins in hopes of getting adequate numbers (about 20) to feed within 2 h. This produced sample sizes ranging from 14 to 31 for each of our 16 separate assays.

Bioassay procedures

Our goal was to test extracts and compounds from chemical separations in an artificial diet that was similar to *Fucus* in nutritional value. This was attempted by incorporating metabolites into an agar-based diet containing freeze-dried, powdered tissue from the palatable green alga *Ulva*. This approach was not entirely successful because an artificial diet holding an algal dry mass/volume equal to an average *Fucus* plant would not harden appropriately. Thus, we had to make our diet so that the dry mass of *Ulva* per milliliter of food was only 50% of the dry mass of *Fucus* per milliliter of *Fucus*. This means that the reward (=dry mass of alga) per bite was artificially low for our test food. Because we initially added *Fucus* extracts at their natural volumetric concentrations (i.e., extracts from 20 ml of *Fucus* were placed in 20 ml of our artificial diet), the concentration of chemical deterrents per concentration of food value, could have been double the natural concentration. To counter this, once a deterrent compound had been isolated and identified, we determined its natural concentration in *Fucus* using HPLC and re-tested the compound at its natural concentration per dry mass of alga (i.e., the compound contained in a milligram of dry *Fucus* would be placed on a milligram of dry *Ulva*, with this then imbedded in the agar-based food).

Although agar may bind, or leach, some phlorotannins (Steinberg 1988; Pavia and Toth 2000) or other polar metabolites, all previous assays demonstrating feeding deterrent effects of seaweed phlorotannins have used agar-based artificial foods for their bioassays (e.g., Geiselman and McConnell 1981; Steinberg 1988; Steinberg and Van Altena 1992; Winter and Estes 1992; Pavia and Toth 2000). To keep our assays comparable to these, we also used this proven method. Our artificial diet differed, however, from those used previously in that our diets were more nutritious and more closely approximated the nutritional value of seaweeds. Most agar-based diets used in previous assays of phlorotannins did not contain concentrated algal particles, but instead used only water extracts of ground algae to flavor the agar or sparse particles of algae as feeding stimulants (e.g., Steinberg 1988).

Hay et al. (1994, 1998) give a detailed description of our bioassay procedures . In general, if extracts to be tested were lipid-soluble, they were dissolved in an appropriate amount of solvent (generally ether), poured over freeze-dried powdered *Ulva* until the powder was completely wetted, and the solvent was then removed by rotary evaporation. This resulted in adherence of the extract to the powdered *Ulva*. The *Ulva* was then incorporated into an agar matrix (2 g dry *Ulva* mixed into 10 ml distilled water, 0.36 g agar mixed into 8 ml distilled water and boiled, the two then mixed together as the agar cooled), poured into a mold laid over a piece of window screen, and the mixture allowed to harden onto the window screen material (see diagrams in Hay et al. 1994; 1998). After the agar solidified, the mold was removed, leaving a strip of artificial food adhering to the screen – essentially making an artificial alga with graph-paper-like squares imbedded in it. The strip was cut into individual replicates, offered to herbivores along with a similar control treated with solvent only, and feeding was quantified by counting the number of window screen squares cleared by the herbivore during feeding.

For water-soluble extracts, distilled water was added to the water-soluble extract until it made a volume of 10 ml; this 10 ml was placed on the powdered algae and stirred to assure uniform mixing. This mixture was then added to 8 ml heated water and agar, mixed, and poured onto screens in molds, as with the lipid-soluble extracts described above.

Chemical extraction and separation procedures

For our initial assay of potential chemical defenses in *Fucus*, we placed 20 ml fresh *F. vesiculosus* tissue in 100 ml of methanol and homogenized this with a Brinkman tissue homogenizer. Extraction proceeded for 2 h at 0°C. The extract was filtered through a glass-fiber filter to remove particulates, and the extraction procedure was repeated. The two extracts were combined and methanol was removed by rotary evaporation leaving a small amount of tissue-derived water. The extract was partitioned between water and dichloromethane (DCM). Each of these fractions was incorporated into an artificial diet and offered to sea urchins along with a control treated with solvent only. In the artificial diet, the extract from 20 ml of tissue was incorporated into the same volume of artificial food. Therefore, all components of the extract were at natural concentration by volume (but ≈2 times natural concentration relative to dry mass of seaweed tissue). Whenever a fraction significantly deterred feeding, it was subjected to further partitioning and bioassay-guided separation until we isolated a compound responsible for the effect, or until bioactivity could no longer be detected consistently.

The deterrent, water-soluble fraction was investigated in detail. Eighty milliliters fresh *Fucus* was extracted in 400 ml methanol and partitioned between DCM and water as described above. The water-soluble fraction was then partitioned between ethyl acetate and water; with each of these fractions assayed using sea urchin feeding (both were active, and were investigated further).

Following the ethyl acetate-water partition, one-third of the remaining water-soluble material (1.245 g; from 20 ml plant tissue) was fractionated by size-exclusion column chromatography using Sephadex LH20 eluting with DI water. A total of 19 fractions were collected. Based on NMR spectral data, some of the 19 fractions were recombined to yielded three distinct fractions: (1) a mixture of carbohydrates including sulfated sugars (0.327 g), (2) pure mannitol (0.912 g), and (3) phlorotannins alone (0.0065 g). Each of these fractions was tested in the urchin feeding assay. To determine the effect of the phlorotannins at higher concentrations, the remaining material from the water fraction (from the ethyl acetate-water partition; 2.488 g from 40 ml plant tissue) was fractionated by size-exclusion chromatography as described above to yield a fraction (0.031 g) containing the phlorotannins and a small amount of mannitol. We used this material to test the effects of phlorotannins on sea urchin feeding at 2× natural volumetric concentration (=4× natural concentration relative to dry mass).

The deterrent ethyl acetate fraction from the ethyl acetate-water partition was adsorbed onto silica gel and subjected to vacuum liquid chromatography (flash column). A 60-ml Buchner funnel was filled with 30 ml silica gel and eluted with: (1) 150 ml ethyl ether, (2) 150 ml of 1:1 ethyl ether:ethyl acetate, (3) 300 ml methanol. Each of these fractions was then tested in the sea urchin feeding assay.

A small portion of the deterrent methanol fraction was assessed by silica gel thin-layer chromatography (TLC) using 9:1 ethyl ether:methanol as a solvent. Three compounds were visualized when the TLC plate was developed with dilute sulfuric acid and heated. It was apparent that a red charring compound and a green charring compound with similar Rf values (≈0.35) would be difficult to separate by silica gel chromatography, so the deterrent methanol fraction was fractionated by size-exclusion (LH20) chromatography eluting with methanol. The green charring compound traveled very slowly over LH20, allowing us to separate this compound from the remainder of the fraction. The green charring

compound versus a mixture of the red charring compound plus a compound that charred brown were then tested in the urchin feeding assay.

The mixture of the red and brown charring compounds was deterrent and so was further separated by silica gel vacuum liquid chromatography. The column was eluted with an ether-methanol gradient (Fig. 1). Six 50-ml fractions were collected and recombined based on TLC to yield pure red charring and brown charring compounds. As only the red charring compound significantly deterred urchin feeding, we investigated its structure using NMR and other spectral methods.

Many investigators studying phlorotannins extract with 7:3 methanol:water. To determine if our procedure using 100% methanol might have been less efficient at extracting phlorotannins, we took two similar wet masses of *Fucus* tips from each of five separate plants, extracted one tip in 100% methanol and the other in 7:3 methanol:water and compared their phlorotannin yields using the Folin-Denis procedure with phloroglucinol as the standard.

To determine whether the southern population of *Fucus* we used for this investigation was also representative of *Fucus* populations in more northerly regions, we used TLC to determine if the deterrent metabolite from our North Carolina site was also apparent in the extract of *Fucus* from Appledorn Island, Maine. We also used feeding bioassays to determine if the ethyl acetate-soluble, galactolipid fraction from Maine plants deterred sea urchin feeding, as had been the case for plants from our North Carolina population.

HPLC quantification of the galactolipid

Ten pieces (1.502–1.598 g wet mass) of North Carolina *Fucus* from separate plants were placed in separate 10-ml volumes of methanol, ground with a Brinkman tissue homogenizer and allowed to extract for 30 min at 0°C. The samples were centrifuged, the supernatant decanted, and the plant material extracted in an additional 5 ml methanol. The two extracts were combined, and methanol was removed by rotary evaporation. Each sample was partitioned between 6 ml water and 6 ml ethyl acetate; the water was removed and partitioned again with an additional 3 ml ethyl acetate, which was then combined with the ethyl acetate from the first partition. The water was discarded. Most ethyl acetate was removed using rotary evaporation, and residual solvent was evaporated under a stream of nitrogen. Each extract was dissolved in 2 ml ethyl acetate and applied to a small column containing 500 mg Florisil. The vial containing the extract was rinsed with an additional 1 ml ethyl acetate and this was also added to the Florisil column. Another 3 ml ethyl acetate eluted pigments and sterols. A second fraction eluted with 9 ml of 9:1 ethyl acetate:methanol contained the galactolipid and small amounts of sterols. A third fraction, eluted with 100% methanol, did not contain galactolipids.

The concentration of galactolipid in the second fraction was quantified by HPLC. The HPLC system consisted of a Waters pump (model 510), Spectraphysics autosampler with 500-μl sample loop, Microsorb silica column (4.6×100 mm), and Dynamax refractive index detector (model RI-1). Each sample was dissolved in 200 μl of 2.5% methanol in ethyl acetate (HPLC grade solvents), and 100 μl was injected into the HPLC system. Solvent (2.5% methanol in ethyl acetate) was delivered at 0.85 ml/min. Peak areas were converted to concentrations with a standard curve constructed with concentrations of pure galactolipid ranging from 2 to 12 mg/ml. Once we determined reliable values for natural concentrations of the galactolipid, we conducted urchin feeding assays incorporating the galactolipid at this concentration per unit dry mass of the alga added to the agar-based diet.

Bioassays of urchin feeding were analyzed via Wilcoxon signed-ranks tests using directed *P*-values (P_{dir}) with γ/α=0.8 as suggested by Rice and Gaines (1994). Replicates with no feeding or where both choices were completely consumed were excluded.

Results

The methanol extract of *F. vesiculosus* produced a water-soluble portion that strongly deterred sea urchin feeding (P_{dir}=0.025) and a DCM-soluble portion that had no significant effect on feeding (P_{dir}=0.291; Fig. 1A, B). Partitioning the active, water-soluble fraction between water and ethyl acetate produced a more polar (water) and less polar (ethyl acetate) fraction, both of which strongly deterred sea urchin feeding (P_{dir}=0.022 and 0.034, respectively; Fig. 1C, D). Separation of 1.245 g of the water-soluble portion using LH20 yielded 0.327 g of a deterrent fraction (P_{dir}=0.030; Fig. 1E) containing a mixture of carbohydrates (including sulfated sugars), 0.912 g of a fraction composed almost exclusively of mannitol, which stimulated feeding (P_{dir}=0.013; Fig. 1F), and 0.0065 g of phlorotannin (purity evaluated by NMR) that did not deter feeding (Fig. 1G, H) at either 1× (P_{dir}=0.112) or 2× (P_{dir}=0.200) their natural volumetric concentrations (= 2× and 4× their natural concentration by algal dry mass). There was a small amount of mannitol in this fraction, but its effect was probably negligible as the mannitol was at ≈2% of natural concentration. Recovery of extract mass from this LH20 column was 100%, so compounds were not being lost on the column. Further purification of the deterrent carbohydrate mixture produced fractions with inconsistent activities in bioassays (initially deterrent, but then losing activity). This instability prevented identification of the active component in this mixture.

When the ethyl acetate fraction (Fig. 1D) was separated into three additional fractions along polarity gradients using a silica column, only the most polar fraction was deterrent (Fig. 1I–K; P_{dir}=0.350, 0.327, 0.012, respectively). Further partitioning of this fraction using both LH20 and silica (Fig. 1L–O), produced a single deterrent fraction (P_{dir}=0.028) that was composed entirely of the galactolipid shown in Fig. 2. Quantification of this compound by HPLC demonstrated that the natural concentration of this galactolipid was 0.41±0.01% (mean±SE, n=10) of *Fucus* dry mass. When this compound was tested in our agar-based foods at this natural concentration relative to algal dry mass, it strongly and significantly reduced feeding (P_{dir}=0.037; Fig. 2).

Given that *Fucus* phlorotannins had not deterred feeding (Fig. 1), we wanted to be sure that our extraction in 100% methanol had not been inefficient relative to the more common procedure of using 7:3 methanol:water. One-hundred percent methanol was as effective as 7:3 methanol:water at extracting Folin-Denis reactives from fresh *Fucus* [100% methanol=0.86±0.11% dry mass phenolics; 7:3 methanol:water=0.72±0.07% dry mass phenolics (mean±SE); n=5; P=0.304, paired sample *t*-test].

TLC of *Fucus* from Maine versus North Carolina produced the same red-staining spot at the same location on the plate, indicating that the northerly population contained the galactolipid we isolated from North Carolina *Fucus*. As with our assays using North Carolina

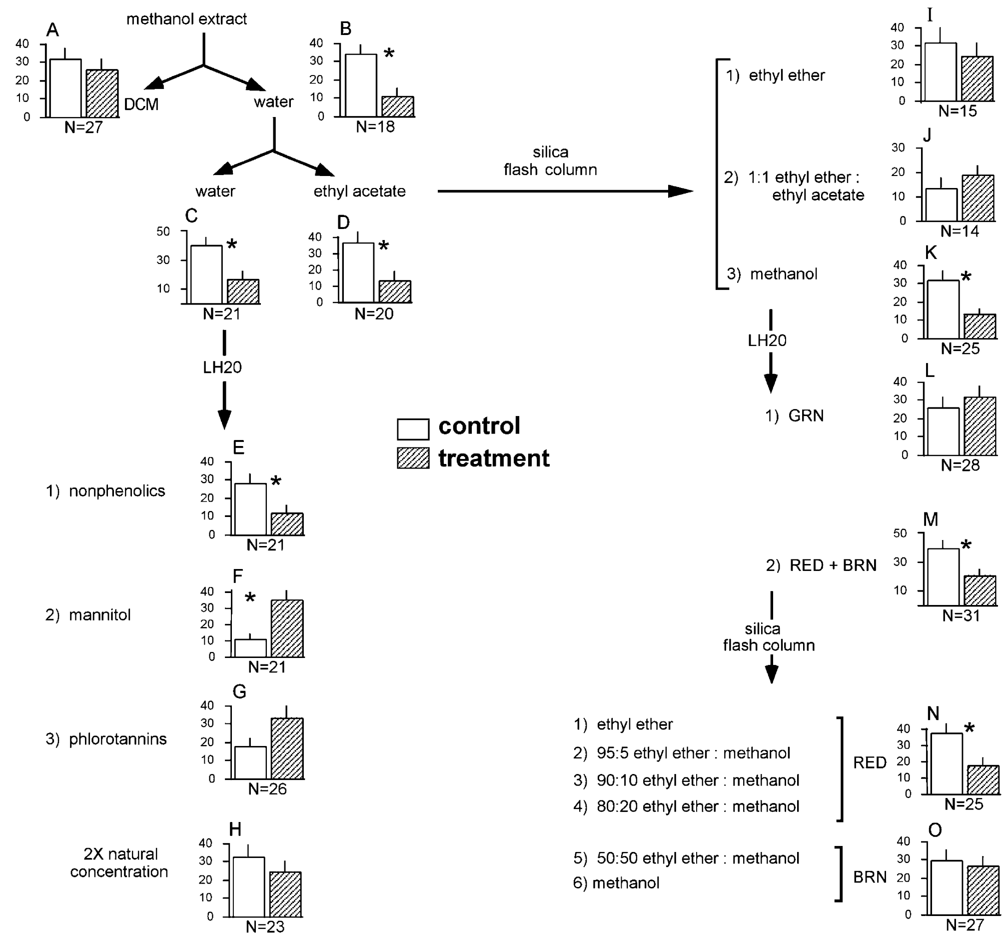

Fig. 1 Bioassay-guided separations of the methanolic extract from *Fucus vesiculosus*. *Fractions that significantly deterred (directed *P*-values (P_{dir}) with γ/α=0.8 as suggested by Rice and Gaines (1994); P_{dir}<0.05) feeding by the sea urchin *Arbacia punctulata*. All *y*-axes represent the number of window screen squares uncovered by sea urchin feeding. *Error bars* represent +1 SE. Sample sizes are given at the *base of each bar*. *P*-values are from Wilcoxon signed-ranks tests. *GRN* green charring compound, *RED* red charring compound, *BRN* brown charring compound; all discussed in the text

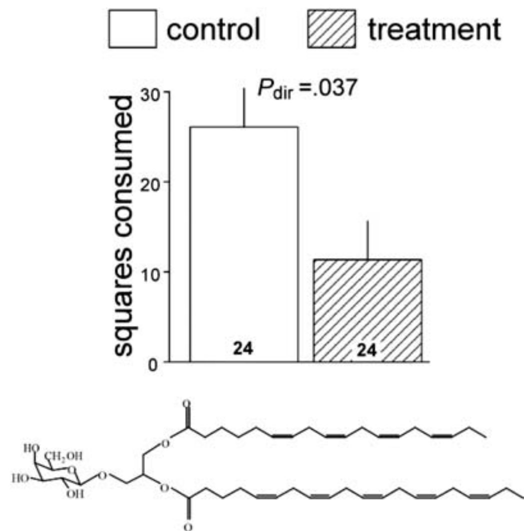

Fig. 2 The effect of a galactolipid (at its natural concentration of 0.41±0.01% of algal dry mass; mean±SE) isolated from *F. vesiculosus* on feeding by the sea urchin *A. punctulata.* Other symbols are as in Fig. 1

plants, feeding bioassays also demonstrated that the (ethyl acetate-soluble) galactolipid fraction from Maine plants (Fig. 1D) also deterred sea urchin feeding when placed into our artificial diet [control, 49.8±5.8 squares consumed; treatment, 13.8±4.2 squares consumed (mean± SE); *n*=28; P_{dir}=0.002, Wilcoxon signed-ranks test.]

Discussion

Bioassay-guided fractionation of the crude chemical extract from *F. vesiculosus* produced a galactolipid that deterred sea urchin feeding at natural concentration, as well as a deterrent fraction containing a mixture of carbohydrates, including sulfated sugars (Figs. 1E, N; 2). We were not able to isolate a deterrent pure compound from this second deterrent mixture; as we began to further purify this extract, deterrence became inconsistent, suggesting chemical instability. The deterrent galactolipid we found in *Fucus* also occurs in a marine dinoflagellate (Kitagawa et al. 1989), and a different galactolipid that deters herbivores is being described from the tropical green seaweed *Bryopsis pannata* (V. J. Paul, personal communication). Thus, galactolipids with feeding deterrent effects are geographically and taxonomically widespread (occurring minimally in pelagic microalgae,

temperate brown and tropical green seaweeds). Glactolipids represent a class of compounds not previously recognized as having a defensive function. Because they are ubiquitous plant metabolites usually associated with primary rather than secondary functions, we would not have detected the ecological role of the compound had we not used bioassay-guided fractionation. Defensive galactolipids could be common, but undetected, in many organisms.

Given that galactolipids are a major source of essential fatty acids for herbivores (Ohlsson 2000), it may seem incongruous that galactolipids can be feeding deterrents. However, it is the specific structure of a compound, not its general class, that determines its bioactivity (e.g., Zucker 1983; Clausen et al. 1990; Boettcher and Targett 1993). Related glycerolipids and fatty acids may function as either feeding attractants or feeding deterrents depending on their molecular structures (Sakata 1989; Sawai et al. 1994). Additionally, some plant galactolipids can form bioactive aldehydes when plants are damaged; these aldehydes could serve as defenses (Metsui et al. 2000).

The phlorotannins from the population of *Fucus* that we investigated had no effect on sea urchin feeding when tested at 100 and 200% of natural concentration by volume, or at 200 and 400% of natural concentration by dry mass (Fig. 1G, H). This could be because the particular phlorotannins in these plants were not deterrent (see variance in tannin effects as a function of structure or size; Zucker 1983; Clausen et al. 1990; Boettcher and Targett 1993) or because concentrations of phlorotannins in these plants were low (about 1% by dry mass). Although phlorotannins in *Fucus*, and other brown algae, can vary considerably across both space and time (Van Alstyne et al. 2001), some previous assays have suggested that concentrations of only 0.5–1% could deter feeding. Folin-Denis measurements of phlorotannin levels in the population we studied have varied from 0.8 to 3.5% of dry mass across different seasons and years, but have not deterred feeding by herbivorous sea urchins or amphipods at any of these concentrations (M. Deal, M. Hay, and J. Kubanek, unpublished data). When we tested phlorotannins from our collection at 4× natural concentration by dry mass, this would have equaled 3.4% by dry mass of algal food. When Pavia and Toth (2000) used methods similar to ours and tested phlorotannins from the related rockweed *Ascophyllum nodosum* (via subtraction of phlorotannins using PVPP), they found significant deterrence at concentrations of only 0.5 and 2.0%. Although less well quantified, Geiselman and McConnell (1981) also suggested that *F. vesiculosus* phlorotannins placed in agar-based foods deterred herbivores at concentrations of only 1% dry mass.

Some variance in phlorotannin-herbivore interactions may occur due to: (1) differential effects of specific phlorotannin molecules (Boettcher and Targett 1993), (2) differences among herbivore species in their susceptibility to phlorotannin-based defenses (Steinberg and van Altena 1992; Steinberg et al. 1995; Pavia and Toth 2000), or (3) interactions between phlorotannin type and gut physiology of the herbivore tested (Targett and Arnold 1998). However, some of the divergent findings in the literature could also result from the variable value of the food in which the phlorotannins are being tested. Compounds that deter feeding in low value foods may have no effect when placed in higher value foods (Duffy and Paul 1992; Hay et al. 1994), and all previous assays demonstrating deterrence of brown algal phlorotannins appear to have been from assays in which the nutritional value of the test food would have been very low compared to the seaweeds from which the phlorotannins were extracted. As examples, Geiselman and McConnell (1981) ground palatable algae in water then added this mixture (with the water diluting the algal material) to additional water and agar to make the food in which their algal extracts were tested. Steinberg et al. (1995) and Pavia and Toth (2000) apparently used similar procedures. Steinberg (1988) ground one part palatable alga in three parts of water, filtered out the solid algal pieces and used this flavored water to make assay food. Winter and Estes (1992) used similar procedures. In all of these assays, the nutritional value per volume of food would have been very low relative to the original alga. The algal dry mass per volume of our assay food was only 50% of the value found in *Fucus*, but this value per volume would have been much higher than in foods used in previous investigations and might explain some of the variance in results.

The first experiment assaying for seaweed chemical defenses against herbivory was conducted with *F. vesiculosus* (Geiselman and McConnell 1981). These authors started their investigation using bioassay-guided fractionation, but discarded this in favor of focusing on phlorotannin-containing fractions. They left other fractions uninvestigated, even though some appeared to deter herbivore feeding. Since this initial study, there have been scores of papers measuring phlorotannins, and often correlating these with herbivore feeding preferences (see review by Steinberg 1992; Van Alstyne et al. 2001). However, there are relatively few studies directly testing purified phlorotannins, partially purified phlorotannin-rich extracts, or equivalent crude extracts from which phlorotannins have been removed to determine their effects on herbivore feeding choices (e.g., Steinberg 1988; Steinberg and van Altena 1992; Steinberg et al. 1995; Pavia and Toth 2000). Thus, the roles of phlorotannins in seaweed-herbivore interactions have most often been studied correlatively, less often been investigated experimentally, and to our knowledge never before studied using bioassay-guided investigations that assay all extracts for deterrence rather than focusing primarily on phlorotannin-containing fractions. The latter approach allows for discovery of additional bioactive metabolites or for finding synergistic or additive interactions among compounds.

The recognition that phlorotannin-producing brown seaweeds can also contain polar, non-phenolic metabolites that deter herbivores adds to our understanding of the potential complexities of seaweed chemical defenses.

Both direct tests of how various phenolic-rich extracts affect herbivore feeding (Van Alstyne and Paul 1990; Steinberg and van Altena 1992; Boettcher and Targett 1993; Steinberg et al. 1995; Pavia and Toth 2000) and correlative tests of how herbivores choose among species of seaweeds that differ in phenolic content (Steinberg 1985; Denton et al. 1990; Denton and Chapman 1991; Steinberg et al. 1991; Targett et al. 1995) have produced variable results. Portions of this variance may be explained by some species and extracts containing other polar deterrents (such as the galactolipid, and possibly sulfated sugars) that confound assessments of the phlorotannins. The discovery of non-phenolic metabolites in *Fucus* that strongly deter feeding at low concentrations, suggests that one should be cautious about assuming that variance in phlorotannin levels is responsible for coincident variance in palatability.

Many ecologically important compounds may remain undiscovered in seaweeds that contain phlorotannins, or other known metabolites, if these species are studied using chemical investigations of known compounds rather than bioassay-guided discovery of ecologically active metabolites. Given the paucity of thorough, bioassay-guided investigations reported in the literature, it is presently impossible to assess whether situations like the one reported here are common or rare. However, if deterrent, but undetected, compounds are common in seaweeds, then these uninvestigated metabolites could confound the numerous investigations of seaweed chemical defenses that are based on correlative information alone.

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References

Appel HM, Govenor HI, D'Ascenzo M, Siska E, Schultz JC (2001) Limitations of Folin assays of foliar phenolics in ecological studies. J Chem Ecol 27:761–778

Boettcher AA, Targett NM (1993) Role of polyphenolic molecular size in reduction of assimilation efficiency in *Xiphister mucosus.* Ecology 74:891–903

Bolser RC, Hay ME (1996) Are tropical plants better defended? Palatability and defenses of temperate vs. tropical seaweeds. Ecology 77:2269–2286

Bryant JP, Chapin FS, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40:357–368

Clausen TP, Provenza FD, Burritt EA, Reichardt PB, Bryant JP (1990) Ecological implications of condensed tannin structure: a case study. J Chem Ecol 16:2381–2392

Coley PD, Bryant JP, Chapin FS (1985) Resource availability and plant antiherbivore defense. Science 230:895–899

Cronin G, Hay ME (1996) Susceptibility to herbivores depends on recent history of both the plant and animal. Ecology 77:1531–1543

Deal MS (1997) The causes and consequences of within-species variation in seaweed chemical defenses. PhD dissertation. University of North Carolina at Chapel Hill, Chapel Hill, N.C.

Denton AB, Chapman ARO (1991) Feeding preferences of gammarid amphipods among four species of *Fucus.* Mar Biol 109:503–506

Denton AB, Chapman ARO, Markham J (1990) Size-specific concentrations of phlorotannins (anti-herbivore compounds) in three species of *Fucus*. Mar Ecol Prog Ser 65:103–104

Duffy JE, Hay ME (1994) Herbivore resistance to seaweed chemical defense: the roles of herbivore mobility and predation risk. Ecology 75:1304–1319

Duffy JE, Hay ME (2001) Ecology and evolution of marine consumer-prey interactions. In: Bertness M, Hay ME, Gaines SD (eds) Marine community ecology. Sinauer, Sunderland, Mass., pp 131–157

Duffy JE, Paul VJ (1992) Prey nutritional quality and the effectiveness of chemical defenses against tropical reef fishes. Oecologia 90:333–339

Feeny P (1976) Plant apparency and chemical defense. Recent Adv Phytochem 10:1–40

Fritz RS, Simms EL (eds) (1992) Plant resistance to herbivores and pathogens: ecology, evolution, and genetics. University of Chicago Press, Chicago, Ill.

Geiselman JA, McConnell OJ (1981) Polyphenolics in brown algae *Fucus vesiculosus* and *Ascophyllum nodosum*: chemical defenses against the marine herbivorous snail *Littorina littorea*. J Chem Ecol 7:1115–1133

Glombitza KW, Rosener HU, Koch M (1976) Polyhyudroxy-oligophenyle und phenylather aus *Bifurcaria bifurcata*. Phytochemistry 15:1279–1281

Hay ME, Fenical W (1988) Marine plant-herbivore interactions: the ecology of chemical defense. Annu Rev Ecol Syst 19:111–145

Hay ME, Lee RR, Guieb RA, Bennett MM (1986) Food preference and chemotaxis in the sea urchin *Arbacia punctulata*. J Exp Mar Biol Ecol 96:147–153

Hay ME, Duffy JE, Pfister CA, Fenical W (1987) Chemical defenses against different marine herbivores: are amphipods insect equivalents? Ecology 68:1567–1580

Hay ME, Renaud PE, Fenical W (1988) Large mobile versus small sedentary herbivores and their resistance to seaweed chemical defenses. Oecologia 75:246–252

Hay ME, Kappel QE, Fenical W (1994) Synergisms in plant defenses against herbivores: interactions of chemistry, calcification, and plant quality. Ecology 75:1714–1726

Hay ME, Stachowicz JJ, Cruz-Rivera E, Bullard S, Deal MS, Lindquist N (1998) Bioassays with marine and freshwater macroorganisms. In: Haynes KF, Millar JG (eds) Methods in chemical ecology, vol 2 (bioassay methods). Kluwer, Norwell, Mass., pp 39–141

Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. Q Rev Biol 67:283–335

Kitagawa I, Hayashi K, Kobayashi M (1989) Heterosigma-glycolipids I and II, the new galactolipids containing octadecatetraenoyl and eicosapentaenoyl residues from a raphidophyte dinoflagellate *Heterosigma* sp. Chem Pharm Bull (Tokyo) 37:849–851

Koch M, Glombitza KW, Eckhardt G (1980) Phlorotannins of Phaeophycea *Laminaria ochroleuca*. Phytochemistry 19:1821–1823

Martinez EA (1996) Micropopulation differentiation in phenol content and susceptibility to herbivory in the Chilean kelp *Lessonia nigrescens* (Phaeophyta, Laminariales). Hydrobiologica 326/327:205–211

McInnes AG, Ragan MA, Smith DG, Walter JA (1985) The high molecular weight polyphloroglucinols of the marine brown alga *Fucus vesiculosus* L. ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. Can J Chem 63:304–313

Metsui K, KurishitaS, Hisamitsu A, Kajiwars T (2000) A lipid-hydrolyzing activity involved in hexenal formation. Biochem Soc Trans 28:857–860

Ohlsson LA (2000) Digestion and absorption of galactolipids. In: Christophe AB, De Vriese S (eds) Fat digestion and absorption. AOCS, Champaign, Ill., pp 287–297

Paul VJ, Cruz-Rivera E, Thacker RW (2001) Chemical mediation of macroalgal-herbivore interactions: ecological and evolutionary perspectives. In: McClintock JB, Baker BJ (eds) Marine chemical ecology. CRC, Boca Raton, Fla.,, pp 227–265,

Pavia H, Toth GB (2000) Inducible chemical resistance to herbivory in the brown seaweed *Ascophyllum nodosum*. Ecology 81:3212–3225

Pavia H, Cervin G, Lindgren A, Aberg P (1997) Effects of UV-B radiation and simulated herbivory on phlorotannins in the brown alga *Ascophyllum nodosum*. Mar Ecol Prog Ser 157:139–146

Ragan MA (1985) The high molecular weight polyphloroglucinols of the marine brown alga *Fucus vesiculosus* L.: degradative analysis. Can J Chem 63:294–303

Ragan MA, Craigie JS (1976) Physodes and the phenolic compounds of brown algae. Isolation and characterization of phloroglucinol polymers from *Fucus vesiculosus* (Linnaeus) Can J Biochem 54:66–73

Ragan MA, Glombitza KW (1986) Phlorotannins, brown algal polyphenols. Prog Phycol Res 4:129–241

Rhoades D, Cates R (1976) Toward a general theory of plant antiherbivore chemistry. Rec Adv Phytochem 10:168–213

Rice WR, Gaines SD (1994) "Heads I win, tails you lose": testing directional alternative hypotheses in ecological and evolutionary research. Trends Ecol Evol 9:235–237

Rosenthal GA, Berenbaum MR (eds) (1992) Herbivores: their interactions with secondary plant metabolites. Academic Press, San Diego, Calif.

Sakata K (1989) Feeding attractants and stimulants for marine gastropods. Bioorg Mar Chem 3:115–127

Sawai Y, Fujita Y, Sakata K, Tamashiro E (1994) 20-Hydroxy-4,8,13,17-tetramethyl-4,8,12,16-eicosatetraenoic acid, a new feeding deterrent against herbivorous gastropods, from the subtropical brown alga *Turbinaria ornata*. Fish Sci 60:199–201

Schneider CW, Searles RB (1991) Seaweeds of the southeastern United States. Duke University Press, Durham

Schoenwaelder MEA, Clayton MN (1999) The presence of phenolic compounds in isolated cell walls of brown algae. Phycologia 38:161–166

Steinberg PD (1985) Feeding preferences of *Tegula funebralis* and chemical defenses of marine brown algae. Ecol Monogr 55:333–349

Steinberg PD (1988) The effects of quantitative and qualitative variation in phenolic compounds on feeding in three species of marine invertebrate herbivores. J Exp Mar Biol Ecol 120:221–237

Steinberg PD (1992) Geographical variation in the interaction between marine herbivores and brown algal secondary metabolites. In: Paul VJ (ed) Ecological roles of marine natural products. Comstock Press, New York, pp 51–92

Steinberg PD, van Altena I (1992) Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. Ecol Monogr 62:189–222

Steinberg PD, Edyvane K, de Nys R, Birdsey R, van Altena IA (1991) Lack of avoidance of phenolic-rich brown algae by tropical herbivorous fishes. Mar Biol 109:335–343

Steinberg PD, Estes JA, Winter FC (1995) Evolutiuonary consequences of food chain length in kelp forest communities. Proc Natl Acad Sci USA 92:8145–8148

Strong DR, Lawton JH, Southwood R (1984) Insects on plants: community patterns and mechanisms. Harvard University Press, Cambridge, Mass.

Targett NM, Arnold TM (1998) Predicting the effects of brown algal phlorotannins on marine herbivores in tropical and temperate oceans. J Phycol 34:195–205

Targett NM, Boettcher AA, Targett TE, Vrolijk NH (1995) Tropical marine herbivore assimilation of phenolic-rich plants. Oecologia 103:170–179

Van Alstyne KL (1988) Herbivore grazing increases polyphenolic defenses in the intertidal brown alga *Fucus distichus*. Ecology 69:655–663

Van Alstyne KL, Paul VJ (1990) The biogeography of polyphenolic compounds in marine macroalgae: temperate brown algal defenses deter feeding by tropical herbivorous fishes. Oecologia 84:158–163

Van Alstyne KL, Dethier MN, Duggins DO (2001) Spatial patterns in macroalgal chemical defenses. In: McClintock JB, Baker BJ (eds) Marine chemical ecology. CRC Press, Boca Raton, Fla., pp 301–324

Winter FC, Estes JA (1992) Experimental evidence for the effects of polyphenolic compounds from *Dictyoneurum californicum* Ruprecht (Phaeophyta: Laminariales) on feeding rate and growth in the red abalone *Haliotus rufescens* Swainson. J Exp Mar Biol Ecol 155:263–277

Yates JL, Peckol P (1993) Effects of nutrient availability and herbivory on polyphenolics in the seaweed *Fucus vesiculosus*. Ecology 74:1757–1766

Zucker WV (1983) Tannins: does structure determine function? An ecological perspective. Am Nat 120:335–365