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Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra*

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Abstract The localisation of halogenated furanones, the biologically active secondary metabolites from the red alga *Delisea pulchra* (Greville), was determined by a combination of fluorescence microscopy, culture studies and quantitative chemical analyses. All types of evidence showed that furanones are localised in the central vesicle of gland cells in *D. pulchra*. These cells release furanones onto the surface of the plant, where they can be quantified using a newly developed surface extraction-technique. Levels of furanones on the surface of the plant were highest near the apical tips ($\simeq$100 ng cm^{-2}), and decreased towards the base of the alga. Variation in furanone levels within the plant and variation in the number of gland cells followed a similar pattern. The localisation of furanones within gland cells in *D. pulchra* and the presence and concentrations of furanones on the surface of the plant are consistent with furanones functioning as antifoulants and in mediating other ecological interactions at the surface of the alga.

Introduction

Secondary metabolites play a major role in mediating ecological interactions for both terrestrial (Feeny 1992) and marine (Hay and Steinberg 1992; Hay 1996) plants. For marine plants such as benthic macroalgae (seaweeds), most evidence for the chemical mediation of ecological interactions comes from studies of plant/herbivore interactions (Hay and Fenical 1988; Hay and Steinberg 1992), where it is clear that algal secondary metabolites play an important role as defences against herbivory. The importance of algal secondary metabolites in other ecological interactions such as deterrence of epibiota (antifouling; De Nys et al. 1995; Schmitt et al. 1995) and bacteria (Kjelleberg et al. 1997), or inhibition of competitors (allelopathy; De Nys et al. 1991), is much less well known (reviewed by Hay 1996).

One important reason for the lack of understanding of some chemically-mediated interactions involving macroalgae is that we know very little about the presentation and the localisation of metabolites in or on seaweeds. Many marine herbivores – particularly ecologically important ones such as echinoids, fishes, and gastropods – consume macroscopic portions of algal thalli (in some cases the entire thallus). Therefore, they will contact metabolites, whether these are contained within the tissues of the alga or are on the surface of the thallus (although some smaller herbivores are capable of making finer scale distinctions: Poore 1994). Interactions such as allelopathy and antifouling, however, are mediated at or near the surface of the plant, and thus metabolites contained within the thallus may be irrelevant to the interaction. For these latter interactions, the placement and presentation of metabolites by the alga will be crucial.

Studies of terrestrial plants highlight the importance of understanding the localisation of secondary metabolites for interpreting their ecological role. Secondary metabolites in terrestrial plants are present in a variety of structures, including vacuoles (Wink 1997), idioblasts, glands, cavities and canals (McKey 1979; Juniper and Southpark 1986; Fahn 1988; Duke et al. 1994). These structures store and transport the compounds, protect the plant against autotoxicity (McKey 1979; Wink 1997) and, in some cases, present metabolites at the surface of the plant. Secondary metabolites on the surface of terrestrial plants have been quantified in a number of studies (Zobel and Brown 1988a; Eigenbrode et al. 1991), and their role as (for example) deterrents of herbivores (Chapman and Bernays 1989) or UV blockers (Zobel and

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Brown 1988b; Stapleton 1992) have been investigated. Not surprisingly, the ways in which secondary metabolites are localised in terrestrial plants strongly influences their ecological effects (Bernays and Chapman 1994), and also the ability of natural enemies such as herbivorous insects to overcome these defences (Dussourd and Denno 1991, 1994; Bernays and Chapman 1994).

Comparable data on the localisation of metabolites is largely lacking for marine algae, although algae also have to guard against autotoxicity and provide for storage of metabolites. In a few instances, secondary metabolites in marine algae are known to be sequestered within membrane-bound vesicles. For example, terpenoid metabolites in the red algal genus *Laurencia* occur in intracellular vesicles termed *corps en cerise* (Young et al. 1980). In the brown algae (Phaeophyta), polyphenolics (''phlorotannins'') are held in membrane-bound structures known as physodes (Ragan 1976), which can occur at the surface of the thallus and release the metabolites into the surrounding water. However, the details of localisation and presentation are unknown for most algal secondary metabolites. Without such information, it is difficult to develop a broader view of the possible ecological roles for these metabolites.

To further understand the ecological role of algal secondary metabolites and, in particular, whether metabolites have the potential to mediate interactions at or near the surface of an alga, we investigated localisation and presentation of secondary metabolites in the subtidal red alga *Delisea pulchra*. *D. pulchra* produces a range of structurally similar metabolites known as halogenated furanones (Kazlauskas et al. 1977; De Nys et al. 1993). These metabolites have a broad range of biological activities, including feeding deterrence of herbivores (De Nys et al. in preparation), inhibition of settlement of fouling organisms (De Nys et al. 1995; Maximilien et al. 1998), and interference with bacterial signal-mediated regulatory systems (Kjelleberg et al. 1997). De Nys et al. (1998) have also recently developed a method for the quantitative measurement of non-polar surface chemistry of algae using *D. pulchra* as one of the model species. Therefore *D. pulchra* and its metabolites provide an excellent opportunity for attempting to relate algal ultrastructure and quantitative chemistry to biological function. In this paper, we describe the localisation of furanones in *D. pulchra* using ultrastructural evidence, epifluorescence microscopy, analytical chemistry, and culture studies. We then relate ultrastructure to quantitative variation in metabolites in and on the alga, and discuss the implications of our results for chemical mediation of surface interactions by marine algae.

Materials and methods

Study organism

Delisea pulchra (Greville) Montagne (Bonnemaisoniales: Rhodophyta), is a sublittoral red alga common near Sydney, Australia. *D. pulchra* produces a range of halogenated furanones, of which four (Fig. 1) constitute >95% of the total amount of furanones in the alga (Kazlauskas 1977; De Nys et al. 1993, 1996b). All plants used in this study were collected between depths of 5 and 10 m from Cape Banks, Sydney (34°00′S; 151°14′E). Plants used for surface-chemistry measurements were transported in seawater on ice, and used immediately on arrival at the laboratory. Plants used for histology and for collection of tetraspores were kept for short periods in aquaria in a 30 000-litre recirculating seawater system.

Light microscopy

Light micrographs were taken of unfixed and unstained hand-cut sections which were washed and mounted in sterile filtered seawater (Millipore, 0.2 μm). Sections and whole-plant mounts were viewed on a Leitz Orthoplan microscope using bright-field optics. Photographs were taken with Kodak T64 colour-reversal film.

Spectrofluorometric analysis of furanones

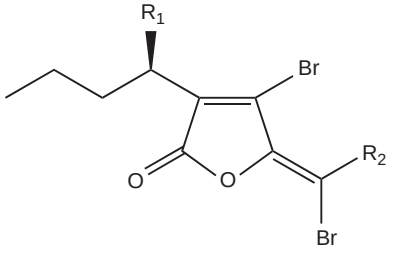
The presence of a series of conjugated double bonds in the structure of furanones causes these compounds to fluoresce when excited by light in the near-UV wavelength. To characterise the fluorescence of furanones (1 to 4 in Fig. 1), they were isolated and purified as described by De Nys et al. (1993), and the fluorescence excitation and emission spectra of each was measured in ethanol (99.7 to 100%) at a concentration of 100 μg ml^{-1}. Fluorescence of the compounds in solution was determined by measuring excitation and emission spectra in the near-UV wavelength range using a Perkin Elmer Luminescence LS 50B Spectrophotometer. To confirm the usefulness of autofluorescence in detecting the presence of furanones, Compounds 1 to 4 were also smeared onto glass slides and autofluorescence was observed using the microscope and filters described in the following subsection.

Fluorescence microscopy

The characteristic autofluorescence of furanones in near-UV light was used to localise these compounds in *Delisea pulchra*. Sections (10 to 20 μm) of fresh *D. pulchra* were cut by hand, washed, and mounted in sterile, filtered (0.20 μm) seawater (SFS) for examination of the localisation of furanones within the thallus. Observations were made using a Zeiss Axiophot microscope with a 360 to 395 nm long-pass filter, dichromic mirror at 395 nm, and band-pass filter of 420 nm. Photographs were taken using Kodak Elite II 400 ASA film.

Distribution of gland cells

Fluorescence microscopy indicated that furanones in *Delisea pulchra* were localised in the large central vesicle of specialised cells known as gland cells (Womersley 1994). Similar cells are also found in other members of the Bonnemaisoniales (Bonin and Hawkes 1988; Womersley 1994). To quantify the distribution of gland cells, the numbers at the surface of the plant were counted within a 100 × 100 μm grid on whole pieces of mounted *D. pulchra*. Gland



	R_1	R_2
Compound 1	H	Br
Compound 2	H	H
Compound 3	OAc	H
Compound 4	OH	H

Fig. 1 *Delisea pulchra*. Structure of four major secondary metabolites analysed by gas chromatography–mass spectrometry

cells were quantified on portions of the thallus (distal, middle and basal thirds of the plant) corresponding to those used for measurements of secondary chemistry. Gland cells were counted from ten haphazardly selected fields from five replicate plants for each of the regions on each plant.

Culture studies

To determine when gland cells appear in the development of the alga, *Delisea pulchra* was cultured from tetraspores in the laboratory. Additionally, cultures studies were used to confirm the presence of furanones in the gland cells. This was done by culturing some thalli in media containing bromide and some in media lacking bromide. Although bromide is not considered to be essential for the growth of red algae (Fries 1966), all furanones in *D. pulchra* contain bromine. Thus, we hypothesised that the absence of bromide would lead to changes in the concentration of furanones in the gland cells and potentially cause changes in the cells themselves without otherwise affecting the plant. Similar manipulation of bromide in culture studies with other members of the Bonnemaisoniales has caused changes to gland cells, and in particular to the large refractile vesicles within these cells (Wolk 1968).

Tetraspores for cultures were obtained from fertile plants of *Delisea pulchra* collected in the field. Fertile axes were excised, steeped in a 1% betadine solution (10% provadine–iodine solution, Faulding Pharmaceuticals, Salisbury, South Australia) diluted 1:10 with SFS for 5 min to kill epiphytes (Xue-wu and Gordon 1987), and washed three times in SFS. The axes were placed in dishes containing SFS and 13 mm-diam, round, cover slips; they were then aerated, and tetraspores were allowed to settle overnight on the coverslips. The following day, the coverslips were examined microscopically for attached tetraspores. Coverslips with attached tetraspores were transferred to 9 cm-diam petri dishes containing 5 ml of either bromide (+) or bromide (−) culture media.

Bromide (−) media consisted of Provasoli's basal salt mixture (Provasoli 1964) enriched with Provasoli's enriched seawater (PES; Provasoli 1968) and germanium oxide (500 μg l^{-1}). Bromide (+) media were the same except for the addition of sufficient NaBr to attain the concentration of bromide ions typical of seawater ($\simeq$65 mg l^{-1}; Burton 1996). The ionic concentration in the bromide (−) media was equilibrated to the bromide (+) media by the addition of NaCl.

The tetraspores were cultured at 18 °C in a 16 h light:8 h dark cycle with media changes every 5 d. Sporelings were examined weekly for the production of gland cells. After 4 wk, the number (density) and size of the gland cells and the size of the refractile vesicle within the gland cells were measured for five plants each from bromide (+) and bromide (−) media. Gland cells in plants grown in Br (+) vs Br (−) media were observed using epifluorescence microscopy.

To further test whether the absence of bromide inhibited the production of furanones, the levels of furanones in these cultured plants were quantified using gas chromatography–mass spectrometry (GC–MS). After 6 wk of growth, the cover slips containing plants from both the bromide (+) (n = 3) and bromide (−) (n = 3) media were air-dried in a desiccator. The plants from each cover slip were scraped off, weighed, and placed into 100 μl glass inserts for GC–MS vials. The plants were extracted by the addition of 10 μl ethyl acetate (EtOAc) containing naphthalene (10 μg ml^{-1}) to the insert, and were mechanically mixed three times for 1 min over a 12 h period. The extracts were directly injected manually into the gas chromatograph–mass spectrometer and analysed as described in the following subsection.

Gas chromatography–mass spectometry

Quantification of furanones by GC–MS followed the method of De Nys et al. (1996a). Gas chromatography was performed with a Hewlett Packard (HP) 5980 Series II gas chromatograph (GC) and a polyamide-coated fused-silica capillary column (BP1, 12 m long, 0.22 mm i.d., SGE Pty Ltd). All injections were performed in the splitless mode with an inlet pressure of 5 psi. The injection port was held at 250 °C and the interface at 300 °C. The GC was held at 50 °C for 1.5 min and ramped at 20 °C min^{-1} to 300 °C, where it was held for 10 min. Helium was used as the carrier gas. Mass spectrometry was performed using an HP5972 mass-selective detector (MSD). Ions characteristic of the internal standard and Furanones 1 to 4 were monitored in the selective-ion monitoring mode. Standards used in the GC–MS analysis were isolated from dichloromethane extract of freeze-dried *Delisea pulchra*. Metabolites were identified by comparison of ^{1}H and ^{13}C NMR data (De Nys et al. 1993). Compounds were quantified by measuring the peak areas for each compound and the internal standard. The ratio of peak areas (compound:internal standard) was calculated for each metabolite and converted to concentration by reference to standard curves.

Quantification of furanones on the surface and within *Delisea pulchra*

We found gland cells on the surface of *Delisea pulchra*, and it is also known that furanones are present on the surface of the alga (De Nys et al. 1998). To determine whether and at what concentrations furanones were released onto the plant surface, and whether intraplant variation in surface furanones paralleled variation in the distribution of gland cells, furanones were extracted and quantified from the surface of the thallus using the methods of De Nys et al. (1998). This was done by splitting the alga into three parts, corresponding to the distal (containing the apical meristem), middle, and basal thirds of the plant. Ten replicate plants were used (n = 10).

Plants used for surface-chemistry measurements were first dried in a salad spinner; then, for each plant, a piece of tissue weighing $\simeq$2 g was removed from the distal, middle and basal regions. Each piece of tissue was then placed in 15 ml of hexane and agitated on a Vortex mixer for 20 s (De Nys et al. 1998); the tissue was then removed, and the hexane was allowed to evaporate. The resulting crude extract was redissolved in 150 μl of EtOAc containing naphthalene as an internal standard (10 μg ml^{-1}) for quantitative analysis by GC–MS.

To compare furanones on the surface with those inside the thallus, each piece of tissue was freeze-dried immediately following the extraction of surface furanones, and exhaustively extracted four times in dichloromethane (De Nys et al. 1996b). Extracts for each piece were pooled, filtered (PTFE 0.5 μm) and air-dried. The resulting crude extract was then dissolved in 2 ml EtOAc, containing naphthalene as an internal standard (10 μg ml^{-1}), for quantitative GC–MS analysis.

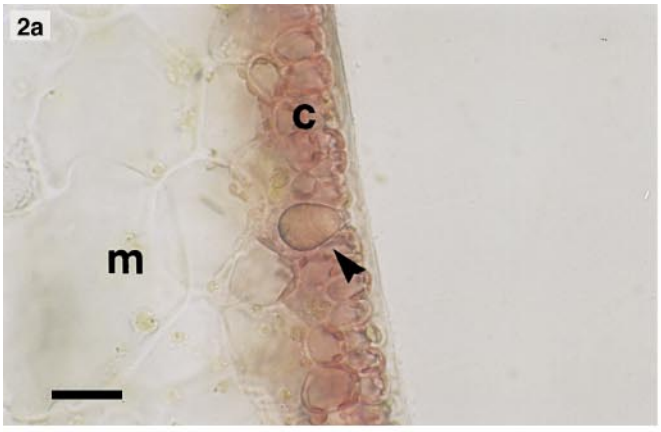
Statistical analyses

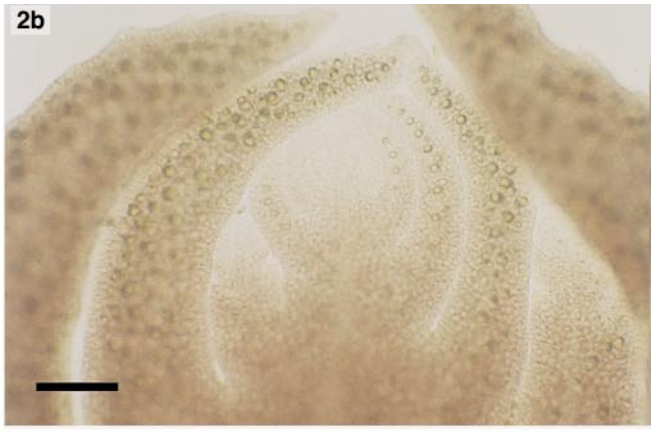
The distribution of gland cells and the levels of metabolites were analysed using analysis of variance (ANOVA) followed by Tukey's multiple-range test where appropriate. Homogeneity of variances was tested with Cochran's test; all variances were homogeneous after log-transformation of the data. As the variation of metabolites and gland cells between parts of an individual plant may not be independent, a blocked design was used to partition the effect of variation among plants from the variation between plant parts. In the two-factor analyses presented in Table 1, the factor "plant" was random and blocked and the factor "position" was fixed. For the analyses that compared individual metabolites the fixed factor "compound" was added to this design.

Results

Light microscopy

The general anatomy of *Delisea pulchra* was as described previously (Bonin and Hawkes 1988; Womersley 1994). *D. pulchra* has a uniaxial construction, in which the axial


2a
c
m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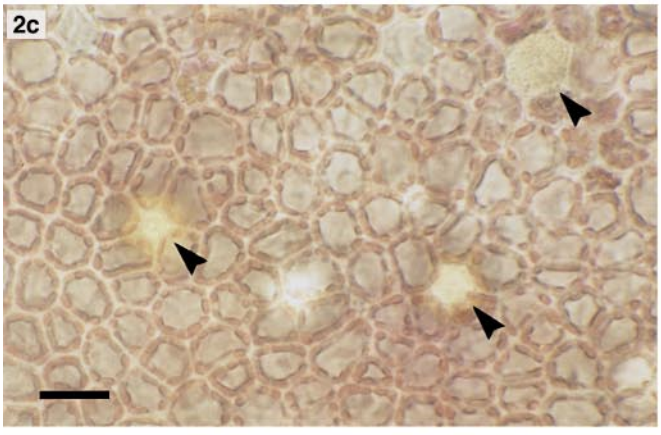
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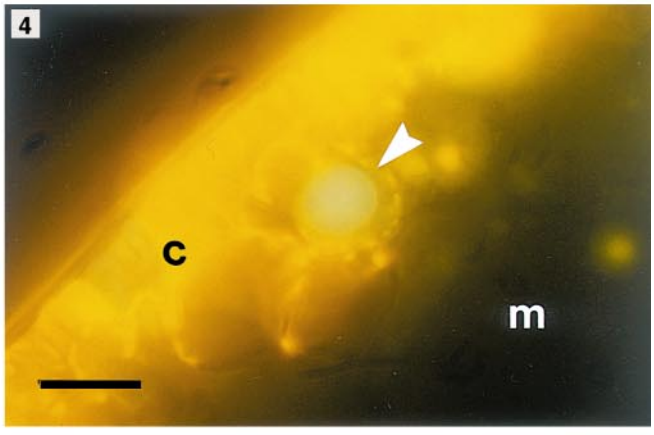
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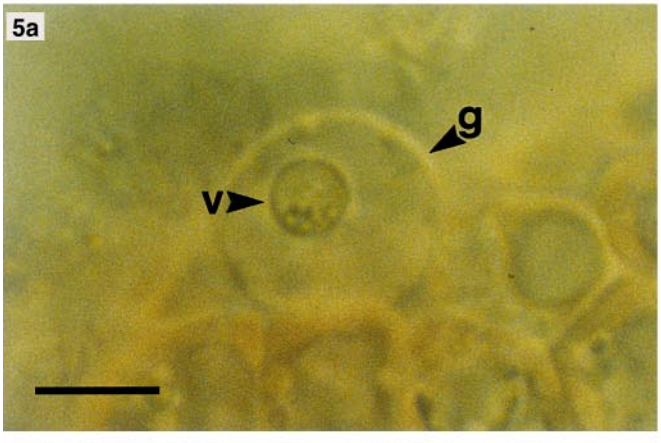
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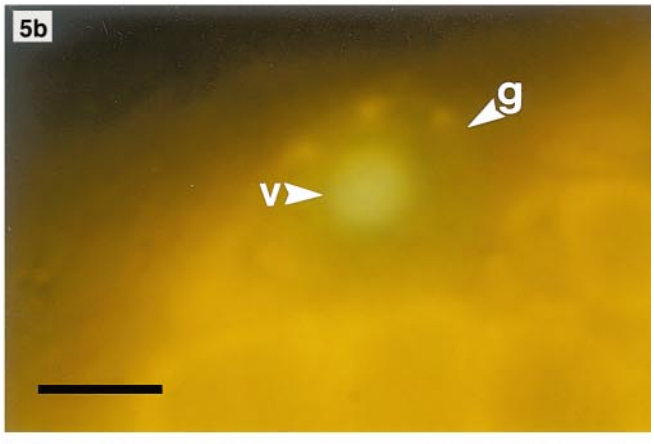
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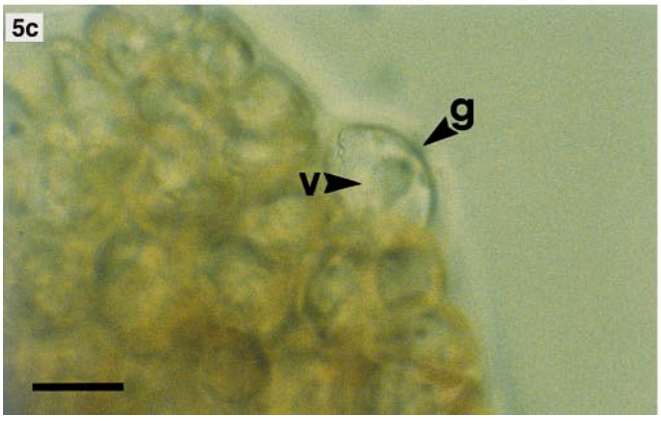
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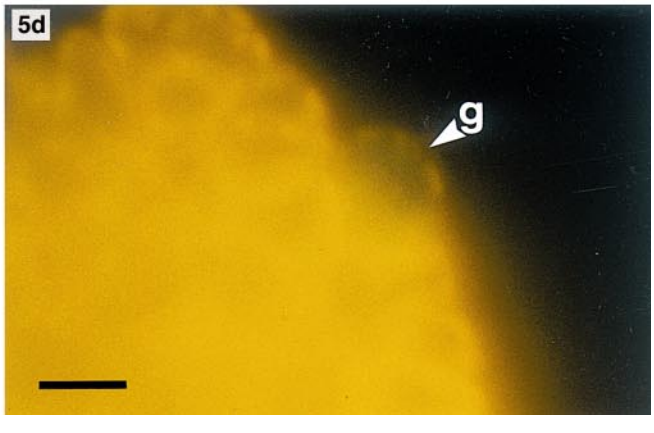
5d
g

Table 1 *Delisea pulchra*. Two-factor ANOVA for log-transformed densities of surface gland cells, concentrations of surface metabolites (*Surface furanones*) and concentrations of total metabolites (*Internal furanones*) within plant tip, middle and basal regions (*position*) (*blocked* = plant; *MS* mean square)

Factor	(*df*)	MS	*F*	*p*
Gland cells				
blocked	(4)	27.777	7.111	0.000
position	(5)	611.468	156.531	0.000
error	(140)	3.906		
Surface furanones				
blocked	(9)	0.696	4.867	0.002
position	(2)	1.645	11.509	0.001
error	(18)	0.143		
Internal furanones				
blocked	(9)	0.019	2.012	0.099
position	(2)	0.439	46.86	0.000
error	(18)	0.009		

cells are ringed by smaller colourless cells, and then by 3 to 9 larger (40 to 60 μm) colourless medullary cells towards the outside of these smaller cells. There were two layers of pigmented cortical cells (3 to 5 μm) on the surface (Fig. 2a). Gland cells (10 μm) were abundant among the pigmented cortical cells (Fig. 2a), and occurred on the surface of all sections of the plant, including the apical tips (Fig. 2b). They were characterised by their lack of chloroplasts at maturity and by a large central vesicle containing a yellow to brown light-refractive substance. Gland cells were visible emergent on the surface of the thallus in transverse sections (Fig. 2a). In surface views, they were visible surrounded by a rosette of cortical cells (Fig. 2c).

Fluorescence microscopy

The four furanones investigated (Compounds 1 to 4: Fig. 1) all had similar excitation and emission spectra (Fig. 3). The excitation peak when irradiated under UV (360 to 410 nm) was at 387 nm. Peak emission when excited at this wavelength was 435 nm, a white-blue colour (Fig. 4). White-blue light was observed when furanones were coated onto glass slides and observed under the epifluoresence microscope. Only the large central vesicle of the gland cells reacted similarly (Fig. 4; see also Fig. 5b).

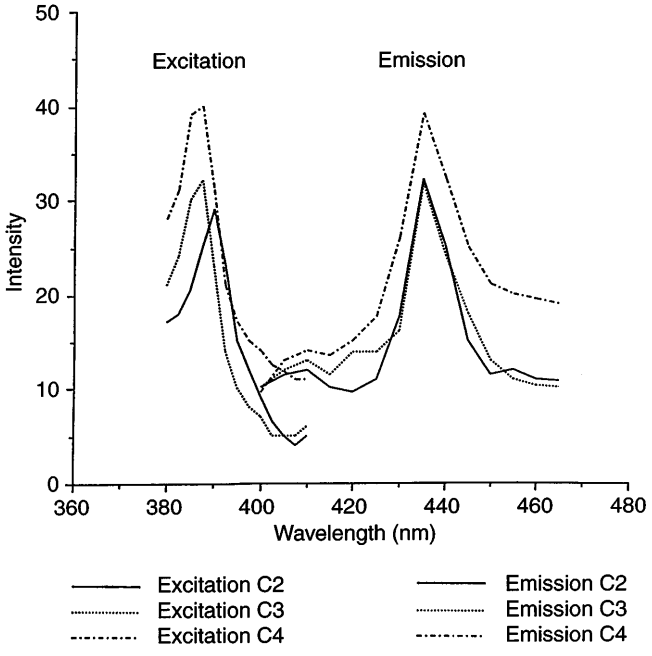

Fig. 3 *Delisea pulchra*. Excitation and emission spectra of three most common brominated furanones. Peak emission (435 nm) for compounds corresponds to white-blue colour (*C* compound)

Culture studies

Gland cells were visible in *Delisea pulchra* cultured from tetraspores after a week of development when the plants were < 0.1 mm in length, indicating that these structures (and the furanones within) appear very early in the development of this alga.

Sporelings cultured in media with bromide vs those grown in media lacking bromide had the same morphology. Gland cells developed in plants from both culture media, and there was no difference in the diameter [mean ± SE for Br (+) = 9.74 ± 0.12 μm, for Br (−) = 10.12 ± 0.24 μm, ANOVA $p = 0.24$] or numbers 56 cells mm^{-2} [61 cells mm^{-2} for Br (+), and 56 cells mm^{-2} for Br (−), ANOVA $p = 0.72$] of gland cells between the two treatments.

However, the size of the large central vesicle within the gland cells of plants grown in Br (−) media (mean diam ± SE, 3.16 ± 0.07 μm) was approximately one-half that of vesicles in plants grown in Br (+) media (6.16 ± 0.12 μm, ANOVA, $p < 0.001$). When observed under epifluorescence microscopy, the vesicle in the gland cells of plants cultured in the Br (−) media did not fluoresce, while those cultured in Br (+) media fluo-

Fig. 2 *Delisea pulchra*. Light micrographs **a** Non-stained, transverse section showing medullary and cortical cells (*arrowhead* indicates gland cells, note absence of chloroplasts in these cells and central inclusion which contains yellowish substance; *c* cortex; *m* medulla); **b** apical tip showing high density of gland cells in growing region of plant; **c** non-stained surface view (*arrowheads* indicate gland cells at surface of alga surrounded by rosette of outer cortical cells). (Scale bars = 10 μm in **a** and **c**, 100 μm in **b**)

Fig. 4 *Delisea pulchra*. Fluorescence micrograph of non-stained transverse section. Autofluorescence characteristic of furanones visible as white-blue, and is apparent in central vesicle of gland cell (*arrowed*) (*c* cortex; *m* medulla). (Scale bar = 10 μm)

Fig. 5 *Delisea pulchra*. Light (**a**, **c**) and fluorescent (**b**, **d**) micrographs of plants grown in bromide (+) and bromide (−) media. **a** Surface of plants producing furanones [bromide (+) medial, showing gland cell (*g*) and its central vesicle (*v*); **b** same plant as in **a**, showing gland cell and fluorescence caused by furanones in central vesicle of the gland cell; **c** surface of plants inhibited from producing furanones [bromide (−) media], showing gland cell and its central vesicle; **d** fluorescence micrograph of the same plant as in **c**, showing gland cell and lack of fluorescence characteristic of furanones in central vesicle of the gland cell. (Scale bars = 5 μm)

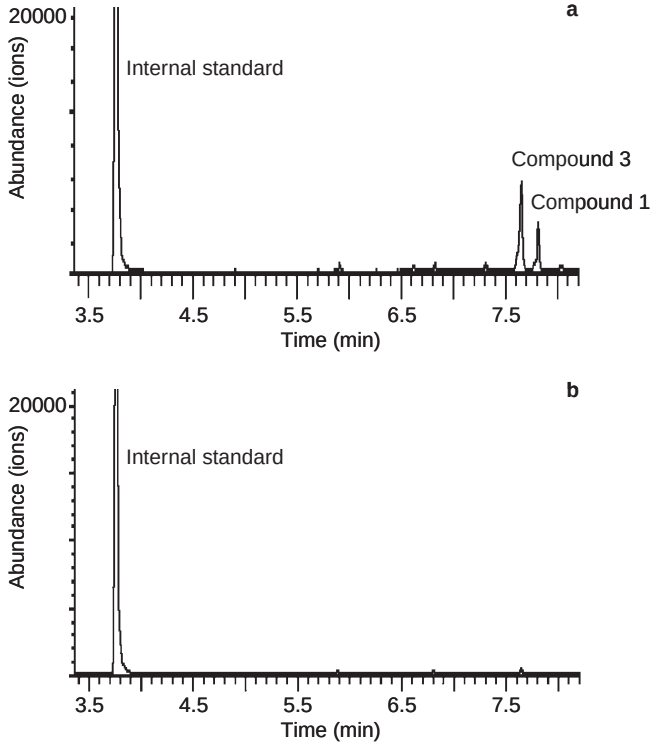

Fig. 6 *Delisea pulchra*. Representative gas-chromatography traces of extracts grown in a medium containing bromide (**a**) and in a medium lacking bromide (**b**)

resced as described above, in second subsection of "Results" (Fig. 5). When plants grown in Br (−) media were analysed by GC–MS, no furanones were detected (Fig. 6b). Plants grown in Br (+) media contained furanones, with Compound 3 the most abundant (104.5 ± 0.2 ng mg^{-1} dry wt), and Compounds 1, 2 and 4 also detectable but at lower levels (Fig. 6a).

Quantification of gland cells and furanones

The density of gland cells on or near the surface of *Delisea pulchra*, the levels of furanones on the surface, and the total amounts of furanones in the thallus were all highest in the tip of the plant (Table 1; Fig. 7). The most dramatic change in the density of gland cells occurred in the top ≃5 mm of the plant (Fig. 2b). Total concentrations of Furanones 1 to 4 on the surface were significantly higher on the tip than the middle and base [ANOVA (Table 1) followed by Tukey's HSD test, $\alpha = 0.05$ (not tabulated)]. Mean total concentrations were 89 ng cm^{-2} on the tip, 38 ng cm^{-2} on the mid region of the plant and 40 ng cm^{-2} on the base. Compounds 3 and 4 were the most abundant metabolites,

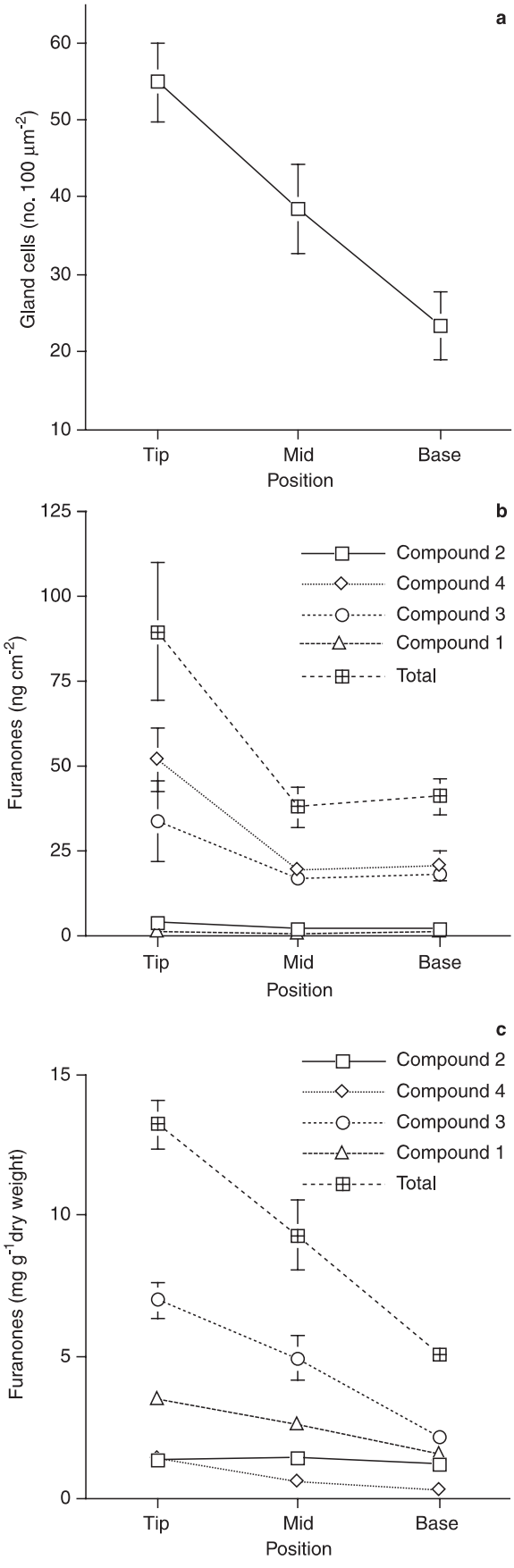

Fig. 7 *Delisea pulchra*. **a** Distribution of gland cells at surface of plant; **b** furanones on surface of plant; **c** furanones within plant. All values are means ±1 SE ($n = 5$ for **a**; $n = 10$ for **b** and **c**)

with mean surface concentrations of 33 and 51 ng cm^{-2} and at the tip of the plant, respectively, decreasing to 18 and 20 ng cm^{-2} at the base. Both compounds were present in significantly higher concentrations than Compounds 1 and 2 in all portions of the plant [three-factor ANOVA (not tabulated) followed by Tukey's HSD test, $\alpha = 0.05$; Fig. 7b].

The concentrations of total furanones within the plants also significantly decreased from the tip (13.2 μg mg^{-1} dry wt) to the base of the plant (5 μg mg^{-1} dry wt) (Table 1, Fig. 7c). Compound 3 comprised >50% of the total concentration at the tip, and was the most abundant metabolite at all positions on the plant [three-factor ANOVA (not tabulated) followed by Tukey's HSD test, $\alpha = 0.05$; Fig. 7c].

Relationship between furanones on plant surface and within plant

To assess the relationship between the concentration of furanones on the surface and within plants, surface and internal concentrations in sections of the thallus were regressed against each other. There was a significant ($p = 0.042$) relationship between total levels of furanones on the surface of the plant and total levels within the plant, but only 10.9% of variance in the surface concentration was explained by within-plant levels of furanones (Table 2). When individual metabolites were analysed separately, only Compound 2 showed a significant relationship between levels on the surface and within the plant, with whole-plant chemistry explaining 34.5% of the variance in surface chemistry (Table 2). There was no relationship between surface and whole-plant levels for Compounds 1, 3 and 4 (Table 2). The absolute amounts of metabolites extracted from the surface of the plant indicated that surface-bound metabolites represented only a small fraction of the total metabolite load of the plant. An average of 0.28% of total extracted furanones from the tip of the plant were on the surface, with surface furanones contributing 0.23 and 0.40% of the metabolite load in the mid and basal regions, respectively.

Discussion

To understand the ecological role of secondary metabolites in marine organisms, we need to understand how compounds are (a) localised within the organisms, and (b) the consequences of this localisation. Using a variety of techniques, we have shown that furanones in the red alga *Delisea pulchra* are contained within specialised cells known as gland cells, similar to those described for other members of the Bonnemaisoniales (Wolk 1968; Womersley 1994). These cells occur both within the thallus and at the surface of the alga. Their presence at the surface results in the release of furanones onto the surface of the alga, presumably through lysis of the cells. Quantification of furanones (also see De Nys et al. 1998) showed that the quantitative distribution of gland cells co-varied with levels of furanones on the surface, with gland cells and furanones decreasing in number or amount from the apical tip to the base of the thallus. However, even with this decrease in concentration towards the base, furanones on the surface of all sections still occurred in concentrations that deter ecologically relevant epibiota (De Nys et al. 1995; Maximilien et al. 1998).

Table 2 *Delisea pulchra*. Log of surface furanones regressed against log of internal furanones for each of Furanones 1 to 4 and total metabolites (* significant at $\alpha < 0.05$)

Compound	R^2	F	p
1	0.075	2.783	0.11
2	0.340	15.960	<0.001*
3	0.000	0.697	0.411
4	0.074	0.3.320	0.079
Total	10.9	4.546	0.042*

While the quantitative distribution of gland cells and surface furanones were clearly correlated, the relationship between levels of metabolites in the whole plant and those on the surface was less strong. Variation in the amounts of Compound 2 on the surface of the plant was significantly and positively related to variation in the whole thallus, as were concentrations of all compounds summed together, but there was no correlation between surface concentrations and whole-thallus concentrations for Compounds 1, 3 and 4. In particular, Compound 4 was relatively more, and Compound 3 relatively less abundant on the surface than in the thallus. These differences may result from differences in the contents of gland cells near the surface vs those more interior to the plant, from interconversion of some metabolites at the surface of the plant, or from differential diffusion of different metabolites from the surface of the plant.

Although obviously important for understanding the ecological roles of algal secondary metabolites, studies on the localisation of secondary metabolites in marine algae are relatively rare. Perhaps the best-studied metabolites in this regard are the brown algal phlorotannins, which are contained within vesicles known as physodes (Ragan 1976; Ragan and Glombitza 1986) and are ubiquitous among the brown algae. Because phlorotannins stain readily and distinctly in histological sections, the distribution of physodes (and phlorotannins) within brown algae, their movement during ontogeny (Clayton and Ashburner 1994), and their ability to lyse and release their contents to the outside environment have all been studied (reviewed by Ragan and Glombitza 1986). Some brown algae, particularly members of the Dictyotales (Hay and Fenical 1988), also produce terpenoid or other non-polar metabolites. However, it is not known whether these metabolites are also contained within physodes.

Among other taxa of algae, terpenoid metabolites in the red algal genus *Laurencia* are contained within subcellular vesicles known as *corps en cerise* (Young et al. 1980). These vesicles have been observed only in the in-

terior of cells, and consequently do not appear to release their contents to the surface of the thallus except when damaged. Concentrations of terpenoid metabolites on the surface of undamaged *Laurencia obtusa*, measured using the same techniques as used here for *Delisea pulchra*, were at most only several nanograms per cm^2 (De Nys et al. 1998). Wolk (1968) has shown that the central inclusion of gland cells in *Asparagopsis armata* and *Bonnemaisonia nootkana* (which both produce brominated secondary metabolites: McConnell and Fenical 1977, 1980) atrophied in the absence of bromide in the culture media. Although the form of bromine in gland cells of *B. nootkana* was not known (Wolk 1968), this morphological change would appear analogous to the shrinkage of the central inclusion in gland cells of *D. pulchra* observed when furanone production was inhibited.

In part, methodological difficulties have hindered investigations of the localisation of metabolites in algae. Some histological techniques cause artefacts in the localisation structures or confound measurements of the compounds of interest. For example, Borowitztka and Pallaghy (*in* Pallaghy et al. 1983) were unable to localise furanones in *Delisea pulchra*, most probably because the fixation techniques used would have extracted these non-polar metabolites from the vesicles during the fixation process. In contrast, the use of epifluorescence microscopy in the present study enabled us to detect the presence of furanones in the plant without disrupting cells. This technique has been extensively applied in terrestrial plants, especially for the localisation of flavonoids and coumarins (Schnabl et al. 1986; Zobel and March 1993), and for detecting polyphenolics in brown algae (Clayton and Ashburner 1994). There is further scope for its use in studying the localisation of non-polar metabolites in other marine algae.

Following Wolk (1968), we also grew *Delisea pulchra* in bromide-free media in order to investigate the effects of bromide on vesicle formation in gland cells and the production of furanones. Given the abundance of brominated secondary metabolites in the red algae (Rhodophyta) in particular, and the apparent lack of a role for bromide in red algal primary metabolism (Fries 1966), growing plants in bromide-free media would also appear to be a useful tool for investigating localisation of secondary metabolites in these algae. Like Wolk's findings for *Asparagopsis armata* and *Bonnemaisonia nootkana*, we found that *D. pulchra* produced gland cells with significantly smaller central vesicles in bromide-free media and produced no detectable furanones in the absence of bromide. Thus, bromide appears to be essential for the production of furanones, but not gland cells. This is important, since it suggests that plants grown in the absence of bromide are morphologically normal. This allows us to manipulate secondary metabolites per se while leaving their storage structures intact.

Localisation of metabolites in benthic marine organisms other than macroalgae is also poorly understood. In sponges, the secondary metabolites aerothionin and homoaerothionin occur in the spherulous cells of *Aplysina fistularis* (Thompson et al. 1984), and avarol is localised to choanocytes in *Dysidea avara* (Uriz et al. 1996). The difference in sites for the storage of these secondary metabolites has been interpreted as indicating different roles for these chemicals. Secondary metabolites contained within spherulous cells have been implicated in surface-mediated ecological interactions, as this cell type is known to lyse on the surface of the sponge (Thompson et al. 1984). Such a function for the metabolites in the choanocytes is unlikely, as these cells are found within the sponge (Uriz et al. 1996). In tunicates, vanadium and sulphuric acid have been localised in cells called vanadocytes. Vanadocytes, like gland cells in *Delisea pulchra* and spherulous cells in *A. fistularis*, are believed to lyse on the surface of the organism, releasing metabolites which can inhibit the settlement of fouling organisms (Stoecker 1978; but see Davis et al. 1989).

The localisation of furanones in and on *Delisea pulchra*, the levels of these metabolites found on the surface of the alga, and their activity at ecologically realistic concentrations against a variety of epibiota (De Nys et al. 1995; Maximilien et al. 1998; Steinberg et al. 1998) are all consistent with a role for these compounds as deterrents of epiphytes on this alga. For example the concentration of total furanones found on the surface of *D. pulchra* here was $\simeq$100 ng cm^{-2} (it can reach $>$500 ng cm^{-2}; Dworjanyn et al. unpublished data). Furanones or crude extracts of *D. pulchra* (containing $\simeq$50% furanones) inhibit attachment of bacteria in the laboratory and in the field at concentrations of $\leq$100 ng cm^{-2} (Maximilien et al. 1998). The settlement and development of spores from *Ulva* sp., an ecologically relevant fouling alga, is significantly deterred by furanones at concentrations as low as 25 ng cm^{-2} (De Nys et al. 1995). Other examples where concentrations of inhibitory metabolites on or near the surface of marine organisms have been measured and then tested against relevant epibiota are rare. Walker et al. (1985) demonstrated that the sponge *Aplysina fistularis* exuded the secondary metabolites aerothionin and homoaerothionin into the water column at a rate of several $\mu g\ h^{-1}$ $sponge^{-1}$; these metabolites are toxic to some invertebrate larvae at 10 $\mu g\ ml^{-1}$. Jennings and Steinberg (1997) measured in situ concentrations of exuded phlorotannins near the surface of *Ecklonia radiata*, and concluded that levels were too low to have strong effects on epibiota. Schmitt et al. (1995), while not quantifying metabolites on the surface of *Dictyota menstrualis* directly, showed that compounds found in surface extracts from this alga were strongly inhibitory against the epibiotic bryozoan *Bugula neretina*.

In contrast to these examples, many other algal (or invertebrate) metabolites may be ineffective as inhibitors of fouling if localisation of the metabolites is such that they do not normally reach the surface of the organism. For example, while terpenoids from *Laurencia* spp. inhibit settlement of epibiota in laboratory assays (De Nys et al. 1996a; Steinberg et al. 1998), these compounds are unlikely to reach the plant surface, as they are contained

within intracellular *corps en cerise*. Concentrations of sesquiterpenes on the surface of *Laurencia obtusa* (De Nys et al. 1998) are 2 to 3 orders of magnitude below concentrations that deter settlement of common epibiota (Steinberg et al. 1998; De Nys in preparation). Thus, an understanding of the localisation of metabolites in an organism should give a clear indication as to the potential for those metabolites to be used in surface-mediated interactions. Metabolites which are contained in structures which do not release them to the exterior of the organism except when damaged may have other functions, e.g. as a wounding response, as a defence against herbivores, etc.

At an even smaller scale, an understanding of the localisation of metabolites is necessary in order to understand how metabolites function against bacterial epiphytes. As Hay (1996) has recently pointed out, the effects of marine secondary metabolites on mediation of interactions involving bacteria is one of the great unknowns of marine chemical ecology. While we quantified furanones on the surface of *Delisea pulchra*, we do not know how these surface concentrations vary, at the scale relevant to bacteria, e.g. microns. Depending on (a) the distribution of gland cells on the surface, and (b) the spread of furanones across the surface after release, there may be "furanone free" pockets on the surface of the alga where bacterial colonisation is not affected by these metabolites. While technically difficult, quantification of metabolites on these small scales is necessary if we are to understand the real ecological role(s) of these compounds.

Finally, an understanding of the localisation of secondary metabolites is central to understanding another topical aspect in chemical ecology – the cost of production of secondary metabolites. In this regard, the culture methods used here, whereby plants can be produced which are apparently normal except in their ability to produce secondary metabolites, may be useful for testing a variety of ecological and evolutionary hypotheses regarding the cost of production of secondary metabolites. "Cost", the negative consequences to other plant functions incurred by producing defensive chemicals, is a major and recurring theme in the plant/herbivore literature (Fritz and Simms 1992). Theories of cost predict that the production of secondary metabolites should be inversely related to growth of the plant (although there are more complex versions of these models which predict non-monotonic relationships, e.g. Herms and Mattson 1992). There are at least two reasons for this: (a) allocation of carbon or other materials to growth vs secondary metabolites is mutually exclusive, and (b) production of metabolites in new, rapidly growing and developing (apical) tissues is constrained because of autotoxicity or other reasons specific to such cells (Cronin and Hay 1996). Evidence for (a) for algae is mixed (Hay and Steinberg 1992; Steinberg 1995; Hay 1996), and it now seems that apical cells or other rapidly developing tissue in algae are also not necessarily constrained in their production of secondary metabolites. For example, gland cells (and consequently furanones) in *Delisea pulchra* are produced in the greatest number at the apical tip (Figs. 2b, 7a). Physodes and the phlorotannins within are found in zygotes and dividing cells of *Durvillaea potatorum* (Clayton and Ashburner 1994), and are most heavily concentrated in apical tips of *Zonaria angustata* (Poore 1994). Thus, chemical defences in rapidly growing tissues need not be transported to these tissues (Cronin and Hay 1996), but can be produced in situ. More generally, these data, for taxonomically very different algae [see also Lindquist and Hay (1995) for chemical defences of invertebrate larvae] and structurally very different metabolites indicate that rapidly growing and developing tissues are not necessarily constrained in their ability to produce effective levels of chemical defences.

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