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ORIGINAL PAPER

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Pseudopterosin biosynthesis—pathway elucidation, enzymology, and a proposed production method for anti-inflammatory metabolites from *Pseudopterogorgia elisabethae*

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Abstract The pseudopterosins are a family of diterpene pentosides isolated from the marine octocoral, *Pseudopterogorgia elisabethae*. These compounds possess non-steroidal anti-inflammatory and analgesic properties which have been shown to be greater than the industry standard, indomethacin. In our investigations, we are interested in examining the biosynthesis and enzymology of these compounds for the development of a biotechnological production method. We have isolated the pseudopterosin diterpene cyclase product, elisabethatriene, using a radioactivity-guided isolation. This has provided us with an assay to isolate the diterpene cyclase enzyme. The amino acid sequence of the purified diterpene cyclase will facilitate cloning and expression of the gene in a suitable host. In addition, we have identified over 25 novel diterpenes from one of our collections of *P. elisabethae*. Several of these compounds appear to be involved in pseudopterosin biosynthesis and are presently being evaluated as potential intermediates. These compounds have also been evaluated for anti-inflammatory activity and some possess greater activity than that of the pseudopterosins. We therefore propose a production method utilizing a combination of recombinant enzyme technology and synthetic methods/biocatalysis in order to produce one or more anti-inflammatory metabolites in *P. elisabethae*.

Keywords Anti-inflammatory · Biosynthesis · Diterpene cyclase · Marine natural product · Pseudopterosin · Terpene

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Introduction

The pseudopterosins (Fig. 1, compounds 1–4) are diterpene glycosides isolated from the marine octocoral, *Pseudopterogorgia elisabethae*, which is found in the West Indian region, the Bahamas, and the Florida Keys [11, 14]. The seco-pseudopterosins (Fig. 1, compounds 5–8) are a related family of compounds initially isolated from *P. kallos* from the Florida Keys [10]. These compounds are of particular interest due to their potent anti-inflammatory and analgesic properties, which have been demonstrated to be greater than the industry standard, indomethacin (IC_{50} 0.5–4.0 mM) [2]. Pseudopterosin A (compound 1) has been found to significantly inhibit phorbol myristate acetate-induced topical inflammation in mice [12] and the methyl ether of this natural product has shown promise as a treatment for contact dermatitis [4]. In addition, these compounds have a commercial market as ingredients in a skin-care product. Since the only present supply of the pseudopterosins is from wild-collected biomass, there is a need for the development of a sustainable supply of these compounds.

Our approach to developing a sustainable supply is to gain a detailed knowledge of the biosynthesis and then design an enzyme-based production method based on key biosynthetic transformations. The plan has therefore been to elucidate the biosynthetic pathway, purify key biosynthetic enzymes, and clone the corresponding biosynthetic genes. The terpene cyclase is one of the key enzymes in terpene biosynthetic pathways, as these generally assemble the entire carbon skeleton in a single step. Since terpene cyclases show only about 6–15% sequence identity [15] and there have been no reported sequence data from marine organisms, we elected to purify and sequence the diterpene cyclase involved in pseudopterosin biosynthesis and use reverse genetics to clone the gene. We previously reported isolation of the diterpene cyclase product, elisabethatriene (Fig. 2, compound 9), which was identified

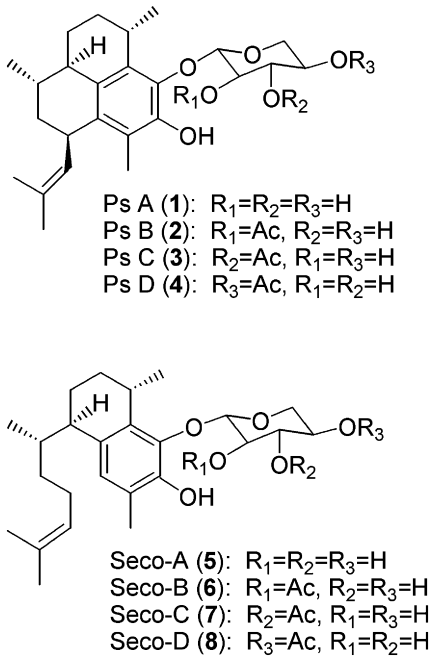

Fig. 1 Structures of pseudopterosins A–D (Ps A–D, compounds 1–4) and seco-pseudopterosins A–D (Seco–A–D, compounds 5–8)

after radioactivity-guided isolation with [1-^{3}H]-geranylgeranyl diphosphate (GGPP) [1]. The production of compound 9 from [1-^{3}H]-GGPP defines an assay to isolate the elisabethatriene cyclase enzyme in aqueous extracts of *P. elisabethae*. We also isolated other biosynthetically related metabolites which possess even greater anti-inflammatory activity than the pseudopterosins and are structurally simpler molecules. Our production method is therefore directed at these new metabolites.

In addition, we are interested in the question of whether the host or a microbial symbiont is responsible for the production of pseudopterosins, as a microorganism could potentially be cultured to produce the desired compounds without substantial collection of the coral and without the need to clone biosynthetic genes. We recently determined the cellular origin of the pseudopterosins and these data will be published elsewhere.

Materials and methods

Identification of the pseudopterosin diterpene cyclase product

Cell-free extracts (CFE) of *P. elisabethae* were prepared by homogenizing flash-frozen *P. elisabethae* collected from Sweetings Cay, Bahamas (at a depth of 5 m) with sodium phosphate buffer (100 mM) containing 5 mM $MgCl_2$, 3 mM EDTA, and 5 mM β-mercaptoethanol. The crude preparation was centrifuged for 2.75 h at 39,000 *g*. Radioactivity-guided isolations to identify the diterpene cyclase product, compound 9, were performed by incubating 100 μCi [1-^{3}H]-GGPP with a portion of the CFE for 24 h at 200 rpm and 29 °C. The incubation was then lyophilized and solvent partitioning was carried out between hexanes and methanol/water (9:1). The least polar radioactive compound from this incubation mixture was purified by normal phase (hexanes to hexanes/ethyl acetate) and subsequent reversed phase (100% methanol) HPLC. Approximately 10 mg of the same compound was obtained from an organic extract (6.60 g) of lyophilized *P. elisabethae* (54 g) using the same procedure; and the structure of compound 9 was established using both 1- and 2-dimensional NMR and mass spectrometry.

To establish the radiochemical purity of compound 9, a derivative was made by reacting compound 9 with SeO_2 (21 μmol) and 84 μl of trimethylsilyl polyphosphate (PPSE) [9]. The structure of the derivative (Fig. 3, compound 10) was determined by NMR and mass spectrometry. The identical reaction was performed with radiolabeled compound 9. Since the specific activity of the derivative remained the same after reaction with SeO_2/PPSE (6.8×10^4 dpm/mg for compound 9, 7.9×10^4 dpm/mg for compound 10), it was determined that the observed radioactivity of the cyclase product was due to compound 9 and not to a minor contaminant.

Confirmation of elisabethatriene as an intermediate in pseudopterosin biosynthesis

^{3}H-Elisabethatriene (compound 9; 6.65×10^4 dpm/mg) was incubated with a CFE made from *P. elisabethae* collected from Sweetings Cay, Bahamas for 24 h at 200 rpm and 29 °C. Pseudopterosins A–D (Fig. 1, compounds 1–4) were purified by normal phase HPLC and radioactivity was measured using a scintillation counter.

Investigation of the chemical variation of *P. elisabethae* from the Florida Keys

P. elisabethae was collected from the Florida Keys at a depth of 25 m. The dried gorgonian was extracted with methanol and then methylene chloride. The crude extract was separated on a silica flash column (200–425 mesh) with a step gradient from hexanes to

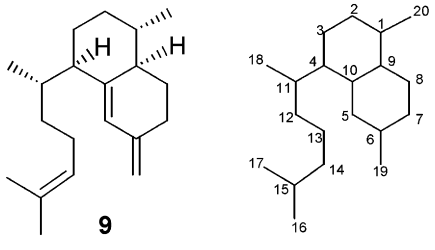

Fig. 2 Structure of elisabethatriene (*compound 9*), the pseudopterosin diterpene cyclase product

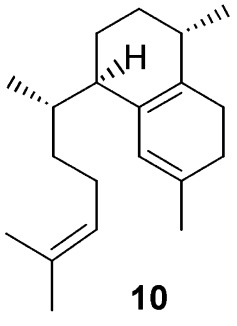

Fig. 3 Structure of derivative of elisabethatriene (*compound 10*) after reaction with SeO_2/trimethylsilyl polyphosphate

ethyl acetate and subsequently from ethyl acetate to methanol. Fractions containing characteristic diterpene resonances in the proton NMR were further purified using reversed phase HPLC. Structures were determined using 1- and 2-dimensional NMR and mass spectrometry. The anti-inflammatory activity of the proposed pseudopterosin and seco-pseudopterosin intermediates was assessed using the mouse ear edema model (25 μg dose/ear, $n = 5$) [7]. The full experimental details of the purification and characterization of all metabolites will be published in due course.

Results and discussion

Radioactivity-guided isolation of elisabethatriene and development of an elisabethatriene cyclase assay

As described previously, we recently isolated compound 9, the pseudopterosin diterpene cyclase product, using a radioactivity-guided isolation. This proved to be a relatively efficient route to the cyclase product as our search simply focused on the characterization of the least polar radioactive metabolite recovered from the GGPP incubation. This isolation was the essential step in the development of an assay to isolate the enzyme involved in the production of compound 9. As described above, terpene cyclases have relatively limited sequence information available and little sequence homology, and thus our approach in the development of a production method is to isolate and sequence the elisabethatriene cyclase in order to develop probes for a cDNA library. Our assumption was that the cyclase product leading to the pseudopterosins would be tricyclic and possess the amphilectane ring system, as it was generally observed that all carbon–carbon bonds were formed in the initial cyclization. However, analysis of 1- and 2-dimensional NMR and mass spectrometry data revealed that the compound was bicyclic, and the structure is shown in Fig. 2 [1]. We also confirmed the intermediacy of compound 9 in pseudopterosin biosynthesis by the incubation of ^{3}H-elisabethatriene (compound 9) with a cell-free extract and subsequent purification of radiolabeled pseudopterosins A–D (Fig. 1, compounds 1–4). One possible mechanism accounting for the formation of compound 9 from GGPP is described in Fig. 4. In this proposed biosynthesis, the initial loss of the pyrophosphate group from GGPP initiates a ring closure, generating a tertiary carbocation following isomerization of the C-2/C-3 double bond from E to Z. Hydride shifts of the α and β protons at C-4 and C-10, respectively, introduce the stereochemistry at C-11 and C-4 and subsequently form an allylic carbocation. After a second ring closure, further rearrangement followed by abstraction of the hydrogen at C-19 accounts for the formation of the bicyclic triene (compound 9).

Efforts to isolate and fully characterize the elisabethatriene cyclase are underway and will shortly be reported with full details. We conclude from assaying protein fractions eluting from a Sephadex G-100 Superfine gel filtration column that the elisabethatriene cyclase is a monomer with a molecular mass of 47 kDa. The molecular mass of the elisabethatriene cyclase is quite distinct from that of the terrestrial diterpene cyclases, taxadiene synthase, abeitadiene synthase, kaurene synthase A and B (all with molecular masses of approximately 80 kDa) [3, 5, 8, 16, 17], and casbene synthase (with a molecular mass of 59kDa) [6]. This suggests that the structure of the elisabethatriene cyclase may be relatively dissimilar from those isolated from terrestrial systems, although we will be able to comment on this in a rigorous fashion only when we have conducted additional experiments.

Variation of natural products chemistry with collection site: exploitation to search for biosynthetic intermediates

Recently, we discovered that *P. elisabethae* inhabits certain locations in the Florida Keys. This coral was previously thought to populate only the Bahamian and West Indian regions. Following detailed chemical investigation of *P. elisabethae* from the Florida Keys, we discovered that this population exhibits a suite of diterpenes distinct from the *P. elisabethae* samples previously analyzed. A detailed analysis of the terpene content of these collections is currently underway, yet this has already resulted in the isolation of over 25 novel compounds. We are in the process of evaluating these compounds as biosynthetic intermediates and can now suggest a plausible biosynthesis of the pseudopterosins

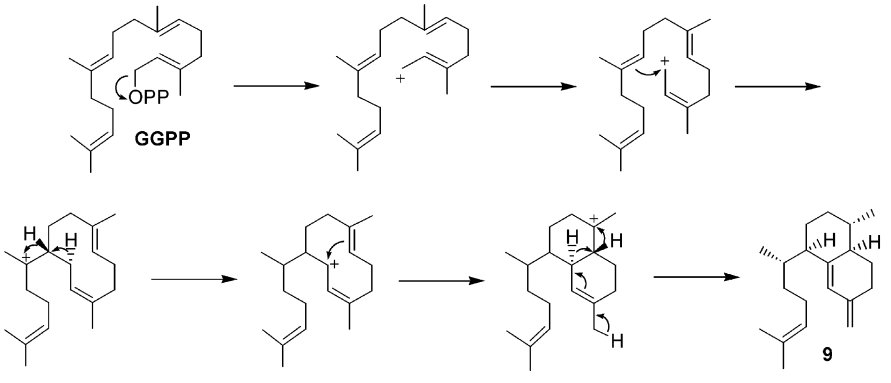

Fig. 4 A potential mechanism accounting for the formation of elisabethatriene from geranylgeranyl diphosphate (*GGPP*)

(Fig. 5). The marine natural products community has long been aware that quantitative and qualitative variations in metabolite composition are often associated with the geographic location of the source organism. Based on our success with this project, we now suggest that this phenomenon can be exploited in searches for biosynthetic intermediates.

In this detailed natural products analysis, we have found that pseudopterosins co-occur with the seco-pseudopterosins, suggesting that these two classes of diterpenes are produced from a single cyclase product. In the proposed biosynthetic pathway, we have confirmed that GGPP cyclizes to form the diterpene cyclase product, compound 9. Elisabethatriene (compound 9) then undergoes aromatization to erogorgiaene (Fig. 5, compound 11) [13]. This aromatic hydrocarbon then presumably undergoes a series of oxidations to form compound 12 or compound 13. The seco-pseudopterosin aglycone (compound 14), which could be readily derived by hydroxylation of compound 12 or compound 13, is then glycosylated at either position to produce one of the seco-pseudopterosins. The biosynthetic pathway to the pseudopterosins appears to diverge at intermediate 13 and we postulate that this compound undergoes an oxidation to compound 15 (Fig. 5) which would then be oxidized to yield an ortho-hydroxyquinone, compound 16. A subsequent ring closure to form compound 17 and aromatization to afford the pseudopterosin aglycone (compound 18), followed by a glycosylation, accounts for the ultimate production of the pseudopterosins. Compounds 16 and 17 have both been obtained in radiolabeled form and, when incubated with a CFE of *P. elisabethae*, both produce radioactive pseudopterosins. Other steps in this postulated biosynthetic scheme are currently under investigation.

Several of the novel diterpenes isolated in this biosynthetic study were screened for anti-inflammatory activity, using the mouse ear edema model. Novel seco-pseudopterosins and one of the biosynthetic intermediates, elisabethadione (compound 16), displayed similar or slightly greater anti-inflammatory activity than the pseudopterosins. We therefore plan to take advantage of the biosynthetically less challenging structure of the seco-pseudopterosins and elisabethadione and now suggest these as attractive anti-inflammatory targets for an enzyme-based production method.

Proposed production method of anti-inflammatory metabolites

A general outline of a suggested production method of the newly identified anti-inflammatory agents that is significantly shorter than the biosynthetic pathway leading to the pseudopterosins is presented in Fig. 6. This proposed production method involves utilizing a recombinant elisabethatriene cyclase to cyclize GGPP to compound 9. Aromatization of compound 9 can be achieved by standard synthetic methods and the

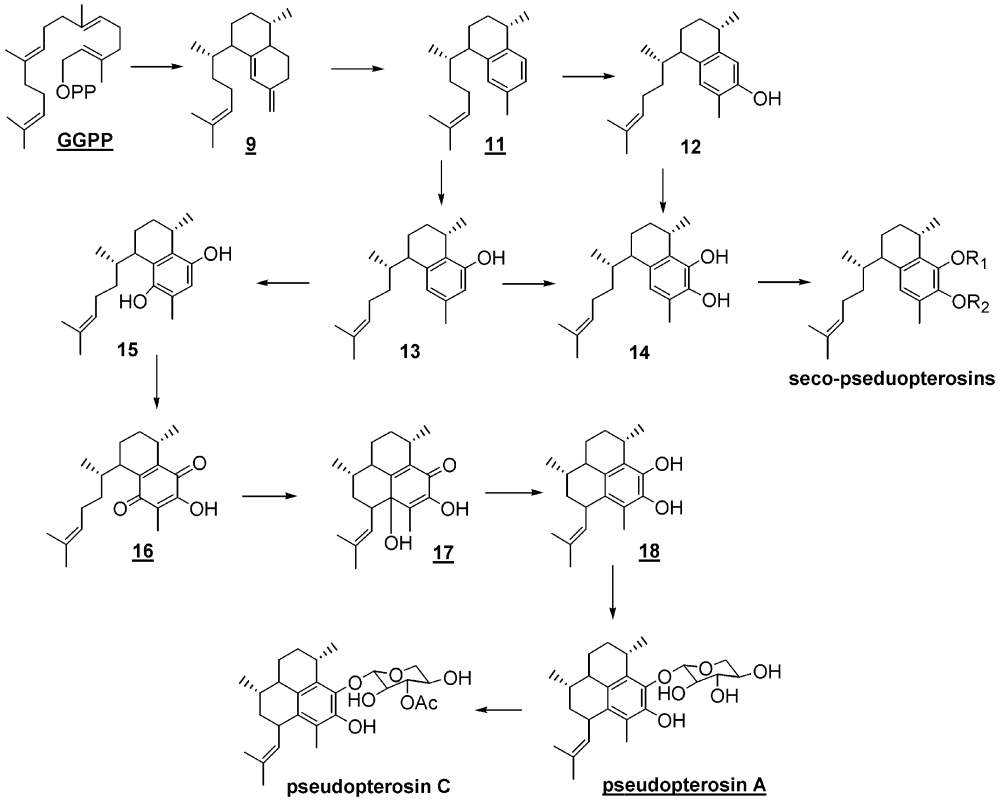

Fig. 5 A plausible biosynthetic pathway of the pseudopterosins and seco-pseudopterosins. *Compound numbers that are underlined* indicate intermediates that have been confirmed to be involved in pseudopterosin biosynthesis using radiolabeling studies

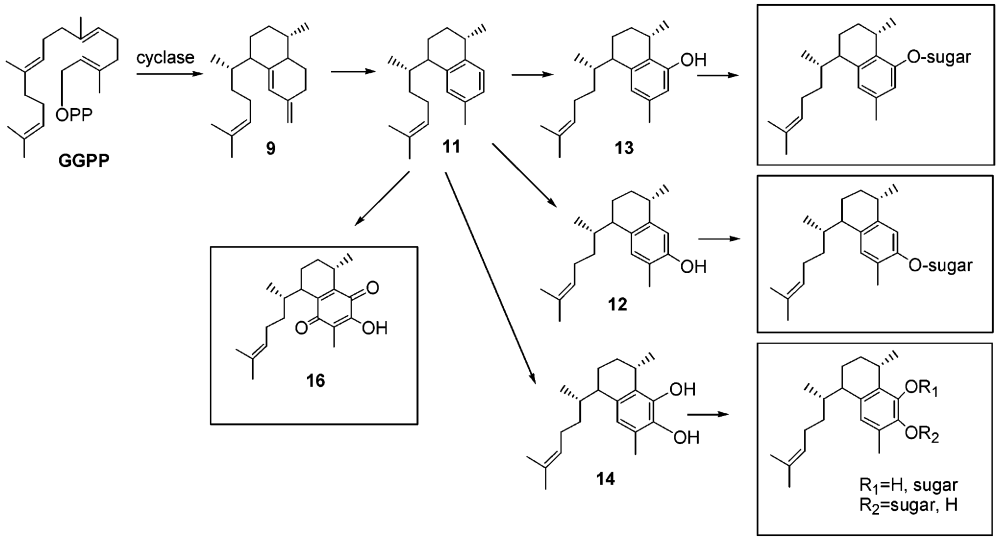

Fig. 6 Proposed production method of anti-inflammatory metabolites

compound can subsequently undergo an oxidation using either chemical or biocatalytic methods to form the seco-pseudopterosin aglycone (compound 14) or a related compound. Glycosylation could then be achieved using either one of the readily available glycosidases or glycosyltransferases, or a recombinant glycosyl transferase from *P. elisabethae*.

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