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Pseudopterosin Biosynthesis in *Symbiodinium* sp., the Dinoflagellate Symbiont of *Pseudopterogorgia elisabethae*

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Summary

Investigations are reported that identify the biosynthetic source and origins of the pseudopterosins, pharmacologically important diterpene glycosides, in the gorgonian coral *Pseudopterogorgia elisabethae*. We report here the isolation of physiologically significant levels of endogenous pseudopterosins A, B, C, and D from purified symbionts identified as the dinoflagellate *Symbiodinium* sp. Biosynthetic studies in photosynthesizing symbiont isolates utilizing ^{14}C-labeled inorganic carbon and the tritiated intermediate geranylgeranyl diphosphate yielded radiochemically pure pseudopterosins A through D and the first committed intermediate, elisabethatriene. The $^{14}CO_2$ uptake into the pseudopterosin pathway occurred at low levels compared to the $^{14}CO_2$ uptake into total lipids, suggesting a large reserve pool of the compounds. These results reveal for the first time that pseudopterosin biosynthesis is occurring within the algal symbiont and suggests the physiological implications of this biosynthesis.

Introduction

The pseudopterosins are effective antiinflammatory and analgesic agents that have important actions on degranulation and release mechanisms in immune cells [1, 2]. They are currently marketed widely as antiirritants in cosmetic formulations. The exact mechanism of action remains elusive but may involve membrane stabilization [3]. The relationship of the antiinflammatory effects of the pseudopterosins to their endogenous function in the coral-symbiont complex is not known. We chose to begin this investigation by determining if pseudopterosin biosynthesis occurs in the dinoflagellate symbiont.

The pseudopterosins are a group of 15 unique tricyclic diterpene glycosides which are isolated from the gorgonian soft coral, *Pseudopterogorgia elisabethae* [4, 5]. Broad applications for therapeutic uses of the pseudopterosins have been limited by supply issues, as collection of the coral from its natural habitat has reached the tonnage level in recent years [6]. Ecological and environmental concerns for the sensitive reef habitats in the Caribbean have initiated numerous efforts aimed at the development of viable production methods for sustainable pseudopterosin supplies [7–10].

Like many reef-dwelling invertebrates, *P. elisabethae* contains intracellular symbionts of the genus *Symbiodinium*. These dinoflagellates contribute substantially to the nutrition of their hosts by translocating photoassimilates, making them major primary producers in reef habitats [11, 12]. There has been some debate in the literature regarding the general contributions of symbiont and host to diterpene biosynthesis, and to this date the involvement of symbionts in terpene production remains unresolved [13–16]. Interestingly, the Australian resin-producing higher plant, *Eremophila serrulata*, has been reported to produce a metabolite that contains the pseudopterosin skeleton [17]. This compound, a tricyclic diterpene phenolic acid, was shown to possess a carbon skeleton of the C-3 epimer of pseudopterosin K [5].

In the study presented in this paper, we provide direct evidence that pseudopterosin A, B, C and D (PsA, PsB, PsC, and PsD) (Figure 1) biosynthesis occurs in the symbiotic partner of P. *elisabethae*. The intracellular symbiont identified as *Symbiodinium* sp. type B1 [18, 19] was isolated from gorgonian tissues and repeatedly purified on Percoll gradients. With this separation and purification technique refined, it was possible to examine symbiont metabolism independent of coral metabolism. We chose to investigate pseudopterosin biosynthesis in *Symbiodinium* sp. cells from inorganic carbon and from the diterpene precursor, geranylgeranyl diphosphate (GGPP), to the first committed intermediate, elisabethatriene, and the final products.

Results and Discussion

Purity and Species Identification

The *Symbiodinium* sp. collected from *P. elisabethae* at Sweetings Cay, Bahamas was identified as the common type "B1," a symbiont also found in numerous other gorgonians and corals from the Caribbean [20]. The ITS 2 sequence of B1 is identical to the informally described species *Symbiodinium burmudense*.

Symbiodinium sp. cells purified by repeated washing and Percoll gradients showed no visible impurities as determined by light microscopy. Furthermore, DNA labeling by DAPI staining failed to detect any bacterial or coral cell contaminants. These findings indicate that our preparation of *Symbiodinium* sp. cells achieved a high level of purity.

Presence of Endogenous Pseudopterosins in *Symbiodinium* sp. and the Intracolonial Distribution

Endogenous levels of pseudopterosins A, B, C, and D in purified *Symbiodinium* sp. were quantified from lipid extracts using HPLC. Compounds were identified by comparison of retention times with those of standards and confirmed by coinjection with authentic standards

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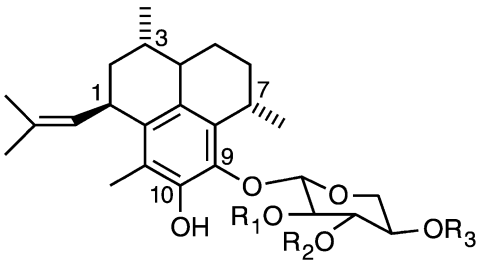

PsA (**1**) $R_1 = R_2 = R_3 = H$
PsB (**2**) $R_1 = Ac$, $R_2 = R_3 = H$
PsC (**3**) $R_2 = Ac$, $R_1 = R_3 = H$
PsD (**4**) $R_3 = Ac$, $R_1 = R_2 = H$

Figure 1. Structures of Pseudopterosins A–D

(Figure 2). The pseudopterosin content was quantified by comparison of the sample HPLC traces with a calibration curve. The concentration of the mixture of pseudopterosin A through D in the purified algal cells was found to be 19 $\pm$ 4 μg/10^6 cells.

We have observed that the concentration of pseudopterosins and other biosynthetic intermediates varies between individual *P. elisabethae*. An analysis of a clipping from an individual specimen of *P. elisabethae* revealed that the pseudopterosins comprised 5% of the lipid extract, whereas an analysis of purified *Symbiodinium* sp. cells from the same individual indicated that these compounds comprised 11% of the algal lipid extract.

An individual specimen of *P. elisabethae* was divided

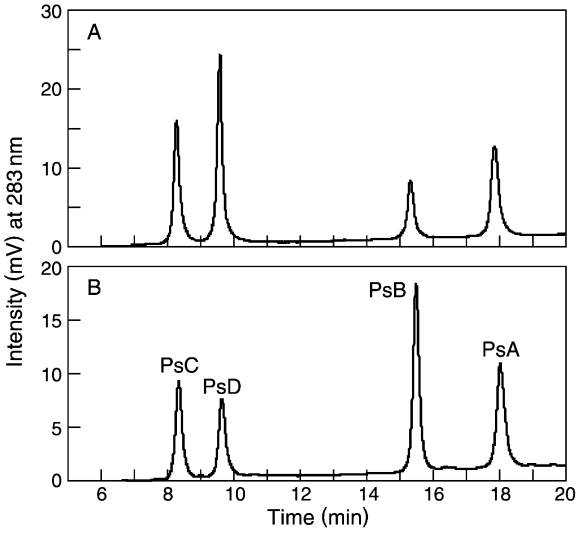

Figure 2. NP-HPLC Trace of the Pseudopterosins

(A) Representative NP-HPLC chromatogram of pseudopterosin A, B, C, and D purified from *Symbiodinium* sp. cells (retention times of 8.27, 9.57, 15.30, and 17.84, respectively).
(B) Pseudopterosin (Ps) standards on NP-HPLC (retention times of 8.33, 9.64, 15.47, and 18.01, respectively).

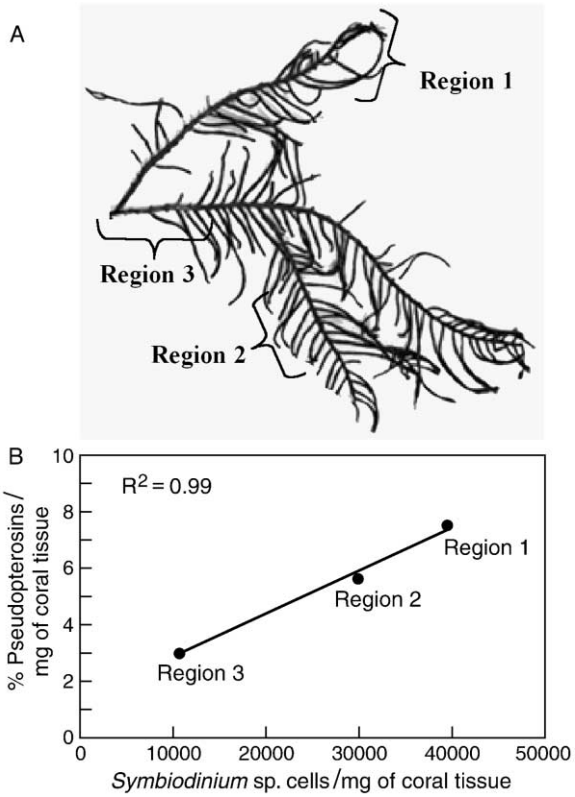

Figure 3. Intracolonial Distribution of Pseudopterosins and *Symbiodinium* sp. Cells

(A) Diagram of *P. elisabethae* colony showing segments.
(B) Correlation between number of *Symbiodinium* sp. cells and pseudopterosin concentration.

into three regions (Figure 3A). The concentration of *Symbiodinium* sp. cells/mg coral tissue, as well as the pseudopterosin concentration, was 3-fold greater in region 1 than in region 3. Figure 3B shows a 99% correlation between the number of *Symbiodinium* sp. cells and pseudopterosin concentration per coral weight, providing evidence that the *Symbiodinium* sp. cells may be involved in pseudopterosin production.

Presence of Endogenous Elisabethatriene in *Symbiodinium* sp.

In previous studies, elisabethatriene (Figure 4) was identified as the diterpene cyclase product, and thus the first committed intermediate of pseudopterosin biosynthesis [10]. In the current study, endogenous elisabethatriene was detected in a hexane extract of purified *Symbiodinium* cells by HPLC. Levels as high as 0.113 μg/10^6 cells (0.034% of lipid extract) were observed. The presence of elisabethatriene in this extract was confirmed by ^{1}H-NMR (Figure 4). ^{1}H-NMR assignments for elisabethatriene were presented elsewhere [10].

The analytical work described above clearly indicates that pseudopterosins are present in the *Symbiodinium* sp. cells, in fact, at a much higher concentration than in the coral tissue. Further, the presence of elisabethatriene in the *Symbiodinium* sp. extract suggests that this dinoflagellate is the biosynthetic source of these potent

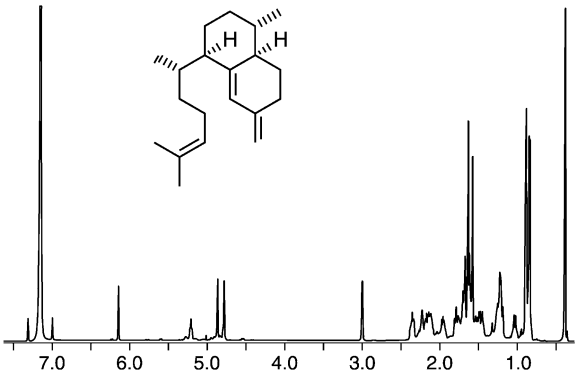

Figure 4. Structure and Proton NMR of Elisabethatriene (on 500 MHz in C_6D_6)

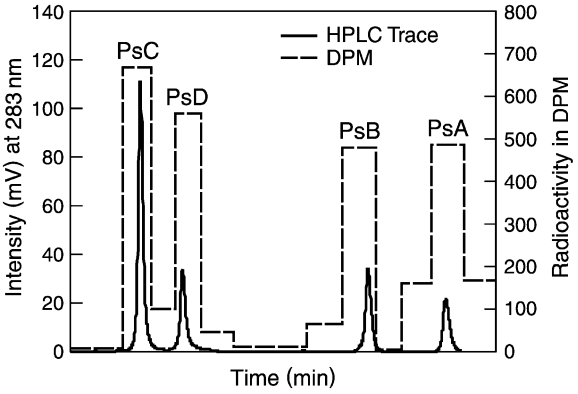

Figure 5. Representative NP-HPLC Trace of Pseudopterosins Produced after 48 Hr Incubation with $NaH^{14}CO_3$ with Associated Radioactivities for Each Pseudopterosin
Standards were added.

antiinflammatory agents. To confirm the algal origin of pseudopterosin biosynthesis, a number of biosynthetic experiments were conducted.

Biosynthesis of Pseudopterosins from Inorganic Carbon

Photosynthetic uptake and biosynthesis of inorganic carbon into the pseudopterosins was observed 48 hr post incubation of *Symbiodinium* sp. cells treated with ^{14}C-labeled $NaHCO_3$ under ambient light. Analogous experiments were carried out in the dark or with cells treated with 100 μM mevastatin to inhibit pseudopterosin biosythesis. In all experiments, cells were extracted and the transformation of $NaH^{14}CO_3$ to total lipids and pseudopterosins was quantified. Incorporation of the label is large into the lipids of the cells (5.06×10^6 dpm), indicating that photosynthesis is readily occurring. The key biosynthetic intermediate, elisabethatriene, had radioactivity of $4.3 \pm 0.54 \times 10^3$ dpm and specific activity of $3.69 \pm 0.2 \times 10^8$ dpm/mmol. Recovered radioactivity of the pseudopterosin pool (Ps A–D) was $2.6 \pm 0.2 \times 10^3$ dpm with specific activity of $5.18 \pm 0.54 \times 10^7$ dpm/mmol.

After initial experiments (data not shown), it was found that increasing incubation time from 24 hr to 48 hr, increasing concentration of cells to 2×10^6 cells/ml, and increasing concentration of added $NaH^{14}CO_3$ to 2.29 μCi/ml increased levels of recovered radioactivity in both total lipids and pseudopterosins relative to earlier experiments. The labeled $NaH^{14}CO_3$ added in this experiment was equivalent to approximately 0.038 μM. The natural occurring levels of inorganic carbon in seawater range from 1800–3200 μM [21], indicating over a 40,000-fold dilution of the label in seawater. Once incorporation occurs, further dilution of the label in the metabolic pool of compounds in the *Symbiodinium* sp. cells takes place. In spite of these dilution factors, significant labeling does occur and is evidence of de novo synthesis of the pseudopterosins in photosynthesizing cells.

The ability of the cells to photosynthesize and incorporate inorganic carbon into the lipid fraction in *Symbiodinium* sp. is evidence of viability of the cells after the separation procedure and their ability to survive in vitro for at least 48 hr. The isolation of ^{14}C-labeled pseudopterosins within the lipid pool is evidence that CO_2 is a substantial carbon source for these compounds. Expressed on a per hour basis, *Symbiodinium* sp. cells produced radioactive pseudopterosins at a rate of approximately 3.6 dpm/10^6 cells/hr, which comprised only 0.05% of the total labeled lipids after 48 hr. This indicates a low turnover rate of the pseudopterosins and a sizeable reserve pool of the compounds that is also reflected in the high endogenous (unlabeled) pseudopterosin concentrations.

Evaluation of Radiochemical Purity

Radiochemical purity of the pseudopterosins was established by HPLC purification and subsequent derivatization. Following initial collection of the pseudopterosin peaks, the radioactivity profile of the eluent was monitored during the second HPLC analysis. In all cases, radioactivity was only associated with the pseudopterosin fractions (Figure 5). To further confirm radiochemical purity of the pseudopterosin compounds, base hydrolysis of Ps B, C, and D yielded Ps A. Four hundred micrograms of standards was added to the cell extract (Figure 6). Specific activity of Ps A, B, C, and D before base hydrolysis was 2.04×10^6 dpm/mmol. Final specific activity of the Ps A was 1.85×10^6 dpm/mmol.

Photosynthetic Inhibition of Ps Biosynthesis

In a separate set of experiments, inhibition of pseudopterosin biosynthesis in *Symbiodinium* sp. cells was analyzed in 24 hr dark- and light-adapted cells and boiled cells (see Supplemental Table S1 at http://www.chembiol.com/cgi/content/full/10/11/1051/DC1). There was a 98% reduction of photosynthesis as measured by ^{14}C incorporation into lipids in dark-treated cells. There was corresponding reduction (100%) in ^{14}C incorporation into the pseudopterosin pool, which demonstrates that the labeling phenomena seen in the light-exposed cells was indeed due to photosynthetic processes and not isotopic exchange. The complete inhibition of pseudopterosin biosynthesis in boiled cells further confirms that the ^{14}C incorporation into the pseudopterosins is due to an enzymatic process.

Metabolic Inhibition of Ps Biosynthesis

Since biosynthesis was blocked in dark-treated and boiled cells, we further investigated pseudopterosin bio-

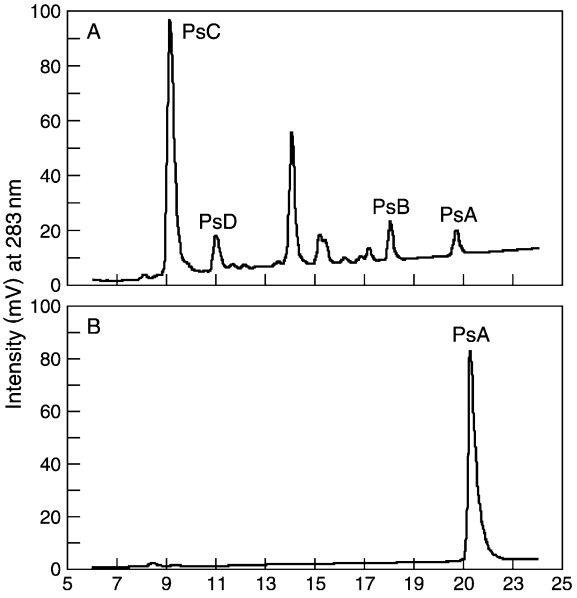

Figure 6. NP-HPLC Trace of Pseudopterosins before and after Base Hydrolysis

(A) NP-HPLC trace of pseudopterosin A–D before base hydrolysis. Only the peaks corresponding to the pseudopterosins were collected and subjected to base hydrolysis.

(B) Reaction mixture after base hydrolysis to yield PsA as only product.

synthesis using the mevalonic acid pathway inhibitor, mevastatin sodium. Mevastatin sodium inhibits HMG-CoA reductase and the biosynthesis of mevalonic acid, which subsequently inhibits the production of cholesterol and other terpenoid products [21]. Statin compounds have been used in algae such as *Haematococcus* and various chlorophytes to discern the origins of terpenoid biosynthetic pathways [22, 23]. In *Symbiodinium* sp. cells incubated with radiolabeled $NaH^{14}CO_3$ for 48 hr, the inhibitory effects of mevastatin can be observed during pseudopterosin biosynthesis (Figure 7). ^{14}C incorporation into the pseudopterosins was reduced significantly ($p < 0.01$) by 55% compared to controls,

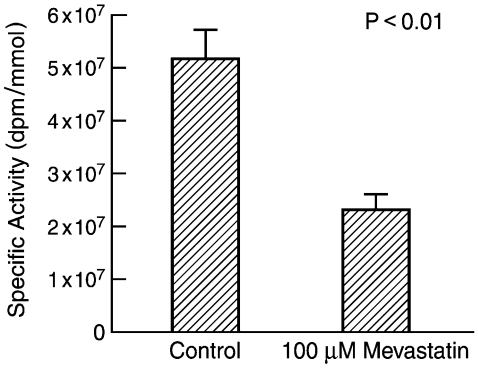

Figure 7. Inhibition of Incorporation of $NaH^{14}CO_3$ into Pseudopterosin Biosynthesis by the HMG-CoA Reductase Inhibitor Mevastatin Sodium Measured by Specific Activity of Ps A, B, C, and D as dpm/mmol ($n = 3$)

while ^{14}C incorporation into lipids was not effected. The effects of mevastatin suggests the involvement of the mevalonic acid pathway in pseudopterosin biosynthesis.

Overall, the data presented here are supported by previous studies conducted in several algal groups. In diatoms [24] and in the unicellular chlorophyte *Acetabularia* [25], it has been demonstrated that CO_2 in the form of $NaHCO_3$ is the main carbon source for isoprenoid biosynthesis. In *Acetabularia*, 48% of the radiolabeled lipids were attributed to prenyl lipids with biosynthesis taking place in isolated chloroplasts. Various cultured phytoplankton species such as the chlorophyte *Dunaliella* and the cryptophyte *Rhodomonas* showed quantitatively similar labeling in terpenoid-based pigments from $^{14}CO_2$ incubations [26]. Rice et al. demonstrated ^{14}C carbonate uptake into intact coral sections of *Pseudoplexaura porosa*, including the labeling of the diterpene crassin acetate [27].

Biosynthesis of Pseudopterosins from Terpene Precursors

In addition to inorganic precursors, the capability of purified *Symbiodinium* sp. cells to metabolize GGPP was tested in two systems: an in vivo system using intact cells and an in vitro system using a cell lysate.

In the in vivo system, purified cells in filtered seawater were incubated with (0.72 μCi) ^3H-GGPP for 48 hr. Purification of the pseudopterosins by HPLC and analysis by scintillation counting indicated that pseudopterosins A–D collectively were radioactive (3.7×10^3 dpm, 5.4×10^7dpm/mmol). This represented a yield of only 0.23% for the biosynthetic transformation of GGPP to the pseudopterosins, suggesting that GGPP transport into intact cells may not be optimal. Elisabethatriene was also found to be radioactive (640 dpm).

The transport of precursors into lipid pools is limited when the lipid is sequestered in subcellular compartments, and for this reason cell-free extracts were developed to increase substrate bioavailability.

In the in vitro experiment, a cell-free extract was incubated with (5 μCi) ^3H-GGPP, $MgCl_2$, and 1% Triton X-100 for 24 hr. Triton X-100 was added to help solubilize GGPP and to optimize substrate micelle concentration. Both substrate availability and yield of the pseudopterosins were increased by 10-fold in the presence of Triton X-100. Following extraction and purification by HPLC, the pseudopterosins were found to be radioactive (6.9×10^4 dpm, specific activity of 4.23×10^9 dpm/mmol).

This represents a yield of 0.62% for the conversion of GGPP into the pseudopterosins, a marked increase from the intact cell yields. In a 45 min incubation, elisabethatriene was also found to be radioactive (1016 dpm). The pseudopterosins and elisabethatriene were not radiolabeled when the cell-free extract was boiled for 1 hr prior to incubation.

Significance

These results provide the first direct evidence of the presence of a pseudopterosin biosynthetic pathway in *Symbiodinium* sp. cells. The chemical analysis of

the purified *Symbiodinium* sp. from the gorgonian *P. elisabethae* indicated a high level of pseudopterosins (11% of lipid content) and the presence of the diterpene cyclase product, elisabethatriene. A very high correlation between the number of *Symbiodinium* sp. cells and pseudopterosin concentration per coral weight provided evidence that the *Symbiodinium* sp. cells are directly involved in pseudopterosin biosynthesis.

The biosynthetic data from ^{14}C-labeled CO_2 confirmed that the algal cells possess all of the biosynthetic machinery required for biosynthesis. The elimination of this biosynthesis in dark-treated cells supports our view that pseudopterosin production is dependent on algal photosynthetic processes. These experiments also revealed that the biosynthetic rate of production of the pseudopterosins is low and that the products are stored within the symbiont cell in large quantities, as reflected by the large intracellular standing pool of extractable pseudopterosins. The accumulation of pseudopterosins within the symbiont cells indicates that the compounds may have a physiologically important role and may be stored for cell signaling purposes.

Pseudopterosin biosynthesis in the symbiont cell also utilizes organic diterpene precursors, as shown in the biosynthetic experiments with tritium-labeled GGPP. Under our experimental conditions, this synthesis may follow the mevalonic acid pathway, as suggested by the reduction of pseudopterosin biosynthesis by the HMG-CoA reductase inhibitor mevastain. It is possible that the mevalonate and nonmevalonate pathways are both functioning within the *Symbiodinium* cell; resolving this issue requires additional studies [28].

Overall, our results demonstrate that the symbiotic dinoflagellate *Symbiodinium* sp. in *P. elisabethae* is the source of the antiinflammatory pseudopterosins. Our findings provide the important knowledge necessary to develop strategies for the production of the pseudopterosins and to define their role in the complex symbiont-coral community.

Experimental Procedures

P. elisabethae was collected in May 2001 at Sweetings Cay in the Bahamas at a depth of 10 m. All trans geranylgeranyl diphosphate [1-^{3}H] triammonium salt 60 Ci/mmol and $NaCO_3$ [^{14}C] 50–60mCi/mmol were purchased from American Radiolabeled Chemicals, St. Louis, MO. Millipore Steritop filters (0.22 μm) were purchased from Fisher Scientific. Percoll and Triton X-100 were purchased from Sigma-Aldrich Chemicals. Mevastatin was purchased from Biomol. DNeasy plant mini prep kit was purchased from Qiagen Corporation, Santa Clarita, CA.

HPLC purifications were performed using either a Perkin Elmer Series 400 pump connected to a PE 235 diode array detector with Vydac C18 or silica columns, or a Hitachi L-6200A Intelligent Pump connected to a L-4200 UV-Vis detector with Varian Chrompack C18 or silica columns. ^{1}H-NMR spectra were recorded in C_6D_6 on an Inova Varian 500 spectrometer at 500 MHz.

Isolation and Purification of *Symbiodinium* sp. from *P. elisabethae*

Flash frozen or live coral were homogenized in a blender with 0.22 μm filtered seawater and 10 mM EDTA and filtered through four layers of cheesecloth. Algal symbionts were pelleted out by centrifugation at 250 × g and subsequently washed ten times with 40 ml clean filtered seawater and pelleted by centrifugation at 750 × g. *Symbiodinium* sp. cells were further purified on a Percoll step gradient of 20%, 40%, and 80% two or more times until <1% impurities were seen using light microscopy. DNA staining using DAPI detected on epifluorescence microscopy was used to detect contaminants due to bacterial or coral cells. Cells isolated from live coral were diluted to a final concentration of 4×10^5 or 2×10^6 cells/ml using a hemocytometer and maintained in filtered seawater or used.

DNA from purified symbionts was extracted using the DNeasy plant mini prep kit. As described by LaJeunesse [18], denaturing gradient gel electrophoresis (DGGE) was then used to analyze the internal transcribed spacer 2 (ITS 2) sequences to identify the symbiont type occurring in the samples of *P. elisabethae*.

Extraction of Endogenous Pseudopterosins and Elisabethatriene from *Symbiodinium* sp.

Purified *Symbiodinium* sp. cells were lyophilized and extracted with ethyl acetate and filtered through a 1 cm silica column. Pseudopterosins A–D were purified by normal phase HPLC with a hexane/ethyl acetate gradient (60:40 to 100% ethyl acetate in 30 min) using UV detection at 283 nm. Peak areas corresponding to the pseudopterosins were used to quantify amounts. Elisabethatriene was extracted from lyophilized cells using hexanes and purified on reverse-phase HPLC (isocratic in methanol) at 240 nm. NMR conditions are published elsewhere [10].

Cell Lysate Preparation

Purified cells were resuspended in chilled 0.02 M Tris buffer with 3 mM EDTA, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol and lysed in a French press at 1200 psi followed by addition of 1% Triton X. The homogenate was centrifuged at 1000 × g for 10 min. Protein concentration was determined by Bradford assay.

In Vitro and In Vivo Incubation with Radioactive Substrates and Radioactive Measurements

Cell lysates were incubated with 5 μCi GGPP for 24 hr at 29°C at 200 rpm. Twelve milliliters of live cells diluted to 2×10^6 cells/ml was incubated with 0.72 μCi GGPP. Nine milliliters of 2×10^6 cell/ml was incubated with $NaHCO_3$ (specific activity of 2.29 μCi/ml) for 48 hr at room temperature under constant artificial light source. Data presented consist of mean and standard error of three independent experiments.

Cell lysates and intact cells were extracted using HPLC grade chloroform and ethyl acetate. Crude extracts were partitioned between methanol/water (9:1) and hexanes followed by partitioning between methanol/water (1:1) and chloroform. Radiochemical purity of all compounds was ensured by purification two times through HPLC. During the second HPLC run, fractions were collected at minute intervals and counted in a LKB Wallac 1219 Rackbeta liquid scintillation counter. Efficiency for ^{14}C and ^{3}H was checked using internal standards. Radioactivity (in dpm) includes subtraction of background and was, at a minimum, double background levels.

Base hydrolysis of Ps B, C, and D was accomplished by addition of 1 ml of 5% potassium hydroxide in methanol to approximately 200 μg of pseudopterosins A–D. The reaction was stirred overnight at room temperature. To quench the reaction, 3 ml of water was added, and the pH was adjusted to 7. Ps A was extracted using chloroform, dried over anhydrous sodium sulfate, and evaporated under N_2 gas.

Acknowledgments

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