Salomon CE, Faulkner DJ.. Localization studies of bioactive cyclic peptides in the ascidian Lissoclinum patella. J Nat Prod 65: 689-692

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · JUNE 2002

Impact Factor: 3.8 · DOI: 10.1021/np010556f · Source: PubMed

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Localization Studies of Bioactive Cyclic Peptides in the Ascidian *Lissoclinum patella*

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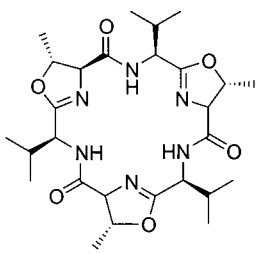
Received November 7, 2001

A Palauan specimen of the ascidian *Lissoclinum patella* contained the cyclic peptides patellamides A−C. In contrast to previous reports concerning the location of cyclic peptides in *L. patella* and *L. bistratum*, it has been demonstrated that these cyclic peptides were not located in the symbiotic cyanobacterium *Prochloron* sp. but were instead distributed throughout the ascidian tunic. The relevance of these results with respect to the biosynthetic origins of the patellamides is discussed.

The isolation of marine natural products that are structurally similar to those found in terrestrial microbes is commonly cited as "circumstantial evidence" for a symbiotic source of the compounds. A comparison of the cyclic peptides from ascidians with those isolated from terrestrial cyanobacteria reveals many similarities, and in one instance the same cyclic peptide was isolated as westiellamide (**1**) from the cyanobacterium *Westiellopsis prolifica*[1] and as cycloxazoline (**1**) from the ascidian *Lissoclinum bistratum*.[2] In the case of the tropical ascidian *Lissoclinum patella*, the hypothesis that the cyclic peptides are produced by symbionts is further supported by the consistent presence of the cyanobacterium *Prochloron* sp. within the ascidian tissues. Due to these observations, the biosynthetic source of the peptides in ascidians has been the subject of much speculation and several studies that produced inconsistent results.[3−7]

Degnan et al. studied the localization of the peptides by isolating *Prochloron* cells from a *L. patella* colony and comparing the secondary metabolites of the cyanobacterial cells with those of the remaining *Prochloron*-free animal.[3] They reported that lissoclinamides 4 and 5, ulithiacyclamide, patellamide D (**2**), and ascidiacyclamide (**3**) could be isolated from the cyanobacteria in equal or greater amounts on a weight-to-weight basis than were found in the intact colony.[4] Using the same techniques, Degnan et al. also studied the location of secondary metabolites in the related ascidian *L. bistratum*, which also lives symbiotically with *Prochloron* sp. on the same reef. *L. bistratum* produced both cyclic peptides and the macrocyclic ether bistramide A (= bistratene A) (**4**), which was not found in the cyanobacteria and was assumed to be produced by the ascidian.[4] In contrast, a second study of *L. bistratum* by Biard et al. demonstrated that bistramide A (**4**) was localized in isolated *Prochloron* cells at concentrations up to 4 times higher (% dry weight) than were found for the intact association.[5] Due to these contrasting results, coupled with our own preliminary studies, we decided to reexamine the location of the metabolites in Palauan samples of *L. patella* using careful dissection, cell and tissue preservation, microscopy, and NMR analysis. Our current studies indicate that the peptides are not stored in the *Prochloron* cells and are instead present in significant quantities throughout the ascidian tunic.



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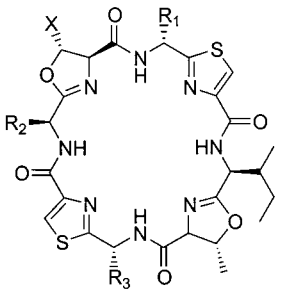
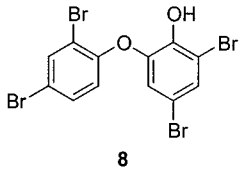


	X	R_1	R_2	R_3
2	Me	D-Ala	L-Ile	D-Phe
3	Me	D-Val	L-Ile	D-Val
5	H	D-Val	L-Ile	D-Val
6	Me	D-Ala	L-Leu	D-Phe
7	Me	D-Ala	L-Val	D-Phe



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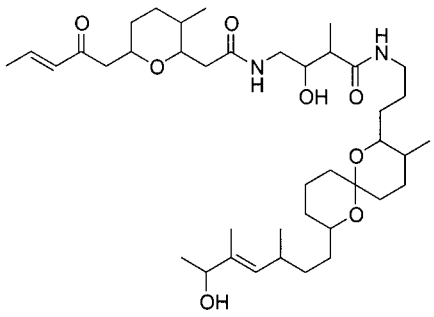

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Results and Discussion

Specimens of *Lissoclinum patella* were found in shallow water near the entrance of Mutmalachel Lake in Palau. A single ascidian colony was carefully removed from the coral rubble substrate, placed underwater in a plastic bag, and immediately returned to the Coral Reef Research Foundation laboratory. At the laboratory, a portion of the sample was dissected as described below and the remainder was frozen for chemical studies. The bulk sample was worked up using standard procedures to obtain patellamides A (**5**, 0.05% dry wt), B (**6**, 0.02% dry wt), and C (**7**, 0.1% dry wt), which were identified by comparison of their ^{1}H NMR and mass spectral data with literature values.[8]

Clean, intact *Prochloron* cells were obtained from sliced tunic using gentle irrigation followed by repeated centrifu-

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10.1021/np010556f CCC: $22.00
Published on Web 04/26/2002

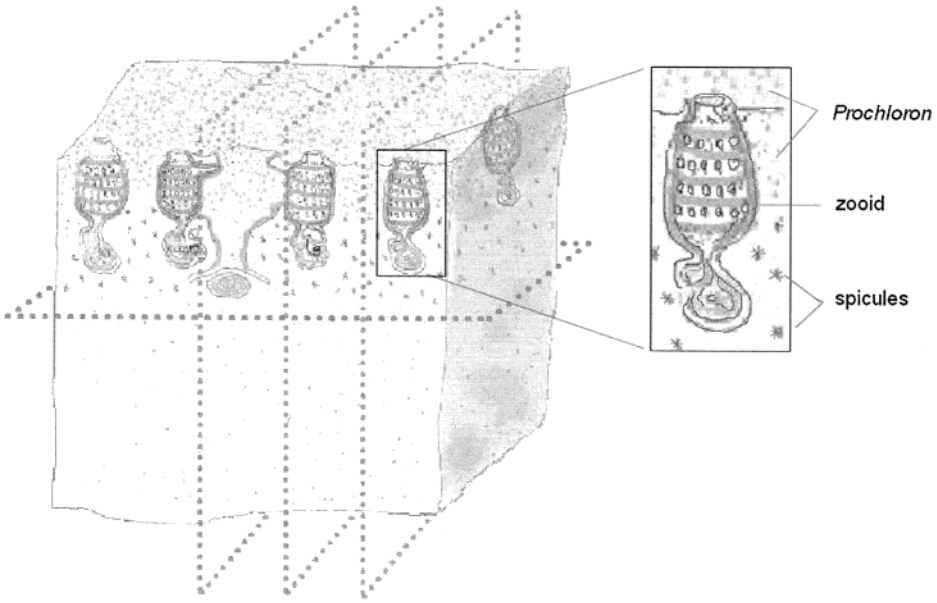

Figure 1. Illustration of the sectioning of *L. patella* tissue and (inset) the locations of spicules and *Prochloron* cells with respect to the zooids.

gation and resuspension in calcium/magnesium-free artificial seawater until free of cellular debris. Although some *Prochloron* cells have been reported to live intracellularly,[9] microscopic examination of the irrigated tunic showed it to be relatively free of cyanobacteria (<5% remaining). The "*Prochloron*-free" tissue was then dissected into an "upper" portion that contained the zooids, a few *Prochloron* cells, calcareous spicules, and epibonts and a "lower" portion that consisted of tunic that was completely free of zooids, *Prochloron* cells, calcareous spicules, and epibionts (Figure 1). Two subsamples of each of the three fractions were fixed in formaldehyde or gluteraldehyde for microscopy, while the remainder was frozen for chemical analysis.

Each of the fractions was extracted with 1:1 methanol/dichloromethane. ^{1}H NMR analysis of extracts of the *Prochloron* cells (17 mg), upper tunic (301 mg), and lower tunic (77 mg) indicated that the peptides were not present in significant quantities in the cyanobacterial cells but were distributed almost equally in the upper and lower tunic (Figure 2). The absence of patellamide signals in the spectrum of the *Prochloron* extract is unlikely to be due to the small amount of material available because signals due to other compounds were clearly observed. Their presence in the lower tunic is significant because examination by light microscopy showed that the lower tunic is quite sparse and filled with large channels and contains primarily fibroblast-like cells. Bacteria could not be detected in a section of the lower tunic by TEM analysis.

The results of this study show that peptides cannot always be extracted from *Prochloron* cells isolated from *L. patella*. We believe that the careful separation of *Prochloron* cells from all other material may be the key step in obtaining this result. The data suggest that either the ascidian itself is producing the compounds or the cyanobacteria synthesize the peptides and then immediately exude them into the ascidian tunic and cloacal cavities. We cannot exclude the latter scenario because it is known that antibiotic-producing bacteria can actively pump compounds out of their cells into the culture medium, presumably to avoid self-toxicity.[10] Although secondary metabolites are not generally found in the media of cultured cyanobacteria, Unson et al. reported the exudation of a brominated diphenyl ether **8** by the cyanobacterial symbiont *Oscillatoria spongeliae* in the sponge *Dysidea herbacea*.[11] However, in that case the brominated diphenyl ether **8** was easily detected in *O. spongeliae* cells as well as being located as extracellular crystals in the sponge matrix.

Other biosynthetic hypotheses include the production of the peptides by eubacterial symbionts living in the tunic. However, so few bacterial cells were observed by TEM in the lower tunic that it is difficult to imagine that they can support the production of the high quantities of compounds found there. Other studies have demonstrated significant concentrations of unicellular eubacteria associated with the branchial bars and incurrent siphons of *L. patella*,[9] but one again needs to explain how the peptides migrate to the lower tunic.

Additional evidence against the production of the cyclic peptides by *Prochloron* is the fact that the cyanobacterium also lives symbiotically with other ascidian species such as *L. voeltzkowi* and *Diplosoma virens* that have not been reported to contain cyclic peptides. This argument has limited validity because the true phylogeny of the associated *Prochloron* as well as the host has yet to be determined. Sequencing of RNA polymerase genes from *Prochloron* obtained from several ascidian species suggests that at least two closely related groups of *Prochloron* live in the same area in Palau.[12]

The true test for the source of the cyclic peptides would be to culture *Prochloron* independent of an ascidian host and to test for the production of metabolites. Despite numerous attempts, however, *Prochloron* has not been cultivated successfully.[13] It might also be instructive to grow *L. patella* without its *Prochloron* symbionts, although it appears that the association is obligate for the ascidian.[13]

It is quite obvious that more research is needed to unambiguously assign the biosynthetic source of the peptides. This paper was the result of a single field experiment that should now be repeated with multiple specimens of *L. patella* and *L. bistratum* from different locations. Localization of the cyclic peptides in specific cells will be helpful unless the cells implicated are known to transport peptides. The best approaches to this problem would be to determine the cellular location of the peptides using an antibody to a specific peptide or to determine the location of the biosynthetic genes, both of which are technically difficult procedures.

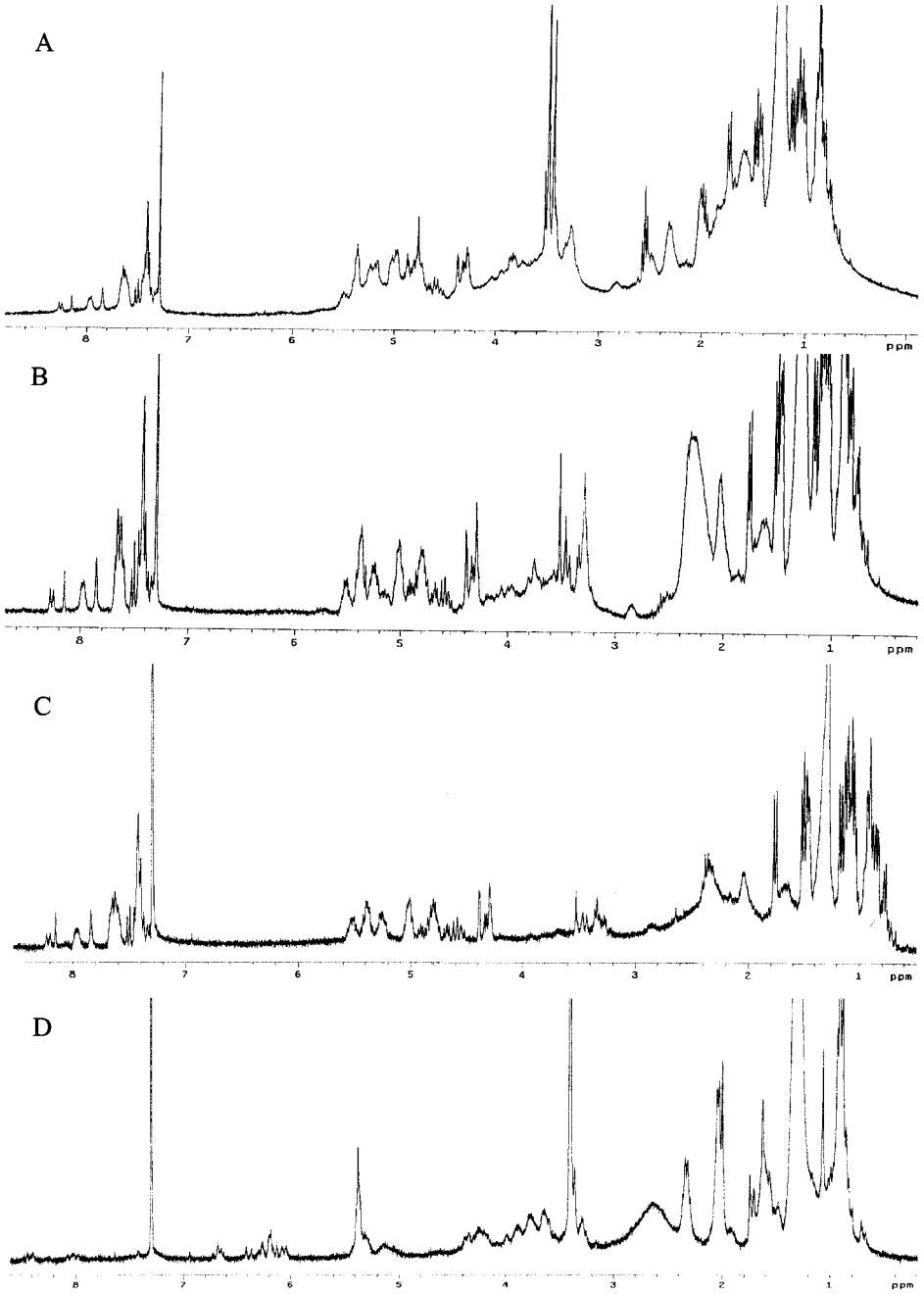

Figure 2. ^{1}H NMR spectra of extracts from *L. patella* sections. A: Whole tissue. B: Upper "zooid" layer. C: Lower "tunic" only layer. D: *Prochloron* cells.

Experimental Section

Animal Material. Specimens of *Lissoclinum patella* Gottschaldt were collected by hand in shallow water (−1 m) near the entrance to Mutmalachel Lake in Palau. They were carefully removed from the rock and coral debris substrate, placed underwater into plastic bags, and immediately brought back to the laboratory at the Coral Reef Research Foundation for dissection and preservation.

Dissection and Preservation. A single specimen was used for this study. The majority of the sample was frozen for later analysis, and small aliquots were preserved in 4% paraformaldehyde/MOPS (morpholinepropanesulfonic acid) (= general fix), ethanol, and 1.2% glutaraldehyde/1.8% formaldehyde in calcium/magnesium-free artificial seawater with 1 M phosphate buffer (PB-CMF-ASW), pH 7.4 (= TEM fix). All preserved samples were stored at 4 °C.

The remaining sample was dissected as follows: A palm size piece of the colony (1−2 cm thick) was carefully sliced into 2−3 mm thick long sections. Each section was turned on its side, and a jet of PB-CMF-ASW was used to dislodge the *Prochloron* cells from the cloacal cavities and upper tunic. The remaining tunic (95% *Prochloron*-free) was cut into an "upper" half that contained the zooids and a "lower" half that consisted of only tunic. These tissue samples were divided into three aliquots that were frozen and preserved in general and TEM fixatives. The *Prochloron* cells were gently centrifuged in 50 mL conical tubes to concentrate the cells and then further washed and recentrifuged several times to obtain a clean cell suspension. The cells were preserved in the same manner as the above tissue samples.

Chemical Analysis. Upon return to the lab at SIO, the "upper" tunic, "lower" tunic, and *Prochloron* cell pellet samples that had been preserved in 4% paraformaldehyde were washed

of fixative with several changes of ASW and freeze-dried. These samples and frozen nonpreserved samples were extracted with 1:1 MeOH/CH_2Cl_2 (10 mL) and sonicated for 30 min. The extracts were filtered, dried in vacuo, weighed, and analyzed by ^{1}H NMR spectroscopy in $CDCl_3$. The frozen and preserved samples gave essentially identical results except that some additional signals due to the fixative were observed in the spectrum of the preserved sample.

Isolation of Patellamides A−C. The frozen whole tissue was extracted as in previous studies.[8] Briefly, the wet tissue was extracted in methanol and the extract was filtered and dried. The extract was subjected to solvent partitioning between EtOAc and H_2O, and the EtOAc partition was further purified by RP-HPLC (75% MeOH/H_2O) to yield the known compounds patellamides A (**5**, 0.05% dry wt), B (**6**, 0.02% dry wt), and C (**7**, 0.1% dry wt), which were identified by ESIMS and ^{1}H NMR spectroscopy.

Acknowledgment. We thank the Republic of Palau for a research and collecting permit and the Coral Reef Research Foundation for logistical support. This research was funded by NSF grant CHE-9816169 and an ARCS Fellowship to C.E.S.

References and Notes

(1) Princep, M. R.; Moore, R. E.; Levine, I. A.; Patterson, G. M. L. *J. Nat. Prod.* **1992**, *55*, 140−142.
(2) Hambley, T. W.; Hawkins, C. J.; Lavin, M. F.; Watters, D. J. *Tetrahedron* **1992**, *48*, 341−348.
(3) Degnan, B. M.; Hawkins, C. J.; Lavin, M. F.; McCaffrey, E. J.; Parry, D. L.; van den Brenk, A. L.; Watters, D. J. *J. Med. Chem.* **1989**, *32*, 1349−54.
(4) Degnan, B. M.; Hawkins, C. J.; Lavin, M. F.; McCaffrey, E. J.; Parry, D. L.; Watters, D. J. *J. Med. Chem.* **1989**, *32*, 1354−9.
(5) Biard, J. F.; Grivois, C.; Verbist, J. F.; Debitus, C.; Carre, J. B. *J. Mar. Biol. Assoc. U.K.* **1990**, *70*, 741−746.
(6) Muller, W. E. G.; Maidhof, A.; Zahn, R. K.; Conrad, J.; Rose, T.; Stefanovich, P.; Muller, I.; Friese, U.; Uhlenbruck, G. *Biol. Cell* **1984**, *51*, 381−388.
(7) Sings, H. L.; Rinehart, K. L. *J. Ind. Microbiol. Biotech.* **1996**, *17*, 385−396.
(8) Ireland, C. M.; Durso, A. R., Jr.; Newman, R. A.; Hacker, M. P. *J. Org. Chem.* **1982**, *47*, 1807−1811.
(9) Swift, H.; Robertson, D. L. *Symbiosis* **1991**, *10*, 95−113.
(10) Martin, J. F.; Demain, A. L. *Microbiol. Rev.* **1980**, *44*, 230−251.
(11) Unson, M. D.; Holland, N. D.; Faulkner, D. J. *Mar. Biol.* **1994**, *119*, 1−11.
(12) Palenik, B. S.; Swift, H. *J. Phycol.* **1996**, *32*, 638−646.
(13) Lewin, R. A.; Cheng, L. *Prochloron: A Microbial Enigma*; Chapman and Hall: New York, 1989.

NP010556F