*Mar. Drugs* **2003**, *1*, 54- 65

*Marine Drugs*
**ISSN 1660-3397**
www.mdpi.net/marinedrugs/

# Pseudopterosin Biosynthesis: Aromatization of the Diterpene Cyclase Product, Elisabethatriene

**Amber C. Kohl and Russell G. Kerr***

Department of Chemistry and Biochemistry, and Center of Excellence in Biomedical and Marine Biotechnology, Florida Atlantic University, Boca Raton, Florida 33431, USA.
http://www.science.fau.edu/chemistry/kerr_group, Tel.: +1 (561) 297-3356, Fax: +1 561) 297-2759
* Author to whom correspondence should be addressed; E-mail: rkerr@fau.edu

*Received: 15 October 2003 / Accepted: 13 November 2003 / Published: 26 November 2003*

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**Abstract:** Putative precursors in pseudopterosin biosynthesis, the hydrocarbons isoelisabethatriene (**10**) and erogorgiaene (**11**), have been identified from an extract of *Pseudopterogorgia elisabethae* collected in the Florida Keys. Biosynthetic experiments designed to test the utilization of these compounds in pseudopterosin production revealed that erogorgiaene is transformed to pseudopterosins A-D. Together with our previous data, it is now apparent that early steps in pseudopterosin biosynthesis involve the cyclization of geranylgeranyl diphosphate to elisabethatriene followed by the dehydrogenation and aromatization to erogorgiaene.

**Keywords:** Biosynthesis, diterpene, elisabethatriene, *Pseudopterogorgia elisabethae*, pseudopterosin.

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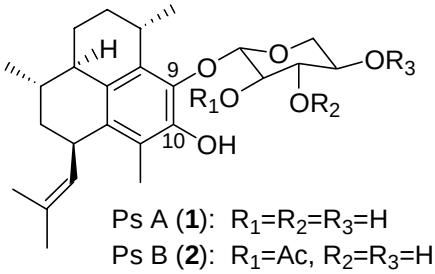
## Introduction

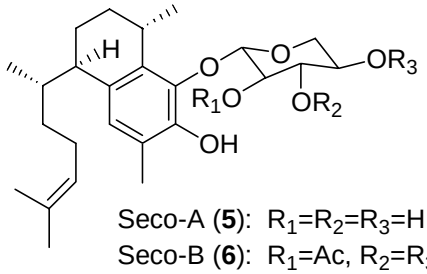
The pseudopterosins are a class of diterpene glycosides isolated from the sea whip *Pseudopterogorgia elisabethae* [1-3]. As with many marine natural products, diverse congeners of the pseudopterosins are found in different locations and there are presently fifteen known pseudopterosin derivatives (A-O). All of the known pseudopterosins contain the amphilectane skeleton with a glycosidic linkage at either C-9 or C-10.

The identity of the sugar and the degree of acetylation account for the additional structural variation of this family of diterpenes. Pseudopterosins A-D (**1**-**4**), from Sweetings Cay in the Bahamas, possess the amphilectane skeleton with an attached xylose sugar which is acetylated at different locations (Figure 1).

**Figure 1.** Structures of pseudopterosins and seco-pseudopterosins A-D.




Ps A (**1**): $R_1=R_2=R_3=H$
Ps B (**2**): $R_1=Ac$, $R_2=R_3=H$
Ps C (**3**): $R_2=Ac$, $R_1=R_3=H$
Ps D (**4**): $R_3=Ac$, $R_1=R_2=H$



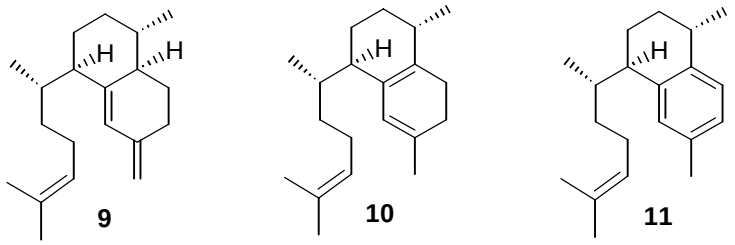

Seco-A (**5**): $R_1=R_2=R_3=H$
Seco-B (**6**): $R_1=Ac$, $R_2=R_3=H$
Seco-C (**7**): $R_2=Ac$, $R_1=R_3=H$
Seco-D (**8**): $R_3=Ac$, $R_1=R_2=H$

The seco-pseudopterosins A-D are a related group of compounds (**5**-**8**) belonging to the serrulatane class of diterpenes initially isolated from *Pseudopterogorgia kallos* in the Florida Keys [4]. More recently, novel seco-pseudopterosins were reported to co-occur with pseudopterosins in *P. elisabethae* [3]. The pseudopterosin and seco-pseudopterosin classes of diterpenes exhibit potent anti-inflammatory and analgesic activity [3, 5]. The pseudopterosins are pharmacologically distinct from typical NSAIDs and they appear to act by a novel mechanism of action [6, 7]. The commercial market for the pseudopterosins,

presently as ingredients in a skin cream, indicates a need for the development of a sustainable supply of these compounds. Consequently, a continuing goal in our laboratory is to elucidate all steps in the biosynthetic pathway leading to the pseudopterosins.

We recently confirmed the identity of the diterpene cyclase product leading to the pseudopterosins as elisabethatriene (**9**) (Figure 2).

**Figure 2.** Structures of plausible initial intermediates in the pseudopterosin biosynthetic pathway.




This hydrocarbon, with the serrulatane skeleton, was isolated from extracts of *P. elisabethae* collected in the Florida Keys and in Sweetings Cay, Bahamas. The utilization of **9** in pseudopterosin biosynthesis was confirmed through biosynthetic experiments [8]. Erogorgiaene (**11**) was recently reported from a collection of *P. elisabethae* off Colombia [9]. Given the structure of the pseudopterosin class of diterpenes and our report of the transformation of **9** to **1-4**, it seems reasonable to suggest that **11** is an intermediate in this biosynthetic pathway. Further, it seems plausible that an endocyclic isomer of **9** such as **10** could be an intermediate in the conversion of **9** to **11**.

This report describes the results of experiments directed at testing the hypothesis that isoelisabethatriene (**10**) and erogorgiaene (**11**) are early intermediates in pseudopterosin biosynthesis. Our approach was to identify these in our *P. elisabethae* extracts through the synthesis of standard samples of **10** and **11** from **9** and if present, test the compounds as metabolic intermediates.

## Results and Discussion

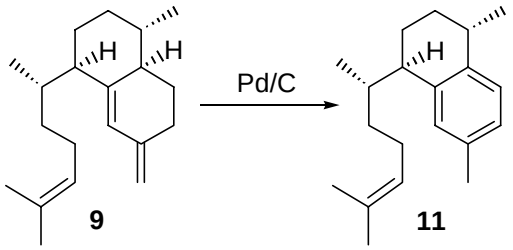
### *Identification of Plausible Biosynthetic Intermediates*

We have observed that the Bahamian populations (e.g. Sweetings Cay) of *P. elisabethae* contain much higher concentrations of pseudopterosins than *P. elisabethae* from the Florida Keys; the latter, however, exhibits a greater diversity of diterpene chemistry [10]. A preliminary survey of the non-polar fraction of an extract of *P. elisabethae* from Sweetings Cay indicated a lack of hydrocarbons that were structurally related to the pseudopterosins and therefore, not likely involved in pseudopterosin biosynthesis. Given

the diverse diterpene chemistry of Floridian specimens of *P. elisabethae*, we examined these collections for the presence of hydrocarbons such as **10** and **11**.

To aid in the search for erogorgiaene (**11**), we synthesized a standard sample of **11** from **9** using Pd/C under nitrogen [10] (Scheme 1). We utilized this sample of **11** as a standard to screen for the presence of this compound in a hexane extract. This analysis revealed the presence of compound **11** in 0.2% of the 100 g extract. NMR analysis of this HPLC fraction confirmed the purity and identity of erogorgiaene.

**Scheme 1.** Aromatization of **9** with Pd/C under nitrogen**.**




Similarly, a standard sample of **10** was synthesized from **9**, as previously described [8], and this was used to monitor its presence in Floridian *P. elisabethae* extracts. We began the search for naturally occurring compound **10** in *P. elisabethae* by obtaining a crude organic extract and partially purifying the extract using a silica flash column. After eluting the silica column with 100% hexanes, analysis of this non-polar fraction by HPLC indicated the presence of a compound with the same retention time and UV absorption profile ($\lambda_{max}$ = 245) as synthetic **10**. Subsequent NMR analysis confirmed the purity and identity of naturally occurring **10**. Interestingly, neither **10** nor **11** were present in isolable quantities in extracts of Bahamian samples of *P. elisabethae*.

The identification of compound **10** in the Florida Keys' *P. elisabethae* represents the isolation of a novel marine metabolite which may be involved in pseudopterosin biosynthesis. Additionally, the natural occurrence of compounds **10** and **11** in the Florida Keys' *P. elisabethae* indicates that this specific population of the coral may be more useful for the identification of putative biosynthetic intermediates than the previously analyzed Bahamian populations. While we confirmed the presence of compounds **10** and **11** in *P. elisabethae*, the presence alone is, of course, not sufficient to confirm their involvement in pseudopterosin biosynthesis. This question was addressed by conducting various incubation experiments and using derivatizations to rigorously confirm radiochemical purity.

*Purification of $^{3}$H-elisabethatriene*

Our approach for the biosynthetic experiments was to utilize a cell-free extract of *P. elisabethae* to produce $^{3}$H-labeled **9** from [1-$^{3}$H]-geranylgeranyl diphosphate (GGPP) and then use this to test for the

conversion of **9** to **10** and **9** to **11**. Thus, a protein preparation of *P. elisabethae* was incubated with 50 μCi of [1-$^3$H]-GGPP. After extracting with hexanes, the organic fraction was partially purified through a small silica column and elisabethatriene (**9**) was rigorously purified by reversed phase HPLC and a portion subjected to scintillation counting. Compound **9** was generated in a radiochemical yield of 0.6% (300,000 dpm) and shown to be radiochemically pure as previously described [8]. This low yield is expected for a reactive intermediate and greater radioactivity was observed for more polar metabolites including the pseudopterosins.

Purified $^3$H-labeled **9** (300,000 DPM) was reincubated with a cell-free extract of *P. elisabethae* (Florida Keys) for 1 hour. The quenched incubation mixture was partially purified by elution through a silica column with 100% hexanes and the resulting non-polar fraction subjected to repeated HPLC fractionation. The radioactivity of fractions corresponding to compounds **9-11** was measured using a scintillation counter (Table 1).

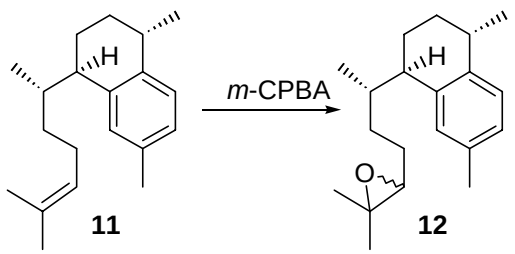
**Table 1.** Recovered Radioactivity of Compounds **9-11** after Incubation of $^3$H-labeled **9** with a Cell-free Extract of *P. elisabethae* (Florida Keys).

| Compound | Radioactivity (DPM) |
|---|---|
| **9** | 48,740 |
| **10** | Background |
| **11** | 2,060 |

The isolated **10** was not radioactive and consequently was deemed not to be involved in the pseudopterosin biosynthetic pathway. The lack of radioactivity in **10** could be explained by the fact that there could be two different diterpene cyclases in the Floridian populations of *P. elisabethae* yielding two different cyclase products. This is consistent with the wide variety of diterpenes present in the Florida samples of this gorgonian [10].

As described in Table 1, **9** was transformed to **11** with a radiochemical yield of 0.7% (2,060 dpm). Radiochemical purity was established for erogorgiaene by conversion of the recovered **11** to an epoxide and monitoring the specific activity of the substrate **11** and the epoxide product **12** (Scheme 2).

**Scheme 2.** Synthetic derivatization of **11** to establish radiochemical purity.



Conditions for this derivitization were established with non-radioactive erogorgiaene (**11**). The reaction of **11** with *meta*-chloroperoxybenzoic acid (*m*-CPBA) proceeded in excellent yield. Importantly, compound **12** eluted more than 10 min. earlier than **11** on reversed phase HPLC, thus ensuring a simple purification of the product. The HREIMS of **12** established a molecular formula of $C_{20}H_{30}O$. The $^1$H-NMR spectrum of **12** was similar to that of **11**. One variation between the NMR spectra of the two compounds was that the olefinic proton in **11** [δ5.27 (1 H, br t)] was replaced with a signal at δ2.57 (1 H, dd) for **12**. Additionally, the two signals for the olefinic methyl groups in **11** [δ1.60 (3 H, br s) and 1.71 (3 H, br s)] were shifted upfield to δ1.14 (3 H, br d) and δ1.17 (3 H, br s) for **12**.

Following the purification of radioactive **11** from the incubation with $^3$H-labeled **9**, $^3$H-labeled **11** (4.27 x $10^6$ dpm/mmol) was treated with *m*-CPBA in $CHCl_3$ and the peak corresponding to the epoxide (**12**) was purified by reversed phase HPLC. Compound **12** was found to have the same specific activity (4.94 x $10^6$ dpm/mmol) as **11** (4.27 x $10^6$ dpm/mmol). The lack of change of specific activity in this derivatization reaction establishes the radiochemical purity of compound **11** and thus confirms that erogorgiaene is derived from elisabethatriene (**9**).

*Evaluation of $^3$H-erogorgiaene in Pseudopterosin Biosynthesis*

The data presented above demonstrated that elisabethatriene (**9**) is transformed to its aromatic derivative **11** in *P. elisabethae*. To test for the conversion of **11** to the pseudopterosins, and thus confirm the involvement of **11** in the pseudopterosin biosynthetic pathway, $^3$H-labeled **11** (5,130 DPM) was incubated with a Bahamian *P. elisabethae* cell-free extract [8]. Radioactive **11** was obtained from the incubation of [1-$^3$H]-GGPP and also by oxidation of **9** [11]. Following incubation, pseudopterosins A-D (**1-4**) were rigorously purified by HPLC. Liquid scintillation counting of the pseudopterosins from the HPLC separation indicated transformation of $^3$H-**11** to **1-4**. The overall radiochemical yield of the four pseudopterosins was 5.7% (290 DPM). The radiochemical yield of the pseudopterosins obtained in this experiment is an order of magnitude higher than that reported for the conversion of GGPP to **9** [8]**.**

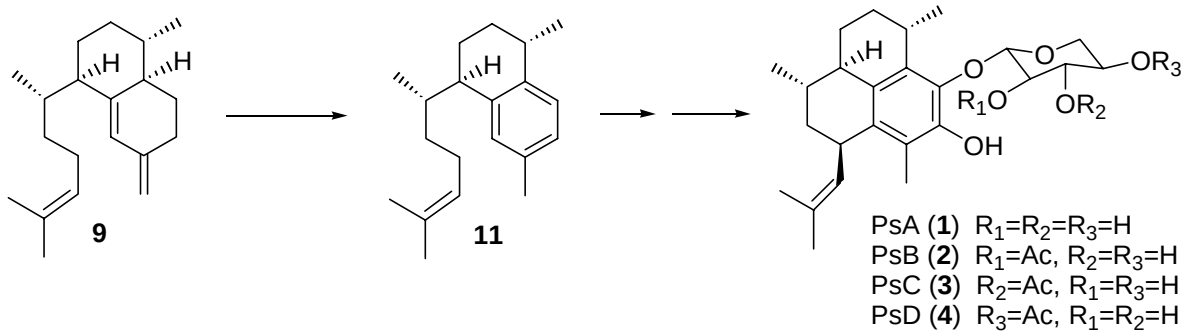
## Conclusions

These data suggest that in pseudopterosin biosynthesis, erogorgiaene is produced from elisabethatriene by a dehydrogenation and spontaneous aromatization (Scheme 3). It is conceivable that isomers of elisabethatriene, other than **10,** could be involved in this pathway. However, lack of such radioactive fractions in the HPLC analysis suggests that this is not likely.

The lack of transformation of elisabethatriene to the isomeric **10** suggests that the conversion of **9** to **11** involves a dehydrogenation which is then presumably followed by a spontaneous aromatization. In addition, utilization of the chemical variance of different populations of *P. elisabethae* for the isolation of

biosynthetic intermediates and subsequent radiolabeling experiments proved to be invaluable for this investigation.

**Scheme 3.** Intermediacy of erogorgiaene in pseudopterosin biosynthesis.




**Acknowledgements**

We thank the National Science Foundation (MCB-0119011) and a National Institutes of Health Minority Predoctoral Fellowship (5 F31 GM64005-02, A. Kohl) for funding this research as well as the Florida Institute of Oceanography for providing ship time aboard the R/V Bellows and the Keys Marine Laboratory for use of their facilities. This is contribution number P200307 from the Center of Excellence in Biomedical and Marine Biotechnology.

**Experimental Section**

*General*

[1-$^3$H]-Geranylgeranyl diphosphate (60 Ci/mol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals and reagents were purchased from Fisher Scientific, Sigma Chemical Co., or Aldrich Chemical Co. The $^1$H-NMR spectra (one- and two-dimensional) were recorded in $C_6D_6$ on a Varian 500 NMR spectrometer at 500 MHz. Mass spectral measurements were conducted at the Midwest Center for Mass Spectrometry at University of Nebraska-Lincoln. TLC was performed using silica gel, $GF_{254}$ pre-coated plates and HPLC was performed using either on an HP 1090 or using a Perkin Elmer Series 410 pump with a Perkin Elmer LC-30 RI detector.

*Collection and Identification of Pseudopterogorgia elisabethae*

*P. elisabethae* from Sweetings Cay, Bahamas was collected by SCUBA in the month of May in 1998-2002 at depths of 10-15 m. Specimens of the gorgonian were identified by standard morphological analysis and pseudopterosins A-D identified by analyzing the crude extract by analytical TLC. Samples were flash frozen with liquid nitrogen and stored at -80°C. *P. elisabethae* from the Florida Keys was collected by SCUBA in 1999-2001 from Tennessee reef at a depth of 25 m. Organisms were flash frozen with liquid nitrogen and stored at -80°C. Specimens collected from Florida were identified as *P. elisabethae* by Fredrick M. Bayer, Department of Invertebrate Zoology, Natural Museum of Natural History, Smithsonian Institution, Washington, D.C. A voucher specimen (USNM100430) has been deposited in this institute.

*Cell-Free Extract Preparation*

Flash frozen *P. elisabethae* (100 g) was homogenized with buffer [20 mM Tris-HCl (pH 7.7) containing 3 mM EDTA, 5 mM β-mercaptoethanol, and 5 mM $MgCl_2$] in a commercial blender. The buffer was added to the blender and was the coral blended in the presence of liquid nitrogen. After homogenizing, the cell-free extract was centrifuged to remove insoluble debris. Initially, centrifugation was conducted at 9,700 g for 10 min. The pellet was discarded and the supernatant was centrifuged again at 39,000 g for 20 min. The pellet was discarded and the supernatant centrifuged one last time at 39,000 for 30 min. A portion of the cell-free extract (40 mL) was incubated with radiolabelled compounds.

*Identification of erogorgiaene* (**11**) *in P. elisabethae collected in the Florida Keys*

Elisabethatriene (**9**) (0.5 mg) was reacted with a spatula tip of Pd/C in 500 μL of triethylene glycol dimethyl ether (triglyme), and the mixture refluxed under nitrogen for 3 hours. Following the reaction, the sample was filtered and the solvent evaporated under nitrogen. Analysis was then performed by reversed phase HPLC using a diode array detector (λ = 215 nm) with 100% methanol (2 mL/min.). To identify the compound in the Florida Keys' *P. elisabethae* extract, the hexane layer (~6 g) obtained from partitioning a crude methylene chloride/ethyl acetate extract with hexanes and methanol/water (9:1) was passed through a small silica column with 100% hexanes. The solvent was evaporated using a rotary evaporator and fractionated by HPLC using a diode array detector (λ = 215 nm) with 100% methanol (2 mL/min.). The peak corresponding to a retention time of 22 min. was subjected to NMR analysis.

*Identification of compound* **10** *in P. elisabethae collected in the Florida Keys*

A crude methylene chloride/methanol extract of *P. elisabethae* (Florida Keys) was partially purified through a silica column eluted with 100% hexanes. The sample was further purified using preparative TLC with hexanes as the eluent and the band corresponding to an $R_f$ of approximately 0.6 was isolated. Further fractionation was achieved by reversed phase HPLC using a refractive index detector and 100% methanol (2 mL/min.). The peak corresponding to a retention time of 27 min. was subjected to NMR analysis. Compound **10**: UV (MeOH) $\lambda_{max}$ 245 nm; $^1$H-NMR (500 MHz, $C_6D_6$) δ 6.44 (1 H, s), 5.23 (1 H, t), 3.02 (1 H, m), 1.72 (3 H, s), 1.68 (3 H, s), 1.57 (3 H, s), 1.02 (3 H, d, $J$ = 6 Hz), 0.90 (3 H, d, $J$ = 6 Hz).

*Purification of $^3$H-labeled 9 from incubation of [1-$^3$H]-GGPP*

Two separate reactions were conducted by incubating 20 μCi of [1-$^3$H]-GGPP with each of two 500 μL aliquots of a Sweetings Cay *P. elisabethae* cell-free extract for 1 hour at 29°C and 200 rpm. A Sweetings Cay *P. elisabethae* cell-free extract that had been partially purified by DEAE-cellulose anion-exchange chromatography was also incubated with 10 μCi of [1-$^3$H]-GGPP for 1 hour at 29°C and 200 rpm. The samples were extracted with hexanes and passed through a small silica pasteur pipet column (5 cm). Elisabethatriene (300,000 DPM) was rigorously purified by reversed phase HPLC using a refractive index detector and 100% methanol (2 mL/min.). The radioactivity was measured using a liquid scintillation counter.

*Incubation of Florida Keys' P. elisabethae Cell-free Extract with $^3$H-***9** *and Purification of $^3$H-***11** *and $^3$H-***10**

$^3$H-Elisabethatriene (300,000 DPM) was collected from HPLC purification and the solvent was evaporated under a stream of $N_2$. Following the addition of 1 mL of assay buffer and 0.05% Tween 20, the sample was sonicated for 10 min. and cell-free extract was added to a total volume of 40 mL. The sample was then incubated at 29°C and 200 rpm for 1 hour. The sample was lyophilized and partitioned between hexanes and methanol/$H_20$ (9:1). After partial purification through a silica column (7 cm) with 100% hexanes, the sample was purified using reversed phase HPLC with 100% methanol (2 mL/min.). Compounds **9-11** were collected in separate vials and the solvent evaporated under $N_2$. Ten percent of each sample was then reinjected and fractions were collected before and after each peak. The solvent was evaporated from the fractions and they were subjected to liquid scintillation counting. For compound **10**, another 40% was added to the sample and the liquid scintillation counting repeated. The remaining amount (90%) of **9** (48,740 DPM) and **11** (1,860 DPM) was then reinjected to purify further.

*Derivitization of* **11** *to* **12**

*meta*-Chloroperoxybenzoic acid (*m*-CPBA, 76.2%, 3.5 mg) was dissolved in dry chloroform (5 mL). A portion of this mixture (1 mL, 4.6 μmol, 1.04 mg *m*-CPBA) was pipetted into a small separatory funnel and added to a stirred vial of **11** (4.6 μmol, 1.25 mg) over 10 minutes. The reaction was conducted at 0°C for 2.5 hours. The reaction mixture was concentrated under $N_2$ and purified by reversed phase HPLC with 100% methanol (2 mL/min.) to afford **12** (1.25 mg) as a colorless oil. Compound **12**: UV (MeOH) $\lambda_{max}$ 220 nm; HRMS calcd for $C_{20}H_{30}O$ ($M^+$) 286.2297, found 286.2297; $^1$H-NMR (500 MHz, $C_6D_6$) δ 7.12 (1 H, d), 7.06 (1 H, s), 6.95 (1 H, d, *J* = 7.5), 2.88 (1 H, m), 2.83 (1 H, m), 2.65 (1 H, m), 2.57 (1 H, dd), 2.21 (3 H, br s), 1.22 (3 H, d, *J* = 6.5), 1.17 (3 H, br s), 1.14 (3 H, br. d, *J* = 3.5), 0.67 (3 H, d, *J* = 3 Hz), 0.66 (3 H, d, *J* = 3 Hz). EIMS *m/z* $[M]^+$ 183 (28), 175 (31), 174 (31), 173 (67), 159 (83), 157 (100), 145 (28), 69 (33).

*Synthesis and Purification of Radioactive* **12**

$^3$H-Labeled **9** (45.6 μg, 169 nmol, 1,420 DPM) was treated with *m*-CPBA (39.7 μg, 175 nmol) in dry $CHCl_3$ at 0°C for 2.5 hours as described previously. The reaction mixture was purified by reversed phase HPLC as described (methanol, 2 mL/min.) to afford 8.8 nmol (5.2%) of **12** and the radioactivity measured. Quantities of the compounds were obtained by integration of HPLC peaks.

*Synthetic Conversion of* **9** to **11**

$H_5PMo_{10}V_2O_{40}^-$ (60 mg) was stirred with 1,2-dichloroethane (1 mL) and tetraglyme (65 μL) in a 2 mL conical vial at 70°C for 5 min [12]. Elisabethatriene (**9**) (0.46 mg) was reacted with the mixture under oxygen atmosphere for 1.5 hours. The 1,2-dichloroethane was evaporated and the mixture partially purified through a small silica pasteur pipet column with hexanes. Reversed phase HPLC of the reaction (methanol, 2 mL/min.) showed the presence of erogorgiaene in approximately 80% yield.

*Synthesis and Purification of Radioactive* **11**

$^3$H-**9** (48,740 DPM) purified from the incubation with [1-$^3$H] GGPP was treated with $H_5PMo_{10}V_2O_{40}^-$ (26.5 mg) in tetraglyme (55 μL) and 1,2-dichloroethane at 70°C for 2 hours as described above. The sample was purified as described in the previous section and 5% was reinjected along with a small amount of "cold" erogorgiaene (**11**). Fractions were collected before and after the erogorgiaene peak and subjected to liquid scintillation counting. The other 95% was reinjected separately for purification.

*Incubation of Cell-free Extract with $^{3}$H-***11** *and Purification of $^{3}$H-[***1-4***]*

$^{3}$H-**11** (5,130 DPM) was added to a plastic conical vial using hexanes and the solvent evaporated. Following the addition of glycerol (4 mL, 10% v/v) and Tween 20 (20 μL, 0.05%), the sample was sonicated for 10 min. and a Sweetings Cay *P. elisabethae* cell-free extract was added to a total volume of 40 mL. The sample was incubated at 200 rpm and 29°C for 24 hours. Solvent partitioning was performed between hexanes and methanol/water (9:1) and then methanol/water (1:1) and methylene chloride. The pseudopterosins A-D were subsequently purified from the methylene chloride layer by normal phase HPLC with a hexane/ethyl acetate gradient (60:40 to 100% ethyl acetate over 35 min., λ=283 nm). Radioactivity was monitored using a liquid scintillation counter.

*Supporting Information Available.* $^{1}$H-NMR spectra for compounds **10** and **12** (12 pages).

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*Sample availability:* Samples of compounds **9**, **10** and **11** are available from the authors.