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MARINE BIOTECHNOLOGY

Original Contributions

Immunolocalization of the Toxin Latrunculin B within the Red Sea Sponge *Negombata magnifica* (Demospongiae, Latrunculiidae)

O. Gillor,[1] S. Carmeli,[2] Y. Rahamim,[2] Z. Fishelson,[3] and M. Ilan[1,*]

[1]*Department of Zoology,* [2]*School of Chemistry,* [3]*Department of Cell Biology and Histology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*

Abstract: The location of latrunculin B, the major toxin of the Red Sea sponge *Negombata magnifica,* was revealed using specific antibodies. Antibodies from rabbits immunized with a conjugate of latrunculin B with keyhole limpet hemocyanin (KLH) were purified over a latrunculin B–Sepharose affinity column. Analysis of immunohistochemical and immunogold-stained sponge sections, using light and transmission electron microscopy, revealed latrunculin B labeling mostly beneath the sponge cortex at the border between the external (ectosome) and internal (endosome) layers (ectosome-endosome border). The endosome was less labeled than the border. Immunogold localization revealed latrunculin B in the sponge cells but not in its prokaryotic symbionts. Archeocytes and choanocytes were significantly more labeled than other cells. The antibodies primarily labeled membrane-limited vacuoles within archeocytes and choanocytes that are perhaps latrunculin B secretory or storage vesicles. Peripheral latrunculin B may have a role in defense against external epibionts, predators, and competitors.

Key words: Porifera, natural product, antibodies, secondary metabolite, defense.

INTRODUCTION

Many exposed, sessile organisms have developed an array of secondary metabolites (natural products) with potent biotoxic and cytotoxic properties (König and Wright, 1996). It has been hypothesized that such compounds act in a variety of ways, mostly as a defensive mechanism against predators (Pawlik et al., 1995) and pathogens (Becerro et al., 1994), and as an aid to the organism in competition with neighboring benthic organisms (Sears et al., 1990; Willemsen, 1994). Sponges, the oldest and simplest metazoans (Wilkinson, 1983), are one of the richest sources for such metabolites.

Sponge natural products have proved to be an important source of new pharmaceuticals and experimental pharmaceutical compounds (Scheuer, 1990; König and Wright, 1996). Although the structure and toxicity of sponge bioactive metabolites have been extensively studied (e.g., Higa et al., 1994; König and Wright, 1996), their localization within the sponge has rarely been established (Table 1). Localization of metabolites within the sponge is a complex task for several reasons. First, sponges contain within them symbiotic microorganisms (bacteria, algae, fungi, and pro-

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**Corresponding author: telephone 972-3-640-8613; fax 972-3-640-9403; e-mail milan@post.tau.ac.il*

Table 1. Secondary Metabolites Localized in Sponge Cells

Secondary metabolites	Cell type	Sponge species	Reference
Aerothionin & homoaerothionin	Spherulous cells	*Aplysina fistularis*	Thompson et al., 1983
Avarol	Spherulous cells	*Dysidea avara*	Müller et al., 1986
Avarol	Choanocytes	*Dysidea avara*	Uriz et al., 1996b
Crambine & crambescidins	Spherulous cells	*Crambe crambe*	Uriz et al., 1996a
Diisocyanoadociane	Spherulous cells & archeocytes	*Amphimedon* sp.	Garson et al., 1992
Latrunculin B	Choanocytes & archeocytes	*Negombata magnifica*	The present study

tozoa). Bioactive metabolites extracted from sponges might have been produced, therefore, either by the sponge itself or by symbiont cells (Thompson et al., 1983; Müller et al., 1986; Garson et al., 1992; Kobayashi and Ishibashi, 1993; Unson and Faulkner, 1993; Uriz et al., 1996a, 1996b). Second, sponges, unlike other metazoans, lack distinct organs, excluding the option of isolating tissues for chemical and biological analyses (e.g., Harvell and Fenical, 1989). Finally, the ability of sponge cells to differentiate into all cell types and redifferentiate (Wilkinson, 1983) makes it difficult to separate and identify the cells producing the bioactive metabolites.

Nonetheless, localization of bioactive substances in marine invertebrates is essential for understanding their biological and ecological role and to enable their production for biotechnological applications (Osinga et al., 1998). For example, secondary metabolites localized near the organism's surface have been suggested to deter predators, as in the nudibranch *Chromodoris* (De Silva et al., 1991) and sponges (Uriz et al., 1996a), or to have antibacterial properties, as in gorgonian corals (Kim, 1994) or echinoderms (Bryan et al., 1996). Identification of cells producing secondary metabolites is important to enable biotechnological production of the compounds for two reasons. One is that these secondary metabolites often are found in minute quantities. For example, 12 mg of halichondrin B (a cytotoxic agent first isolated from the sponge *Halichondria okadai*) was isolated from 600 kg wet weight of sponge. The other reason is that the sponge's numerous microbial symbionts have been frequently suggested to be the producers of the natural products extracted from sponges (reviewed by Kobayashi and Ishibashi, 1993).

When the secondary metabolite possesses a unique characteristic (such as a halogen element, which is rare or absent from primary metabolites), it is relatively easy to identify its producer (Thompson et al., 1983). When such a unique element is absent, however, other techniques such as cell dissociation on a density gradient followed by chemical extraction of each cell fraction have been applied (Müller et al., 1986; Garson et al., 1992; Uriz et al., 1996a). In the present research we used immunolocalization methods to uncover the producer of the bioactive metabolite latrunculin B in the Red Sea sponge *Negombata magnifica.*

Immunolocalization is a well-developed method used to localize secondary metabolites within plant tissue (Weiler et al., 1981), plant cells (Brisson et al., 1992), cyanobacteria (Shi et al., 1995), and dinoflagellates (Anderson and Cheng, 1988; Zhou and Fritz, 1994). Unlike other methods, this one allows not only general localization of the zone where these metabolites are found but also the precise identity of the producing cells and even organelles. The concept of using antibodies to locate and identify the producing cells and organelles in a marine invertebrate is new and has not yet been examined.

Negombata magnifica is a conspicuous bright red branching sponge found in the Red Sea (Ilan, 1995) that was never seen eaten by fish. The sponge "juice" was found to be ichthyotoxic (Neeman et al., 1975). The compounds probably responsible for this activity were identified as latrunculin A and B (Figure 1). Latrunculin B (Lat B) differs from Lat A in containing 14 versus 16 membered macrocyles (Kashman et al., 1980; Groweiss et al., 1983). Latrunculin A was isolated not only from *N. magnifica* but also from other sponges (Kakou et al., 1987; Gulavita et al., 1992) and cnidarians (Mebs, 1994). The toxin causes erratic behavior in fish followed by hemorrhaging, loss of balance, and death. In vitro experiments revealed that the latrunculins disrupt actin filaments (Spector et al., 1983; Coue et al., 1987). The latrunculins have been shown to alter cell shape (Bar-Ziv et al., 1999); disrupt actin microfilament organi-

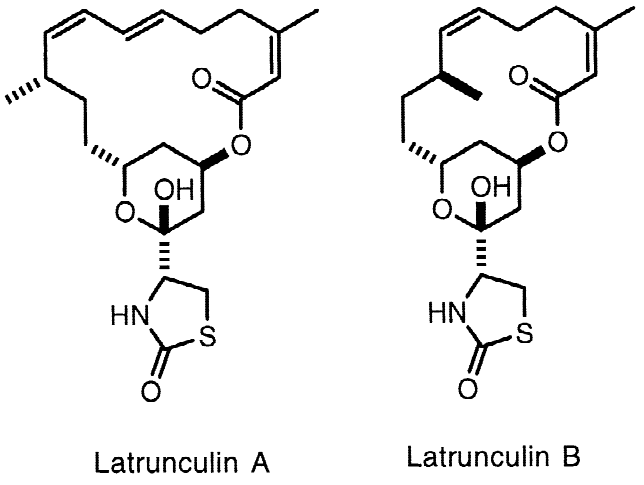


Figure 1. Chemical structure of latrunculins A and B.

zation (Spector et al., 1983, 1989); inhibit the microfilament-mediated processes of meiosis (Forer and Pickett-Heaps, 1998), fertilization, and early development (Schatten et al., 1986; Chen et al., 1999); force development in muscles (Mehta and Gunst, 1999); and even affect protein kinase C signaling (Niggli et al., 1999). These results have raised interest in the potential use of latrunculins as growth inhibitors of some tumor cell lines, which would make them an important tool for pharmacological studies in addition to their ecological role.

The present study was aimed at localizing the producer of Lat B within the sponge *N. magnifica* using methods based on antibodies produced against Lat B. Such localization should assist in the biotechnological production of Lat B as well as in understanding its ecological role and mode of operation. Another goal was to demonstrate the feasibility of the localization method for further utilization with other secondary metabolites.

Materials and Methods

Sampling

In the Gulf of Aqaba *Negombata magnifica* produces Lat B (Ilan, 1995), so our aim was to identify the producer of this compound. Specimens of *N. magnifica* were collected using SCUBA (at 6 to 20 m) from the Gulf of Aqaba and transferred in water to the laboratory.

Specimens used for extraction of secondary metabolites were frozen immediately upon collection at −70°C and transferred to Tel Aviv University. Specimens used for electron microscopy were fixed in 2.5% glutaraldehyde in 0.2 μm of filtered seawater, immediately after collection. Samples for light microscopy were fixed for 24 hours in 4% formaldehyde, then rinsed and transferred to 70% ethanol.

Chemical Extraction

Latrunculin B was isolated from *N. magnifica* as previously described (Kashman et al., 1980). The physical and chemical properties of Lat B are described elsewhere (Groweiss et al., 1983).

Preparation of Latrunculin B–Glutarate–KLH Conjugate

N-*Hydroxymethyl–Latrunculin B* ***(2)***

Because Lat B is a small macrolide, it was considered as a hapten and coupled to a protein carrier macromolecule (Figure 2). Compound **2** (210 mg) was prepared from Lat B (605 mg) in 32% yield, according to the procedure of Blasberger et al. (1989).

N-*(Hemiglutarate)–Oxymethyl–Latrunculin B* ***(3)***

A solution of compound **2** (109 mg, 0.26 mmol), glutaric anhydride (56.2 mg, 0.52 mmol), and 4-dimethylamino pyridine (8 mg) in dicholoromethane (20 ml) was stirred at room temperature for 18 hours. The solvent was evaporated to dryness, and the residual mixture was chromatographed on a Sephadex LH-20 column eluted with 1:1 MeOH/$CHCl_3$ solvent mixture to yield the pure product, **3** (74 mg, 53%) as a colorless oil. Positive fast atom bombardment mass spectrometry (dithiothreitol/dithioerythritol) *m/z* (relative intensity) 540 (20, MH^+), 464 (100), 434(5), 408(11), 390(32), 372(15), and 345(10).[1] H-NMR δ 5.67 (brs, H-2), 2.65 (m, H-4), 1.97 (m, H-4′), 2.17 (m, H-5), 2.34 (m, H-5′), 5.27 (dt, J = 2.7,11.2 Hz, H-6), 5.04 (brt, J = 10.7 Hz, H-7), 2.66 (m, H-8), 1.12 (m, H-9), 1.73 (brd, J = 12.9 Hz, H-9′), 1.50 (m, H-10), 1.40 (m, H-10′), 4.27 (brt, J = 11.0 Hz, H-11), 1.50 (m, H-12), 1.40 (m, H-12′), 5.41 (brs, H-13), 1.90 (m, H-14), 2.17 (brd, J = 14.9 Hz, H-14′), 3.24 (d, J = 5.9 Hz, 15-OH), 4.02 (dd, J = 2.1,9.4 Hz, H-16), 3.34 (dd, J = 12.0,2.1 Hz, H-17), 3.51 (dd, J = 12.0,9.4 Hz, H-17′), 1.90 (brs, 19-CH_3), 0.95 (d, J = 6.4 Hz, 20-CH_3), 5.44 (d, J = 10.2 Hz, NCH), 5.64 (d, J = 10.2 Hz, NCH′), 2.41 (t, J = 7.1 Hz, glut.-H_2-2), 1.95 (tt, J = 7.1 Hz, glut.-H_2-3), 2.41 (t, J = 7.1 Hz, glut.-H_2-4), 4.25 (brs, 5-OH); ^{13}C-NMR δ 162.8 (s, C-1), 115.3 (d, C-2), 158.0 (s, C-3), 33.2 (t, C-4), 24.2 (t, C-5), 124.9 (d, C-6),

133.2 (d, C-7), 26.4 (d, C-8), 28.4 (t, C-9), 28.7 (t, C-10), 60.1 (d, C-11), 32.7 (t, C-12), 66.1 (d, C-13), 28.7 (t, C-14), 96.7 (s, C-15), 61.4 (d, C-16), 24.3 (t, C-17), 171.9 (s, C-18), 21.4 (q, C-19), 19.7 (q, C-20), 66.3 (t, CH_2N), 169.8 (s, glut.-C-1), 30.2 (t, glut.-C-2), 17.2 (t, glut.-C-3), 30.4 (t, glut.-C-4), and 174.1 (s, glut.-C-5).

N-*(Hemiglutarate)–Oxymethyl–Latrunculin B–KLH Conjugate (4)*

The desired conjugates **4** and **5** were prepared according to Weiler et al. (1981). Tributylamine (18 μl) and isobutylchloroformate (9 μl) were added to *N*-(hemiglutarate)–oxymethyl–latrunculin B **(3)** (30 mg, 0.056 mmol), in dry dioxane (1.5 ml). After 30 minutes, formation of the less polar mixed anhydride was confirmed by thin-layer chromatography. The reaction mixture was then added dropwise over 5 minutes to a stirred solution of KLH (30 mg) in 25 mM borate buffer, pH 9 (30 ml), and dioxane (15 ml) at 4°C. After 24 hours at 4°C, the solution was dialyzed (SpectraPor 4 12,000–14,000 membrane), initially against 25 mM borate buffer, pH 8, and then against deionized water (×4). Lyophilization yielded 32 mg of **4** as white fluffy solid. The conjugate Lat B–glutarate–BSA, **5,** was prepared in a similar manner.

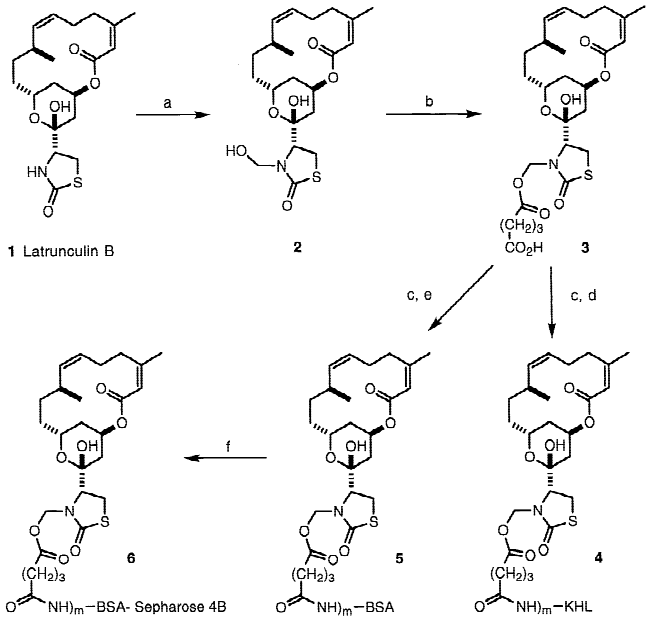


Figure 2. Preparation of latrunculin B conjugate with either keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) and the affinity column: **(a)** aq. CH_2O, EtOH; **(b)** glutaric anhydride, CH_2Cl_2, 4-dimethylaminopyridine; **(c)** isobutylchloroformate, *n*-Bu_3N, dioxane; **(d)** KLH, borate buffer, pH 8, dialysis; **(e)** BSA, borate buffer, pH 8, dialysis; cyanogen-bromide-activated Sepharose 4B, 2 hours.

Preparation of Antibodies Against the Conjugate Lat B–KLH

Three female rabbits were immunized with the Lat B–KLH conjugate (1 mg each) using complete Freund's adjuvant (Sigma) for the first injection and incomplete Freund's adjuvant for four booster injections. The rabbits were bled before the immunization and 1 week after the fourth one. All handling of the rabbits followed national animal care ethical regulations. The serum was separated by conventional methods. Only a small portion of the antibodies were directed against Lat B, whereas the majority were directed against the carrier protein KLH, as revealed by the immunoblot evaluation. Preimmune and immune sera were tested for their ability to bind the Lat B–BSA conjugate by dot blotting on nitrocellulose membranes (Harlow and Lane, 1988).

Affinity Purification of Anti–Lat B Antibodies

Lat B–BSA (3 mg) was first coupled to cyanogen-bromide-activated Sepharose 4B (Sigma) (Figure 2, and **5 and 6**). The Sepharose–Lat B resin was packed in a small (11-ml) column and equilibrated in 0.1 M Tris-HCl (pH 8). After loading of the immune serum, the column was extensively washed with equilibration buffer and then eluted with 0.1 M glycine-HCl (pH 2.9) to remove bound antibodies. Fractions (0.3 ml each) were collected, and the pH was neutralized immediately with 1 M Tris-buffered saline (TBS, pH 7.3). The eluate was analyzed in a spectrophotometer (Uvikon 931) at 280 nm. Fractions 3–9 (with the highest amount of protein) were further analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Harlow and Lane, 1988) and found to be rich in IgG. The purified antibodies were assayed by dot blotting on dots of Lat B–KLH and KLH on nitrocellulose membranes and found to be reactive with Lat B but not with KLH. Furthermore, Western blot of BSA versus BSA–Lat B with the anti–Lat B antibodies labeled the conjugate but not the BSA (data not shown). Thus, we concluded that these antibodies are specific to Lat B.

Indirect Immunolabeling of Paraffin Sections

Formaldehyde-fixed samples were dehydrated in a graded ethanol series, embedded in paraffin, sectioned, and spread

over poly-L-lysine-coated glass slides (Sigma). The 6-μm sections were deparaffinized, hydrated, washed with PBS (pH 7.5; NaCl 300 mM, KCl 5.5 mM, KH_2PO_4 0.3 mM, Na_2HPO_4 15.5 mM), and then quenched with 3% sheep serum (Sigma) diluted in PBS. The sections were incubated for 1 hour with 30 μg/ml purified anti–Lat B serum diluted in 3% sheep serum. The control was incubated only with 3% sheep serum and thereafter treated similarly. Following rinses with PBS (3 × 10 minutes), sections were treated for 1 hour with 1:800 diluted secondary antibody (goat anti-rabbit IgG) conjugated to biotin (Sigma) in 3% sheep serum. After additional PBS rinses (3 × 10 minutes), the sections were incubated for 1 hour with extra avidine–peroxidase (Sigma) diluted 1:150 in 3% sheep serum, and rinsed (3 × 10 minutes) in PBS. The labeling was detected with 65% diaminobenzidine and 0.035% H_2O_2 diluted in PBS.

Immunogold Labeling for Transmission Electron Microscopy

Glutaraldehyde-fixed samples were dehydrated in graded ethanol series, infiltrated, and embedded in LR White resin (London Resin Co.) at 4°C. Resin polymerization was performed at 60°C for 24 hours. Ultrathin sections (~60 nm) were prepared with LKB III Ultratom using a glass knife, and sections were mounted on Fomvar-coated gold grids. The sections were incubated with 3% BSA for 15 minutes followed by 30 minutes of incubation with 30 μg/ml purified anti–Lat B serum diluted in PBS. After excess serum was rinsed with PBS (8 × 2 minutes), sections were treated with gold-conjugated goat antirabbit secondary antibody (1:50 dilution) for 30 minutes, and rinsed again (PBS 8 × 2 minutes). The sections were poststained with 2% uranyl acetate (2 minutes) followed by lead citrate (1 minute). Sections were analyzed in a transmission electron microscope (Phillips 410 TEM). The sponge cells were characterized and identified according to Simpson (1984) and De Vos et al. (1991). Briefly, choanocytes have a circular set of microvilli with a central flagellum and a basal, usually large, nucleus. In TEM the nucleus has a typical pattern of a perimeter that is more electron-dense, with some denser areas within it. Phagosomes are also another constituent of the choanocyte cytoplasm. Archeocytes are large cells roughly twice the size of choanocytes. They have a large nucleus containing a single nucleolus. They also possess cytoplasmic phagosomes and lysosomes.

Morphometric Analysis

The labeling density of sponge cells and their compartments was expressed as the number of gold particles per square micrometer of the sectioned cell and its compartments. The area of the latter was measured directly on randomly obtained TEM micrographs (Hammel and Kalina, 1991; Skutelsky et al., 1995), and the photographed areas of labeled and unlabeled cells were measured using a graphic table (Hewlett Packard 9111A interfaced to Power Macintosh 7100/66AV). All numerical data were examined using Student's *t*-test statistics.

Results

Overall Histological View of *Negombata magnifica*

Two distinct areas appeared in *N. magnifica* sections: the ectosome, with a thickness ranging from 600 to 700 μm, and the endosome (also called choanosome), which varied remarkably in thickness owing to the branching nature of the sponge morphology.

Histological preparations showed that the ectosome of *N. magnifica* is composed of a thick collagenic cortex at the periphery (Figure 3, A). The collagenic layer contained only a few cells, mainly fiber cells, spongocytes, sclerocytes, collenocytes, and lophocytes (termed here skeleton-associated cells). The sponge external surface was free of epibionts.

The endosome-ectosome border (EEB) located just beneath the cortex is a specialized portion of dense mesohyl found to be nearly devoid of choanocyte chambers. The dense cell layer consisted of archeocytes, special cells (spherulous cells, gray cells, and granular cells) and some choanocytes, although in much lower numbers than in the inner section of the endosome (cell definitions after Simpson, 1984).

The inner endosome, in contrast, contained a large number of choanocyte chambers together with organic and inorganic skeletal components (Figure 3, B). In addition to the numerous choanocytes, it contained archeocytes, skeleton-associated cells, and special cells. A small number of microsymbionts, mainly bacteria, were detected in both layers of the sponge.

Indirect Immunolabeling of Paraffin Sections

Sections incubated with the purified anti–Lat B antibody showed significant labeling (Figure 3, B). Control sections

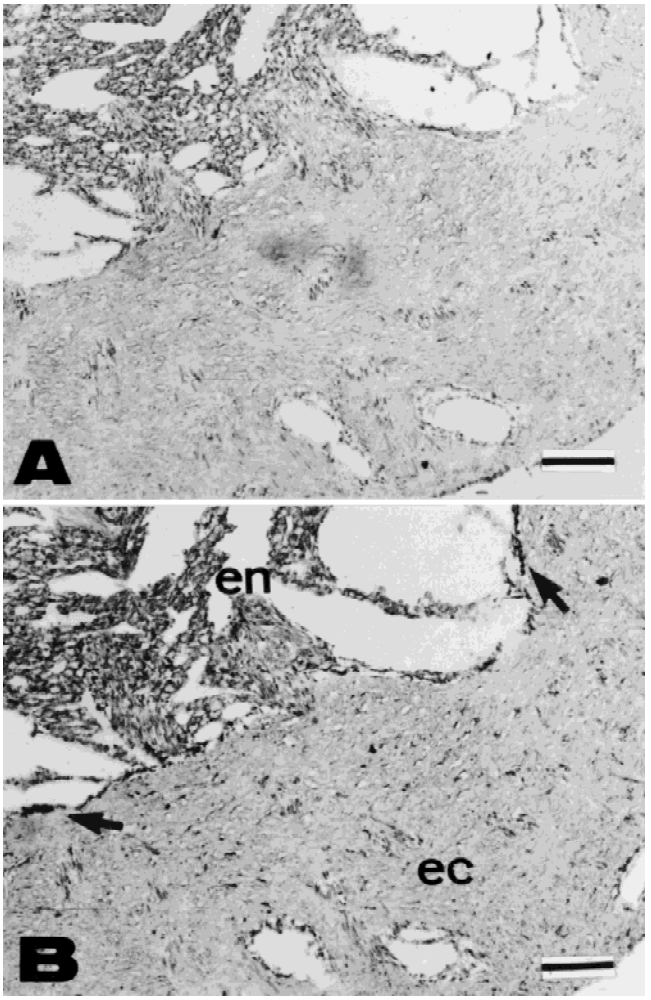


Figure 3. Localization of latrunculin B within *Negombata magnifica* by immunohistochemical tissue labeling with biotin-streptavidin-peroxidase. **A:** Section incubated without the primary antibody. **B:** Section incubated with the primary antibody. Note the heavy label of the thin cell layer (*arrowhead*) beneath the collagenic area *(ec),* compared with the control. The endosome *(en)* is less labeled than the ectosome-endosome border (*arrowheads* point to the dense cell layer at the border.) Both pictures were taken from a light microscope. Scale bar = 100 μm.

incubated with only the secondary and tertiary reagents (goat antirabbit IgG conjugated to biotin and extra avidine–peroxidase, respectively) but without the anti–Lat B primary antibodies were negative (Figure 3, A).

The cortex was poorly labeled, but the thin cell layer just beneath the cortex was heavily labeled (Figure 3, B). This dense cell layer was unevenly labeled: certain cell types were significantly labeled, while other showed pale labeling. Most of the labeled cells were larger and their cytoplasm seemed to be granular. In the inner endosome, both the

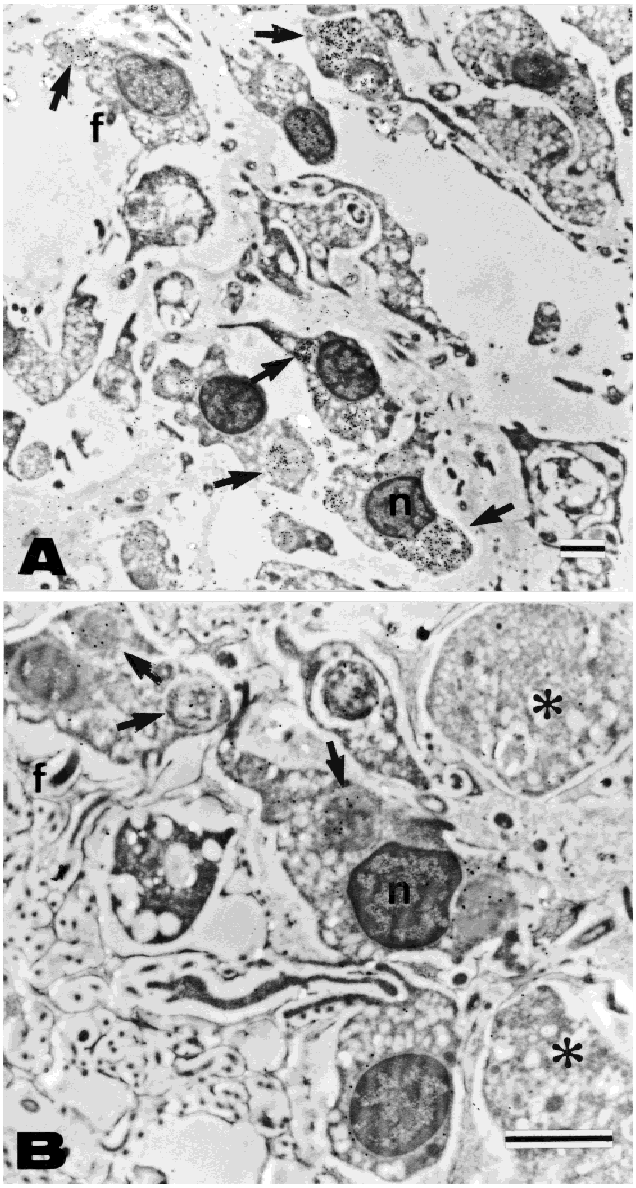
Figure 4. Immunogold labeling of latrunculin B within *Negombata magnifica* cells. **A:** Immunolabeling of the ectosomal cells. The gold particles indicate that latrunculin B is concentrated within vacuoles in the cell cytoplasm. All the marked cells are choanocytes. **B:** Labeling of the endosomal cells. The majority of gold particles are located inside the choanocyte vacuoles (*arrowheads*), and absent from the nucleus *(n)* and cytoplasm. The flagellum *(f)* of several choanocytes can be seen.* Two unlabeled special cells (probably gray cells) located in close proximity to the choanocytes. **C:** Morphometric analysis of gold particles labeling within cells of *N. magnifica* at the ectosome-endosome border (■) and the inner endosome (❑). Values are expressed as the mean number of gold particles per square micrometer ± SE; $n = 35$. Scale bar = 1 μm.

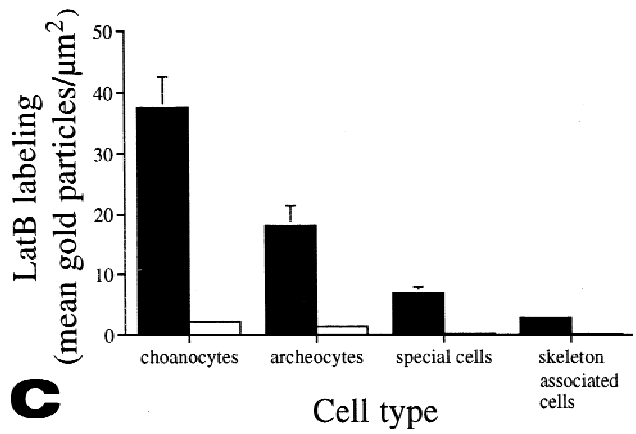
organic skeletal material and the choanocyte chambers were far more labeled than the control sections (Figure 3). The skeletal material was unevenly labeled, with patches of brown color inside the matrix. Overall, most of the endosome appeared to be less labeled than the EEB, which is the thin layer between the two regions (Figure 3, B).

Immunogold Localization of Latrunculin B within *N. magnifica*

Immunogold was used to reveal the sponge cell labeling pattern. At the TEM level gold particles indicative of primary antibody binding were labeled primarily in several types of cells and in specific organelles within these cells. The density of gold particles (expressed as the mean number of gold particles per square micrometer) was significantly higher in EEB than in endosomal cells (Figure 4, A and B, respectively). The corresponding mean values and standard errors were 16.3 ± 7.5 ($n = 38$) for periphery cells and 1.0 ± 0.5 ($n = 68$) for mesohyl cells. The significant standard deviation is due to the difference among the sponge cell types as explained below.

Morphometric analysis showed that the density of gold particles in choanocytes and archeocytes (in both EEB and endosome) was significantly higher than within the skeleton-associated cells and the special cells (Figure 4, C). For instance, in the EEB the mean values and standard errors were 37.6 ± 5.0 ($n = 17$) and 18.2 ± 3.3 ($n = 6$) for choanocytes and archeocytes (respectively), whereas for the skeleton-associated cells and the special cells they were 6.9 ± 1.1 ($n = 5$) and 2.8 ± 0.5 ($n = 10$) (respectively). Although the density of gold particles was higher in the EEB than in the





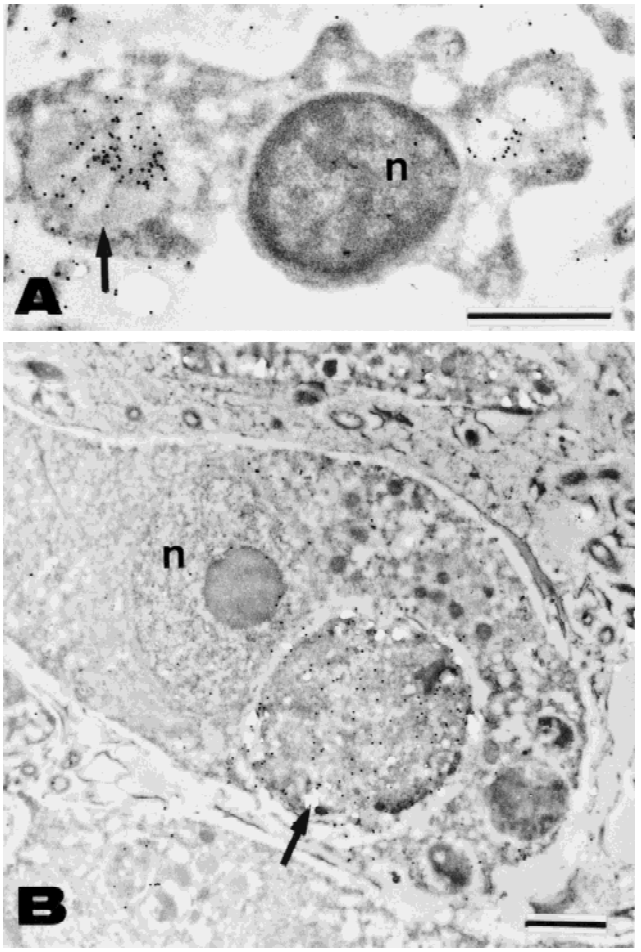


Figure 5. Vacuoles packed with latrunculin B, as indicated by the heavy label *(arrow)*. **A:** Choanocyte. **B:** Archeocytes. *n* = nucleus. Scale bar = 1 μm.

endosome, these differences were not statistically significant.

The bacteria within the sponge tissue were concentrated in limited areas. We therefore measured the bacterial area rather than each bacterium separately. The density of gold particles in bacterial areas as measured in the choanosome was low 0.20 ± 0.02 (n = 5). The density of gold particles appeared to be evenly distributed within the vacuoles of both archeocytes and choanocytes.

Gold particles indicating Lat B presence were found in cell vacuoles within *N. magnifica* choanocytes (Figure 4, A and B, and 5, A) and archeocytes (Figure 5, B). On average, a choanocyte contained 1 to 2 membrane-limited vacuoles, whose interior appeared to be darker and denser than the cell cytoplasm. The mean area of these vacuoles was 0.8 ± 0.1 μm^2 (n = 29), and they were found in 80% of the examined cells. The morphometric analyses showed that the

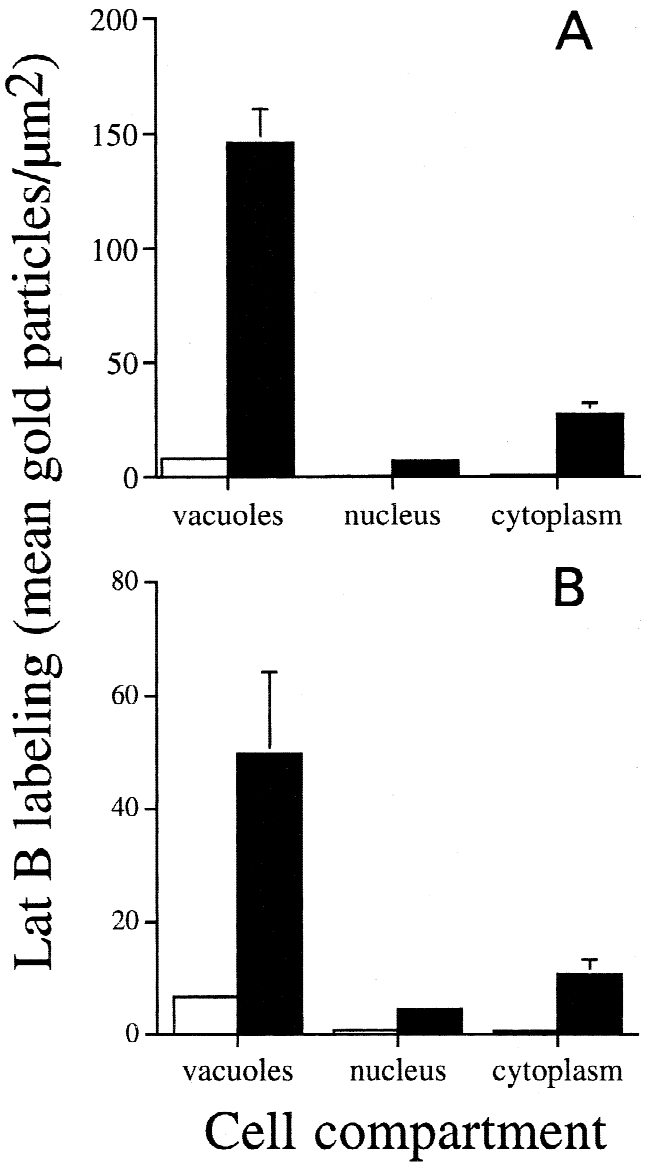


Figure 6. Immunogold labeling of latrunculin B within the organelles of *Negombata magnifica.* **A:** Morphometric analysis of gold particles labeling the choanocytes located in the ectosome-endosome border (■) and the inner endosome (❑); $n = 35$. **B:** Morphometric analysis of gold particles labeling the archeocytes located in the EEB (■) and the inner endosome (❑); $n = 35$. Values are expressed as the mean number of gold particles per square micrometer ± SE.

density of gold particles in those vacuoles within choanocytes (in the choanosome) was significantly higher than in the rest of the cytoplasm and the nucleus (*t* test, $P = .001$; Figure 6, A).

The archeocytes contained a mean of 3 to 4 membrane-limited vacuoles. These vacuoles, which were found in all cells examined in this research ($n = 16$), seemed smaller than the choanocytes vacuoles 0.6 ± 0.1 μm^2. Examination of *N. magnifica* archeocytes photographed in the inner endosome (Figure 6, B), and followed by morphometric analysis, showed that the density of gold particles in the vacuoles was significantly higher than in the rest of the cytoplasm and the nucleus (*t* test, $P = .05$; Figure 6, B). Although the average density of gold particles in archeocytes was lower than within choanocytes in both the endosome and EEB, statistical analysis did not show a significant difference between the cells.

Discussion

The results show that Lat B is localized primarily in the endosomal region of the Red Sea sponge *Negombata magnifica,* which is adjacent to the ectosomal region (EEB). Indirect immunolabeling and gold particles appeared mainly in the endosome and especially at the thin EEB cell layer beneath the sponge cortex (Figure 3, B). Latrunculin B cytotoxicity probably acts against predation, competitors, and epibiotic microorganisms (Neeman et al., 1975; Groweiss et al., 1983; Schatten et al., 1986). We have established the deterrent nature of the total chemical extract of this sponge against predatory fish in ecologically relevant experiments, and the results are to be published separately. It is likely, therefore, that the defensive role of the toxin is best accomplished close to the surface. This assumption correlates well with the localization of toxic secondary metabolites in the ectosome of another sponge, *Crambe crambe* (Uriz et al., 1996a), and in many echinoderms (Bryan et al., 1996), nudibranchs (De Silva, 1991; Fontana et al., 1994), tunicates (Martin and Uriz, 1993), and other marine invertebrates and vertebrates (reviewed by König and Wright, 1996).

The poor labeling of *N. magnifica*'s bacterial symbionts strongly supports the nonsymbiotic origin of Lat B, although it cannot be completely ruled out; Lat B might have been bacterial in origin but later localized and harbored within the sponge cells. In such a case, however, we would have expected to find intenser labeling in bacterial areas. In both the EEB and endosome of *N. magnifica,* gold particles strongly labeled the choanocytes and archeocytes, and to a much less extent special cells and the skeleton-associated cells (Figure 4, B and C). Interestingly, Lat B is also present within the skeletal material and extracellular matrix of the endosome (Figure 3, B). Similar results were obtained by Garson et al. (1992), who localized diisocyanoadociane (a

terpenic toxin) within the Australian marine sponge *Amphimedon* sp. They suggested that some of the toxin might be stored as an extracellular component.

The choanocyte gold labeling is clearly higher than that of the archeocytes in both the EEB and endosome of *N. magnifica* (Figure 4, C). The choanocytes are highly abundant within the sponge, which might explain the high concentration of the toxin (1%–2% of the dry weight) within the sponge (Groweiss et al., 1983). It is highly unlikely that latrunculin is a diet-derived compound because it is found exclusively within the Red Sea *N. magnifica* and not within any other sponge. We conclude that latrunculin is either produced in the choanocytes or stored within these cells. As archeocytes (which are more mobile within the sponge than choanocytes) are located in close proximity to the choanocytes, it is reasonable that they store and mobilize latrunculin throughout the sponge. We suggest, therefore, that choanocytes produce Lat B, which they later transfer to the archeocytes for storage and mobilization. The archeocytes might then transfer the toxin to vulnerable areas within the sponge, such as injured, regenerating or embryo developing sites. Archeocytes were observed to be the first to arrive at an injured area in the sponge (M. Ilan, unpublished data). In a preliminary study of early embryonic stages of *N. magnifica,* two cell types were noted: small cells with a nucleus similar to the choanocytes' nucleus around the embryo surface (where the larval ciliated cells will develop); and within the embryo, large cells that resemble archeocytes. The embryos were not heavily labeled, but the cells that had archeocyte features contained less Lat B than the cells that had choanocyte features (average density of gold particles of 0.8 $\pm$ 0.2 and 1.9 $\pm$ 0.8, respectively).

Ultrastructure analysis showed that Lat B is not distributed evenly in the choanocytes and archeocytes, but rather is concentrated within membrane-bound vacuoles (Figure 6), which contained most of the Lat B present within the cell. A similar confinement of secondary metabolites was found within cells of other sponge species (Table 1). Because Lat B inhibits the polymerization of actin filaments found in cell cytoplasm (Spector et al., 1983, 1989; Schatten et al., 1986), it is reasonable to assume that *N. magnifica* compartmentalizes the toxin in actin-free vacuoles (as the site of either the toxin's synthesis or its storage), away from its own cytoplasmic actin. It is evident that most of the secondary metabolites mentioned in Table 1 are localized in granular cells. Similar to *N. magnifica,* the toxins mentioned in Table 1 are cytotoxic, and their enclosure within membrane-bound cytoplasmic vacuoles might, therefore, be a preventive measure against self-toxination. Production and storage of cytotoxic metabolites inside membrane-bound cytoplasmic vacuoles has already been shown in plants (Roberts et al., 1983; Brisson et al., 1992), cyanobacteria (Shi et al., 1995), and dinoflagellates (Anderson and Cheng, 1988; Zhou and Fritz, 1994). Because Lat B is also highly lipophilic (Groweiss et al., 1983), it may also be expected to be associated with membrane regions in the cells.

In conclusion, immunolabeling of Lat B supported the idea that it is synthesized within *N. magnifica* choanocytes, but stored within its archeocytes and mobilized by them. The choanocytes do not appear to be intermediates in the production of the toxin, but rather may act as its producer. These results, combined with uncovering the biosynthetic pathway of Lat B, may provide a useful framework from which the biological and ecological role of the toxins in the sponge and their biotechnological production could be addressed.

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