

In Vivo and in Vitro Investigations into the Biosynthetic Relatedness of the Pseudopterosins

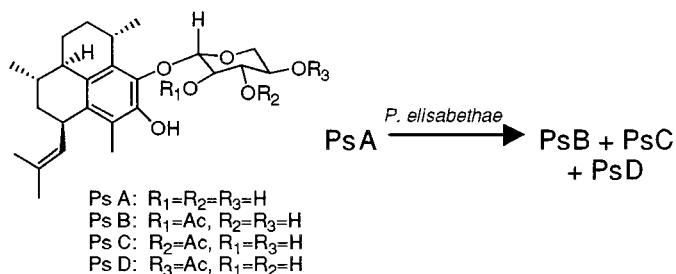
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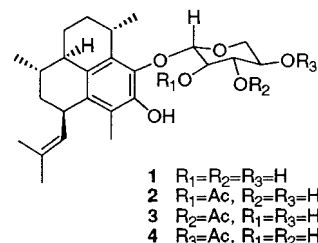
ABSTRACT



Both in vivo and in vitro techniques have been developed to test putative intermediates in the biosynthetic pathway to the pseudopterosins, antiinflammatory compounds isolated from *Pseudopterogorgia elisabethae*. Furthermore, specific activity data we have obtained indicate that pseudopterosin A is a precursor to pseudopterosins B, C, and D. We conclude that in the biosynthesis xylose is attached to the diterpene skeleton to produce pseudopterosin A and is then acetylated to form pseudopterosins B–D.

The pseudopterosins are a class of diterpene glycosides isolated from the marine soft coral *Pseudopterogorgia elisabethae*.¹ These compounds continue to be of great interest due to their potent antiinflammatory and analgesic properties (ED_{50} ca. 3.0 mg/kg) that are superior to existing drugs such as indomethacin.² In addition, the pseudopterosins inhibit pancreatic PLA_2 (IC_{50} 0.5–4 μ m) and are pharmacologically distinct from typical cyclooxygenase NSAIDs.^{1a} A semisynthetic pseudopterosin derivative (pseudopterosin A methyl ether) is currently being evaluated in clinical trials as a treatment for contact dermatitis.³ Presently, there are 12 different congeners, pseudopterosins A–L (Ps A–L), which have been reported in *P. elisabethae* from various geographic locations around the West Indian region.

Our studies focus predominantly on pseudopterosins A–D (1–4), which are the primary metabolites in our collections of *P. elisabethae* from the Bahamas. These compounds are



major metabolites in the gorgonian with 3 being reported to comprise as much as 7.5% of the lipid extract.^{1c} We have recently developed two techniques to test putative intermediates in the biosynthetic pathway. These two methods have allowed us to examine the metabolic relatedness of the four pseudopterosins of interest. Thus, utilizing both in vivo and in vitro systems, we have demonstrated that 1 is a precursor

(1) (a) Look, S.; Fenical, W.; Matsumoto, G.; Clardy, J. *J. Org. Chem.* **1986**, *51*, 5140–5145. (b) Look, S.; Fenical, W.; Jacobs, R.; Clardy, J. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6238–6240. (c) Roussis, V.; Wu Z.; Fenical, W. *J. Org. Chem.* **1990**, *55*, 4916–4922.

(2) Ettouati, W.; Jacobs, R. *Mol. Pharmacol.* **1987**, *31*, 500–505.

(3) Haimes, H.; Glasson, S.; Harlan, P.; Jacobs, R.; Fenical, W.; Jimenez, P. *Inflammation Res.* **1995**, *44*, Suppl. 3W13/17.

to 2–4, indicating that the acetylation of the xylose moiety is the final tailoring step in the biosynthesis of the pseudopterosins.

One of the systems we have developed to test putative biosynthetic precursors is an in vivo method utilizing live coral. To our knowledge this represents the first biosynthetic experiment performed with a live gorgonian. Aboard the research vessel *Bellows* in the Bahamas, four live *P. elisabethae* were obtained by scuba. These gorgonians were placed in four separate plastic vessels, each with the minimal amount of seawater necessary to submerge the coral. The seawater of two of the vessels was treated with 5 μ Ci of 3 H-geranylgeranyl diphosphate (GGPP) while 5 μ Ci of 14 C-xylose was added to each of the remaining two containers. To ensure that the water-soluble xylose was ingested by the filter-feeding gorgonians, it was adsorbed onto a small amount of invertebrate food⁴ before addition to the seawater. The seawater was aerated for 24 h, and the gorgonians were then transferred back into the ocean for an additional 72 h in order to allow for the metabolism of the precursors in a natural environment. The lyophilized coral was extracted with ethyl acetate and methylene chloride. Purification of pseudopterosins 1–4 was achieved by partitioning the crude extract between methanol/water (9:1) and hexanes, followed by partitioning between methanol/water (1:1) and methylene chloride. Subsequent normal phase HPLC of the methylene chloride fraction was performed using a hexane–ethyl acetate gradient. To ensure radiochemical purity of the recovered pseudopterosins, the methylene chloride fraction was subjected to two HPLC analyses. During the first HPLC injection, the pseudopterosin peaks were collected and the solvent was evaporated. The peaks were then reinjected, and fractions were collected throughout the run and counted in a scintillation counter. Figure 1 provides a typical HPLC

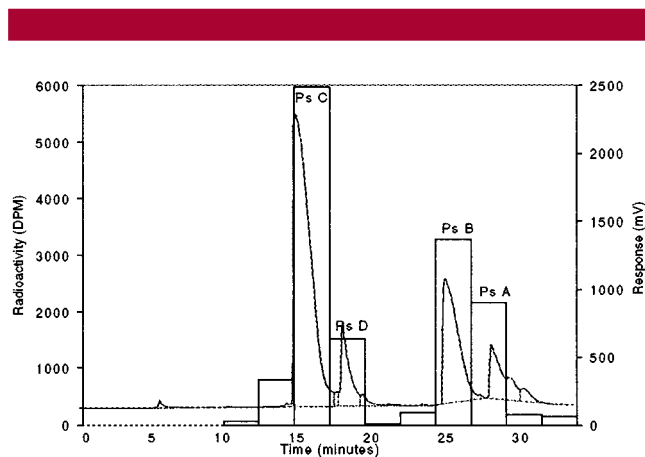


Figure 1. Representative HPLC trace with associated radioactivities

trace with associated radioactivities. Peak areas corresponding to the pseudopterosin fractions were used to calculate specific activity (Tables 1 and 2).

(4) A solution of 14 C-xylose was evaporated in vacuo in the presence of 25 mg of Fritz invertebrate diet. This was deemed to be unnecessary for the water-insoluble GGPP.

Table 1. In Vivo Biosynthetic Feeding Experiment with 3 H-GGPP

compound	recovered radioactivity (DPM)	specific activity (DPM/mmol)	normalized specific activity ^a
Ps A	2170	8.31×10^4	1.00
Ps B	3290	4.00×10^4	0.48
Ps C	5960	2.82×10^4	0.34
Ps D	1530	4.67×10^4	0.56

^a Comparative specific activity data obtained by dividing specific activity data for each Ps by the specific activity data for Ps A.

As shown in Tables 1 and 2, radioactive pseudopterosins were recovered from both the GGPP and xylose feeding experiments, indicating that these are biosynthetic precursors.

Table 2. In Vivo Biosynthetic Feeding Experiment with 14 C-Xylose

compound	recovered radioactivity (DPM)	specific activity (DPM/mmol)	normalized specific activity
Ps A	3370	4.71×10^4	1.00
Ps B	749	2.28×10^4	0.48
Ps C	729	0.44×10^4	0.09
Ps D	590	1.80×10^4	0.38

Fractions collected prior to and following each of the pseudopterosin peaks were at approximately background levels, indicating that the observed radioactivity was due to the pseudopterosins (Figure 1). In each of the xylose and GGPP experiments, the significantly higher specific activity of Ps A indicates that it is a precursor to Ps B–D.

As a result of these findings, we set out to confirm these data in an in vitro system. A cell-free extract was prepared from flash frozen *P. elisabethae* (stored at -80 °C) by homogenizing in a phosphate buffer (pH 7.7 with EDTA and β -mercaptoethanol) with liquid nitrogen in a Waring blender. To remove cellular debris, the homogenate was centrifuged for 15 min at $9000 \times g$. The supernatant was centrifuged at $18000 \times g$ for 3 h and then passed through 0.45μ m nylon membrane filters.

Transformation of geranylgeranylpyrophosphate into the pseudopterosins was carried out by incubating 1 μ Ci of 3 H-GGPP at ambient temperature with the cell-free extract (fortified with 1 mM $MgCl_2$) for 24 h. The pseudopterosins were purified and the radioactivity measured as described for the in vivo experiments. Specific activity data for the in vitro study are presented in Table 3.

In Table 3, the specific activity data supports the findings from the in vivo experiments. In the in vitro incubation, 1.6% of the GGPP was converted to the pseudopterosins as opposed to 0.12% incorporation with the in vivo method. The recovered radioactivity was larger overall in the in vitro experiment, indicating that this system is more suitable for

Table 3. In Vitro Biosynthetic Incubation Experiment Using ^3H -GGPP

compound	recovered radioactivity (DPM)	specific activity (DPM/mmol)	normalized specific activity
Ps A	3170	3.14×10^8	1.00
Ps B	7520	0.21×10^8	0.07
Ps C	19400	0.08×10^8	0.03
Ps D	5260	0.21×10^8	0.07

studying pseudopterosin biosynthesis. Importantly, both of these systems generated data, indicating that pseudopterosin A is a precursor to pseudopterosins B–D. Therefore, we conclude that in the biosynthesis of the pseudopterosins the glycosylation involves the addition of a xylose residue to a

diterpene skeleton to produce pseudopterosin A and subsequently the xylose is acetylated to produce pseudopterosins B–D.⁵ Further experiments to elucidate the detailed biosynthetic origin of the pseudopterosins are currently underway.

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(5) These experiments do not allow us to distinguish between gorgonian metabolism and that from algal symbionts known to be present in *P. elisabethae*.