Mar Biol (2007) 150:967–975
DOI 10.1007/s00227-006-0410-5

RESEARCH ARTICLE

Localization of granulatimide alkaloids in the tissues of the ascidian *Didemnum granulatum*

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Received: 12 September 2005 / Accepted: 20 June 2006 / Published online: 21 July 2006

Abstract Ascidians are a notable source of nitrogen-bearing secondary metabolites with a wide range of biological activities. Although many biologically active compounds have been isolated from ascidians, it is often unclear whether the animal or associated microbial symbionts such as bacteria or fungi are the true biosynthetic source of the metabolites. We have addressed the question of the biosynthetic source of the alkaloids granulatimide and isogranulatimides by localizing these compounds within the ascidian. In this work, we demonstrate that granulatimide is stored in *Didemnum granulatum* tunic bladder cells. Analysis by confocal fluorescence microscopy at the granulatimide emission range indicated the presence of fluorescent cells as highly vacuolated cells found dispersed in the ascidian tunic. Since this is the most exposed ascidian tissue, it is possible that these alkaloids may have a protective role, either as sunscreens and/or as feeding deterrents.

Communicated by M. Kühl, Helsingør

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Introduction

Ascidians are a notable source of nitrogen-bearing secondary metabolites, primarily as alkaloids and modified peptides (Faulkner 2002; Blunt et al. 2005). Such compounds often exhibit an array of biological activities, including cytotoxicity, antibiotic, and immunosuppressive activities, and inhibition of topoisomerases and cyclin kinases (Faulkner et al. 2004). Many biologically active compounds have been isolated from ascidians in the family Didemnidae such as the potent cytotoxic didemnins, aplidine, and the tamandarins (Vera and Joullié 2002). Aplidine has shown great promise in the clinic and is currently being evaluated as a new antitumor lead (Amador et al. 2003; Jimeno et al. 2004; Newman and Cragg 2004a, b).

Although many ascidians have been investigated for the presence of biologically active compounds, it is still not clear if these animals are the true producers of these molecules. Ascidian secondary metabolites may also be biosynthesized by associated microbial symbionts such as cyanobacteria (Sings and Rinehart 1996; Schreiber et al. 1997; Schmidt et al. 2004, 2005; Long et al. 2005), bacteria (Hildebrand et al. 2004; Salomon et al. 2004), or even fungi (Bugni and Ireland 2004). Only a few investigations have been carried out in order to explore the actual origin of ascidian secondary metabolites. Investigations on the biosynthesis of secondary metabolites provided evidence of de novo biosynthesis by ascidians (Steffan et al. 1993; Shen and Baker 1994; Kerr and Miranda 1995; Sakai et al. 1996; Jeedigunta et al. 2000; Hildebrand et al. 2004; Saleh and Kerr 2004). Examples of probable true ascidian secondary metabolites are tambjamines C, E, and F from *Atapazoa* sp. (Lindquist and Fenical 1991),

plicatamide from *Styela plicata* (Tincu et al. 2000, 2003), as well as kuanoniamine D, shermilamine B, and kynuramine from *Cystodytes dellechiajei* (Rottmayr et al. 2001), as in these cases the metabolites appear to be directly associated within the ascidian cells, tissues, or blood. On the other hand, patellamides A–C isolated from *Lissoclinum patella* and initially localized within ascidian tissues (Salomon and Faulkner 2002) have been recently ascribed to *Prochloron* (Schmidt et al. 2004, 2005; Long et al. 2005).

Two new polyheteroaromatic alkaloids encompassing a carbazole-maleimido-imidazole skeleton, granulatimide (Fig. 1a) and isogranulatimide (Fig. 1b), have been isolated from the ascidian *Didemnum granulatum* (Berlinck et al. 1998; Roberge et al. 1998; Britton et al. 2001). Both alkaloids selectively inhibited the G_2 cell cycle checkpoint of $p53^-$ mutated MCF-7 breast cancer cells (Berlinck et al. 1998; Roberge et al. 1998; Britton et al. 2001; Jiang et al. 2004). Isogranulatimide and granulatimide presented a unique biological activity profile, showing potent inhibition of the G_2 checkpoint, and of Chk1, Cdk1, and of glycogen synthase kinase-3b, and less potent inhibitory activity on several other protein kinases (Jiang et al. 2004).

The limited synthetic availability of isogranulatimide and granulatimide may be a drawback for the development of these compounds as drug leads, although efficient synthetic routes for these compounds have been developed (Berlinck et al. 1998; Piers et al. 2000). In order to improve the supply of granulatimide and isogranulatimide, it would be of interest to explore a microbial source for these compounds. Some circumstantial evidence supporting our hypothesis is the fact that many Didemnid ascidians establish symbiotic relationships with cyanobacteria and possibly with bacteria. Additionally, both alkaloids have a structure very similar to that of the microbial-derived staurosporine (Omura et al. 1995), and staurosporine derivatives have been isolated from ascidians (Horton et al. 1994; Schupp et al. 1999, 2002). Therefore, we explored the biosynthetic source of isogranulatimide and granulatimide by localizing the compounds within the ascidian tissues. In the present investigation, we address two questions: (1) Whether granulatimide and isogranulatimide alkaloids are associated with *D. granulatum* tissues or with symbiotic microorganisms, and; (2) Whether both compounds are found in the ascidian tissues, and in what cell type they are located.

Herein we demonstrate that granulatimide and isogranulatimide are associated with *D. granulatum* cells, and that granulatimide is stored in *D. granulatum* bladder cells found in the ascidian tunic.

Materials and methods

Organisms

The ascidian *D. granulatum* Tokioka 1954 (Ascidiacea: Didemnidae) is a semi-encrusting (0.9–1.1 mm thick) colonial animal, usually found growing in vertical rock substrata up to 4 m deep. The color varies from light brown–orange to off-white. Colonies of *D. granulatum* were collected in the São Sebastião channel area (Sao Paulo, Brazil) and brought to the laboratory in ice cooled containers. Freshly collected animals were kept in aquarium for no longer than 24 h until processing.

Tissues

Slices (5 × 5 mm) of freshly collected colonies were fixed using glutaraldehyde 4% in calcium–magnesium free seawater with ethylenediaminetetraacetic acid (EDTA) (CMFSW + E—NaCl 460 mM, Na_2SO_4 7 mM, KCl 10 mM, HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) 10 mM, and EDTA 2.5 mM—pH 8.2; Dunham and Weissmann 1986) for 24 h at 4°C. The fixative was changed to CMFSW supplemented with 10% EDTA (w/v) and the tissues incubated for 1 h at room temperature in order to remove the calcareous spicules. Sections were manually obtained from the decalcified tissues using razor blades. Single individuals were also dissected from these sections and observed under the microscope. To estimate the ratio of organic versus inorganic contents, tissue sections were fixed as described above and dried (80°C–24 h). After weighing, the tissues were decalcified with CMFSW + 10% EDTA, then exhaustively dried and weighed again.

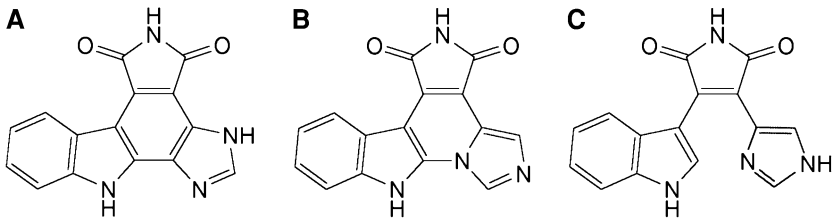

Fig. 1 Chemical structures of granulatimide (**a**), isogranulatimide (**b**), and didemnimide A (**c**)

Cells

Live colonies were cleaned from debris and other organisms and sliced in Petri dishes with CMFSW + E. Cell suspensions were obtained by mechanical dissociation using double parallel fixed razor blades (3 mm apart) to finely chop the colony to less than 0.5 mm fragments. Periodically, the CMFSW + E was collected and new solution was added until the color became clear. The suspension was further dissociated by pipetting and settling for 5 min in 50 ml test tubes, filtering through 50 μm nylon mesh, and washing twice with CMFSW + E by centrifugation (180×*g*—10 min). The cells were either observed immediately or fixed overnight in 4% glutaraldehyde in CMFSW + E at 4°C. Samples of dissociated cells were prepared by adjusting the concentration of the suspension to 5×10^5 cells/ml using a Neubauer chamber. Slides were made using a cytocentrifuge (100 μl per spot—5 min–80×*g*. Citospin 248—FANEM), and fixed with formaldehyde sublimate (1 h), glutaraldehyde 4% in CMFSW + E (overnight 4°C), or air dried. The cytospins were stained with Ziehl's Fuchsin, Mallory's Trichrom, or Toluidine Blue (Behmer et al. 1976). Cell type determination was based on overall morphology following descriptions in the literature (e.g. Hirose et al. 1991, 1994, 1996; Rottmayr et al. 2001).

Cell separation

Discontinuous density gradients were prepared using Percoll (Sigma, St Louis, MO, USA) diluted with CMFSW + E in sets of 10–60% in 10% steps, 40–60, and 60–100%. The gradient was prepared in 15 ml tubes, using 2 ml for each Percoll dilution. Fixed cell suspension were adjusted to a 1×10^8 cell/ml concentration, 2 ml of which were carefully loaded at the gradient top and centrifuged (30 min–1,000×*g*). After centrifugation, the fractions were collected, pelleted, and either stored in dimethylsulfoxide (DMSO) at -20°C for chemical analysis or in CMFSW + E for microscopic observations.

Microscopy

Observation of semi-thin sections and cell counting were conducted using an inverted microscope (Eclipse TE300, Nikon, NY, USA) with phase contrast. Fixed cell suspensions, cytospins, semi-thin sections, and isolated individuals used in fluorescence studies were mounted on slides with antifading (VectaShield, Vector, CA, USA). Live cell suspensions in CMFSW + E were placed in Petri dishes with glass bottoms and observed directly. Fluorescence microscopy was performed using a confocal microscope (LSM-510, Zeiss, NY, USA), with 364 nm excitation wavelength and a BP 385–470 nm emission filter, based on the emission spectra of granulatimide and on the emission spectra of *D. granulatum* methanol (MeOH) crude extract. The observation of granulatimide under the above conditions was confirmed using slides with droplets of a solution prepared with 4% agarose mixed with pure granulatimide (25 μg/ml).

Steady-state fluorescence analysis

Steady-state fluorescence spectra (375–700 nm, 5 nm slits for excitation and emission) of granulatimide, isogranulatimide, and didemnimide A, in DMSO/MeOH (1:2 v/v), were obtained at 25°C in an Hitachi 4500 spectrofluorometer using a rectangular 1 cm path length quartz cell and at an optical density of less than 0.10–0.09 to avoid inner filter effects. The excitation wavelengths were 375 nm for granulatimide and isogranulatimide, and 336 nm for didemnimide A. The fluorescence spectrum of the solvent solution was subtracted to eliminate scattering effect.

Liquid chromatography analysis

The HPLC analyses of extracts from the Percoll fractions and cell pellets were performed as follows. Percoll fractions and cell pellets stored in DMSO were filtered through a cotton plug, which was washed with 5 ml 1:1 MeOH/DMSO to extract solids. The whole extract was evaporated at room temperature in vacuum in a Savant/Speedvac system until dryness. Dried fractions were stored at −20°C until needed. For HPLC analysis, the extracts and standards of granulatimide and isogranulatimide were diluted in 6:4 MeOH/DMSO. A Waters HPLC system including a Waters 600 quaternary pump, Waters 600 pump control system, Waters 2996 photodiode array detector monitored by Waters Millenium 4.00 (2001) software was used for HPLC analyses. The chromatography analysis was performed with a Phenomenex Phenyl-bonded silica gel column (Prodigy 5 μ Phenyl-3, 100 Å), with a gradient of acetonitrile (MeCN) in H_2O as follows: 100% H_2O (1 min.), then a gradient from 100% H_2O to 100% MeCN over 25 min, then 100% MeCN during 5 min. Analyses included the comparison of isogranulatimide and granulatimide retention times and UV spectra with the corresponding peaks present in the DMSO extracts of Percoll fractions and cell pellets.

Results and discussion

The zooids of *D. granulatum* present an average size of 0.9 mm, and are regularly distributed within the colonies (Fig. 2). Therefore, the isolation of specific tissues or organs in sufficient quantity to perform a selective chemical analysis was not practically feasible. The ascidian organic portion – tunic and individual zooids – represents only 15% of the total dry weight. The calcareous skeleton accounts for the remaining 85% of body mass and is formed by star-shaped spicules (about 15 μm diameter), requiring the decalcification of tissues for microscopy.

Clusters of *D. granulatum* granular cells could be observed with a regular distribution both in the upper and lower tunic layers (Fig. 2). Possible cyanobacteria (Fig. 3) were also observed in the lower tunic, either isolated or in clusters of up to eight cells, with an associated fluorescence probably related to the presence of chlorophylls (Bibby et al. 2003). However, the very small amount of these cells within *D. granulatum* tissues precludes the detection of any significant quantity of cyanobacterial secondary metabolites. Although no other microorganisms were observed in association with *D. granulatum* under microscope analysis, the presence of bacterial symbionts has been described in other ascidians (Hirose et al. 1996; Rottmayr et al. 2001; Groepler and Schuett 2003). Nonetheless, HPLC analysis of the fractions obtained after low speed ($180\times g$) centrifugation of the mechanically dissociated cell suspension did not detect any amount of granulatimide or isogranulatimide in the supernatant, but detected only in the cell pellet. This centrifugation speed is enough to settle down ascidian cells and debris but not average sized bacteria or cyanobacteria, and no microorganisms were observed associated with the pellet by microscope analysis. Therefore, we suspected that the alkaloids were restricted to the ascidian cellular fraction.

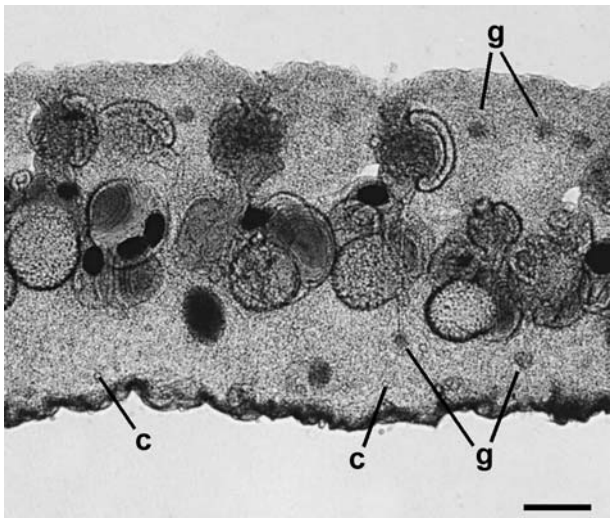

Fig. 2 Decalcified tissue section of *Didemnum granulatum* colony, showing the zooids and clusters of granular cells (*g*). Cyanobacterial cells (*c*) can be observed scattered in the lower tunic area (Unstained phase contrast. Scale bar: 200 μm)

Indeed, HPLC analysis (Fig. 4a–f) of the cell pellet obtained after centrifugation indicated the presence of both granulatimide and isogranulatimide (Fig. 4c). The pellet was further fractionated using the Percoll gradient, in order to concentrate major cell types and enhance the detection of both alkaloids. Microscopic examination of the initial Percoll gradients (10–60% in 10% steps) showed that the cells were distributed in multiple bands from the 30–40% interface to the pellet, and the HPLC analysis detected the presence of granulatimide and isogranulatimide in all these fractions. Although it was initially difficult to establish a clear correlation between the alkaloid concentration and a definite Percoll fraction with a particular cell type, HPLC monitoring of subsequent Percoll fractionations (40–60 and 60–100% gradients) led to the isolation of a cell pellet obtained from the 60–100% fraction, which presented the highest concentration of both alkaloids.

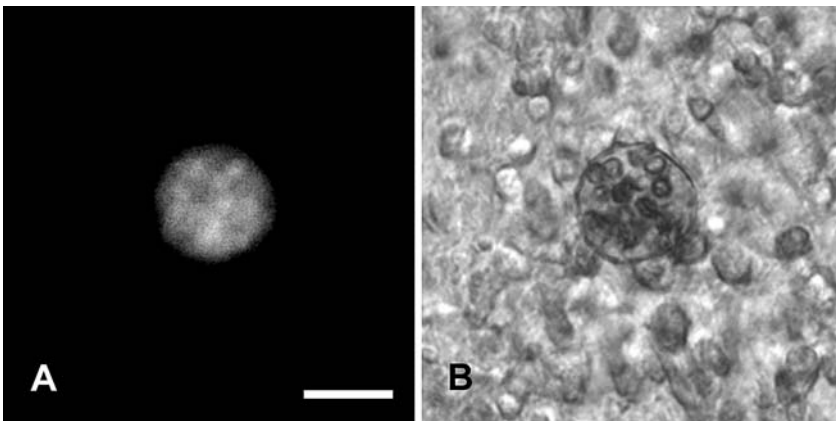

Fig. 3 Single cyanobacteria cell embedded in the *Didemnum granulatum* decalcified tunic matrix: **a** fluorescence associated with cyanobacteria cells; **b** visible. Scale bar: 20 μm

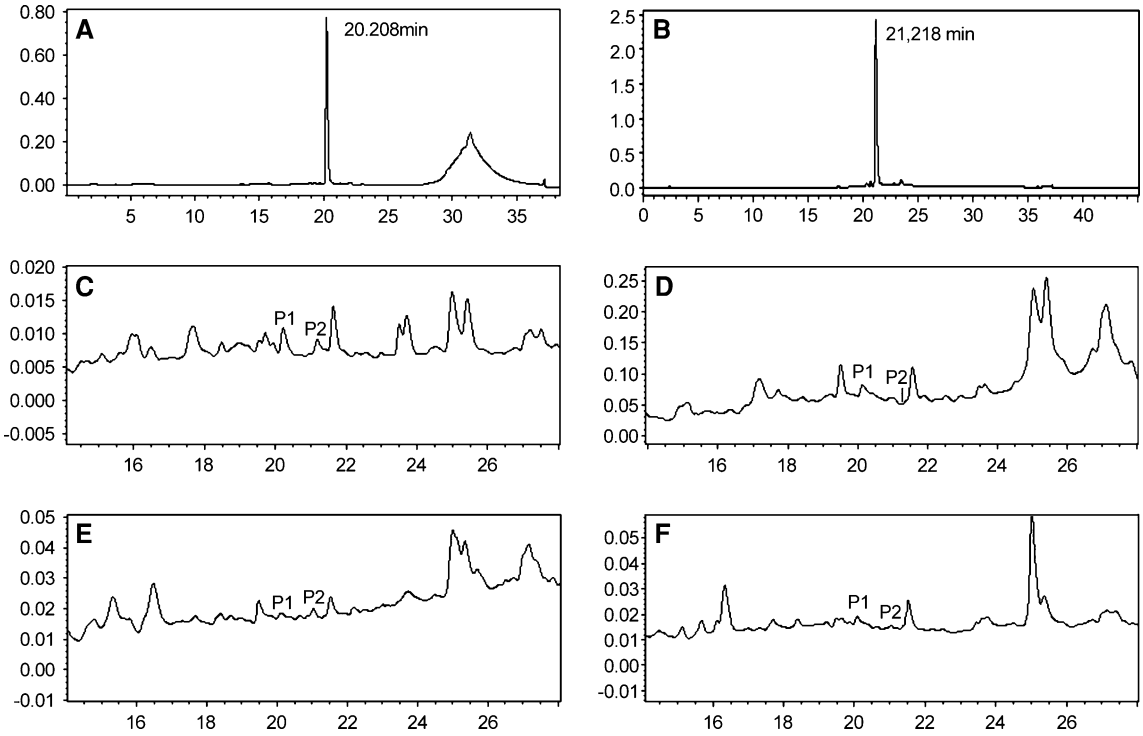

Fig. 4 HPLC analysis of the DMSO extracts of Percoll fractions and cell pellets obtained from *Didemnum granulatum*: **a** granulatimide standard; **b** isogranulatimide standard. **c** analysis of the DMSO crude extract of the cell pellet obtained by centrifugation of the whole tissue dissociate; **d** analysis of the DMSO extract of the 0–60% Percoll fraction; **e** analysis of the 60–100% Percoll fraction; **f** analysis of the centrifugate bottom layer (P1 granulatimide, P2 isogranulatimide. Plots: absorbance units × minutes. Analysis conditions: see Material and methods)

Under fluorescence microscopy, we observed highly fluorescent cells. Since the fluorescence spectrum of granulatimide was intense, with $\lambda^{max}{}_{emis}$ about 510 nm (Fig. 5), the observed fluorescence of the cell pellet obtained from the 60–100% fraction was assigned to the presence of granulatimide. Isogranulatimide presented the weaker fluorescence intensity when compared with granulatimide and didemnimide A (Fig. 1), with a $\lambda^{max}{}_{emis}$ around 565 nm. For didemnimide A, the fluorescence spectrum showed an intense peak around 533 nm. HPLC analysis of the 60–100% fraction indicated that, although in very small amounts, peaks of both alkaloids could be observed by comparing their retention times (granulatimide: 20.2 min; isogranulatimide: 21.2 min) and UV absorption spectra, which are very characteristic for both isogranulatimide [λ_{max} at 233, 289, 304 (shoulder), 332 (shoulder), and 485 nm] and granulatimide [λ_{max} 233, 278, 302 (shoulder), and 392 nm].

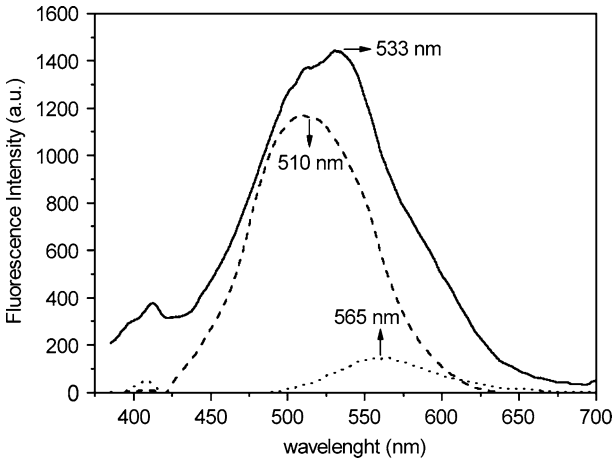

Fig. 5 Fluorescence spectra of granulatimide (dashed), isogranulatimide (dotted) and didemnimide A (solid). The excitation wavelengths were 375 nm for granulatimide and isogranulatimide, and 336 nm for didemnimide A. The emission was scanned from 375 to 700 nm. The $\lambda^{max}{}_{emis}$ are indicated by arrows

Confocal microscopic examination of the dissociated cells revealed that only one cell type shows detectable fluorescence within the granulatimide wavelength range. These are about 5–8 μm in diameter and were identified as bladder cells, due to the possession of a single large homogenous vacuole 5 μm in diameter, peripheric nuclei and reduced cytoplasm in those cells assumed to be in the "mature" stage (Fig. 6). The number and size of the vacuoles in each cell are highly variable. These can be from one to five smaller (Fig. 7a), a single with variable size (Fig. 7b) or up to five or six

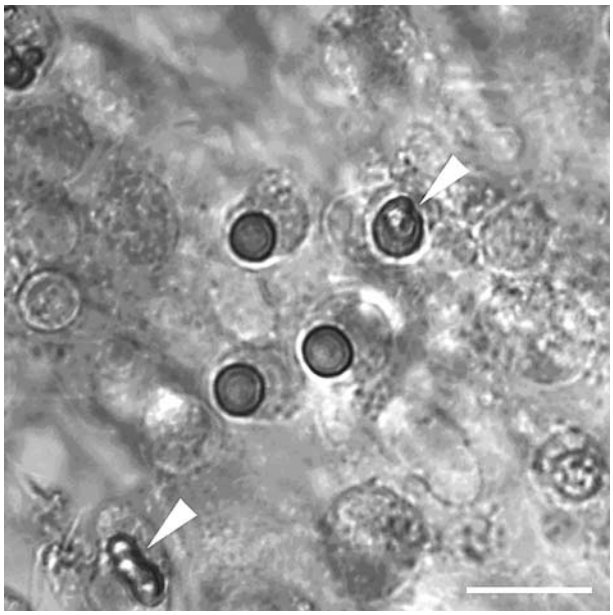

Fig. 6 Tunic bladder cells. The vacuoles are dense and spherical, and irregular shapes (*arrows*) are due to the presence of smaller vacuoles within the same cell (scale bar: 10 μm)

larger vacuoles (Fig. 7c, d). These variations may be the reason why we observed significant differences in the amount of the alkaloids relative to total cell volume, and could be responsible for the distribution of bladder cells in different densities, even in the pellet obtained using 100% Percoll as a bottom layer. The 100% Percoll fraction also concentrated most of the morula cells together with debris (i.e., tunic and spicules fragments).

Ascidian cell type identification is still a debatable science and there is no definitive terminology for ascidian cells. Pigment cells usually have a comparable morphology but are invariably described as possessing pigment granules within the vacuoles (Shirae and Saito 2000; Rottmayr et al. 2001). Similar cells have also been described as morula-like or macro-granular cells (Hirose et al. 1994, 1996). In *D. granulatum* bladder cells, the vacuole content is homogenous, without any inclusion, and even after cell disruption remains as a free-floating oily droplet. No noticeable change of pH, as indicated by phenol red color, was observed during ascidian manipulation. Additionally, microscopic observation of sectioned non-decalcified tissues showed no sign of spicule damage. The existence of cells with different degrees of cytoplasm vacuolization, but presenting the same fluorescence pattern of "mature" *D. granulatum* cells, suggested that those types could be developmental stages, a fact that has been previously observed for other ascidians (Hirose 1992).

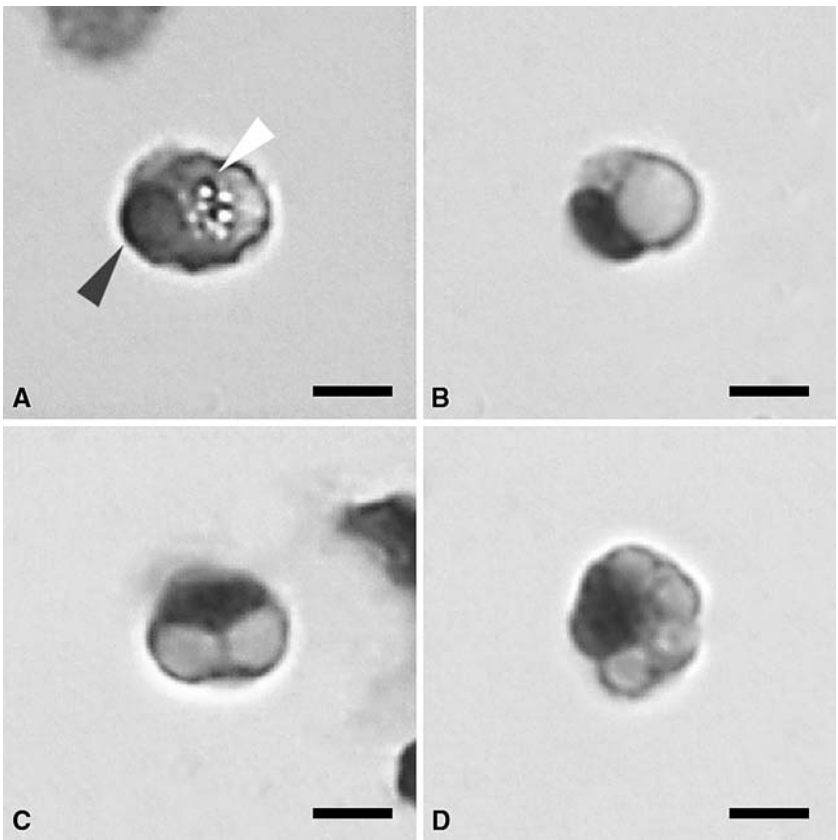

Fig. 7 Isolated tunic bladder cells in cytospins, showing the vacuole size and number variations in different cells. *Black arrow* indicates the nuclei and *white arrow* the vacuoles. The vacuoles can be very minute (as in **a**) or most frequently large (as in **b**). The number can be variable (**c**, **d**) but the maximum cell diameter appears to be approximately constant (*Toluidine Blue*. Scale bar: 5 μm)

Microscopic analysis of decalcified tunic sections showed that the bladder cells were almost all dispersed in the upper tunic area, and never form a distinct organized layer as observed in other colonial ascidians (Hirose 2001; Hirose et al. 2001). Clusters of up to six bladder cells could be observed deeper in this region, usually bearing several smaller vacuoles (Fig. 8), a fact that suggested the existence of proliferation islets. No bladder cells or any other cell types or structures with a similar fluorescence pattern were observed within the tissues from isolated adult zooids.

Previous investigations on the cellular localization of ascidian secondary metabolites presented evidence that the ascidian *C. dellechiajei* violet morphotype accumulates intense colored pyridoacridine alkaloids such as shermilamine, kuanoniamine D, or kynuramine, while *C. dellechiajei* gray–green morphotype did not possess any pyridoacridine alkaloid in pigment cells (Rottmayr et al. 2001). It has been also recently observed that several tropical ascidian species associate with mycosporine-containing *Prochloron* cyanobacteria in their tunics, a fact that indicates that such an association is possibly related to protection against solar radiation (Maruyama et al. 2003; Hirose et al. 2004). A different investigation indicated that secondary metabolites related to patellamides are located within the tunic of *L. patella* (Salomon and Faulkner 2002). But recently it has been verified that pattelamides are produced by associated *Prochloron* cyanobacteria (Long et al. 2005; Schmidt et al. 2005).

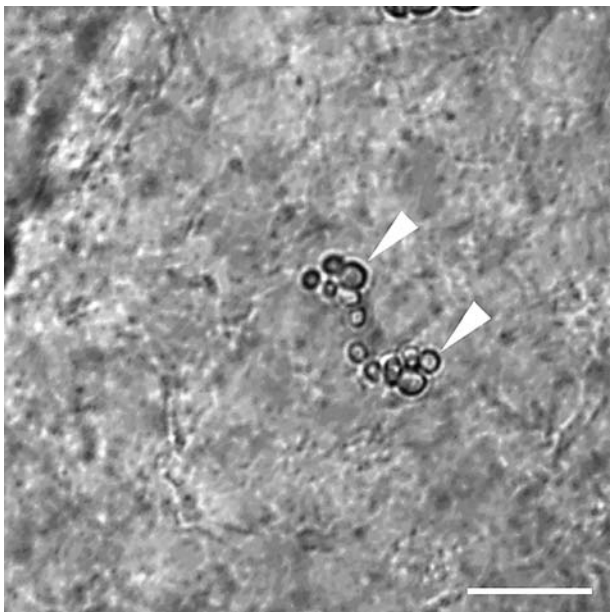

Fig. 8 Cluster of 3–4 bladder cells (*arrows*) in the tunic. These arrangements can be found closer to tunic center, and the cells usually show several smaller vacuoles (scale bar: 10 μm)

The present investigation demonstrated the accumulation of granulatimide and isogranulatimide in bladder cells present in the tunic of *D. granulatum*. The accumulation of these compounds within the ascidian tunic cells may play a role in either photoprotection and/or feeding deterrence. There is evidence that the ascidian hosts are able to protect its symbionts against excessive UV irradiance (Kühl and Larkum 2002; Maruyama et al. 2003; Hirose et al. 2004). The external color of *D. granulatum* is the same color as the crude extract, in which the only colored compounds present are granulatimide, isogranulatimide, and didemnimides A, C, D, and E (Berlinck et al. 1998; Britton et al. 2001). A mixed solution of these compounds in DMSO in natural concentrations has a color very similar to that of *D. granulatum*. Since these alkaloids also have intense radiation absorption within the UV-visible range, it seems likely that a photoprotective role can be suggested for these compounds. In this case, the tunic bladder cells would accumulate these compounds within the colony, below the level of direct observation by fluorescence methods. On the other hand, it has been also verified that structurally related didemnimides display protection against fish predation on the ascidian *Didemnum conchyliatum* (Vervoort et al. 1997, 1998). Therefore, these alkaloids may possess multiple ecological roles.

The presence of alkaloid-containing bladder cells in the upper tunic, in addition to the large amount of non-digestible inorganic spicules, makes the ascidian *D. granulatum* a poor choice for potential predators. As a source of granulatimide, isogranulatimide, and didemnimides, *D. granulatum* has a secondary metabolic profile related to the ascidian *D. conchyliatum*, which is chemically protected against fish predation by the didemnimides (Vervoort et al. 1997, 1998). Additional information about the ecological roles of *D. granulatum* alkaloids may help to explain the accumulation of these substances within *D. granulatum* tissues.

Acknowledgments The authors are particularly grateful to Dr. Christine Salomon (University of Minnesota) for a careful reading of the manuscript and many suggestions to improve it. The authors thank the staff of CEBIMar—USP for the provision of laboratory facilities and technical support during field trips; the staff of the Laboratório de Fisiologia Celular do *Plasmodium* (IB-USP) for the assistance with confocal microscopy; and Gustavo M. Diaz (Universidade Estadual de Campinas, Brazil) for valuable discussions. Financial support was provided by FAPESP as grants to M.R.C. (99/11443-1) and R.G.S.B. (01/06202-7), and as a scholarship to S.P.L. The authors also thank the Universidade de São Paulo within the PROCONTES program to M.H.R.S. and R.G.S.B. All experiments complied with current Brazilian laws. The authors dedicate this work to the late Professor D. John Faulkner (Scripps Institution of Oceanography), *in memoriam.*

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