Pergamon

Tetrahedron 56 (2000) 9569–9574

TETRAHEDRON

Radioactivity-Guided Isolation and Characterization of the Bicyclic Pseudopterosin Diterpene Cyclase Product from *Pseudopterogorgia elisabethae*

Amber C. Coleman and Russell G. Kerr*

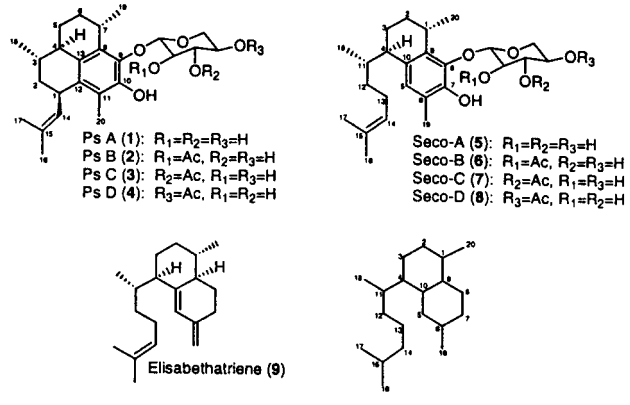
Department of Chemistry and Biochemistry, Center for Molecular Biology and Biotechnology, Florida Atlantic University, Boca Raton, FL 33431, USA

Received 19 May 2000; accepted 26 September 2000

Abstract—The intermediate representing the first committed step in the pseudopterosin biosynthetic pathway has been discovered using a radioactivity-guided isolation. This diterpene cyclase product was identified from a cell-free extract of the marine soft coral, *Pseudopterogorgia elisabethae*, which was incubated with ^{3}H-geranylgeranyl diphosphate. Structural studies of the compound have revealed an unexpected bicyclic skeleton suggesting that the pseudopterosins are related to the *seco*-pseudopterosins through a common bicyclic intermediate. In addition, the intermediacy of this metabolite in pseudopterosin biosynthesis has been confirmed utilizing a cell-free extract of *P. elisabethae*.

Introduction

The pseudopterosins (**1**–**4**) represent a class of structurally distinct diterpene glycosides isolated from the marine octocoral, *Pseudopterogorgia elisabethae*.[1] Different members of the pseudopterosin family have been found in *P. elisabethae* collected in distinct geographic locations of the Bahamas and Bermuda. Currently, there are twelve known derivatives, pseudopterosins A–L (PsA–PsL), and in most cases these are major constituents of the lipid extract. The primary pseudopterosin component in *P. elisabethae* found in the Bahamas, pseudopterosin C, has been reported to occur in 7.5% of the lipid extract.[1a]



The related *seco*-pseudopterosins (**5**–**8**) have been reported from *P. kallos* collected from the Marquesas Keys in Florida.[2]

The pseudopterosins and *seco*-pseudopterosins are anti-inflammatory and analgesic agents with potencies superior to that of existing drugs such as indomethacin.[3] These diterpene glycosides have been reported to inhibit pancreatic phospholipase A_2 (PLA_2, IC_{50} 0.5–4.0 mM) and are pharmacologically distinct from typical cyclo-oxygenase inhibiting NSAIDs.[4] Pseudopterosin A has been found to significantly inhibit phorbol myristate acetate-induced topical inflammation in mice,[4] and the methyl ether of this natural product has shown promise as a treatment for contact dermatitis.[5] Pseudopterosins are also used commercially in an Estee Lauder skin cream. The pseudopterosins used in the skin cream and in biological evaluations have all been obtained by extractions of the harvested coral, thus imposing a potential supply issue.[6]

We recently described in vitro and in vivo systems, which were developed to allow for the elucidation of the biosynthesis of the pseudopterosins.[7] In a continuation of these metabolic studies, we set out to identify the diterpene cyclase product in *P. elisabethae*. Herein, we report the structure of elisabethatriene (**9**), the first committed intermediate in pseudopterosin biosynthesis, which was purified using a radioactivity-guided isolation. To our knowledge, this represents the first account of the isolation of a terpene cyclase product from a marine invertebrate. The isolation of this metabolite suggests that the pseudopterosins and

Keywords: biosynthesis; terpene; marine metabolites; antiinflammatory compounds.

* Corresponding author. Tel.: +516-297-3390; fax: +561-297-2759; e-mail: rkerr@fau.edu

0040–4020/00/$ - see front matter
PII: S0040-4020(00)00930-3

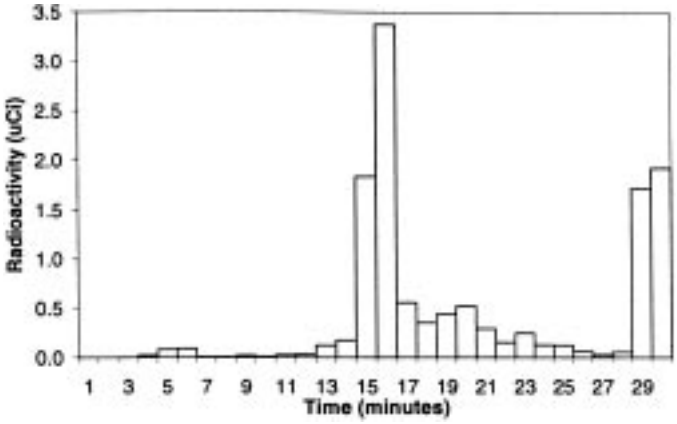


Figure 1. Normal phase HPLC radioactivity profile of hexane fraction from in vitro incubation with ^{3}H-GGPP.

seco-pseudopterosins are derived from a common bicyclic intermediate.

Results and Discussion

Radioactivity-guided isolation and characterization of elisabethatriene

The cyclization of geranylgeranyl diphosphate (GGPP) in diterpene biosynthesis initially generates a carbocation, which can either undergo a proton elimination, or react with water to afford an alcohol. We reasoned that we could gain rapid access to the diterpene cyclase product in *P. elisabethae* by isolating the least polar radioactive metabolite following an incubation of an enzyme preparation of the coral with ^{3}H-GGPP.

A cell-free extract (CFE) was prepared by homogenizing flash frozen *P. elisabethae* with a phosphate buffer in the presence of liquid nitrogen. After pulse blending, the crude extract was centrifuged and the supernatant stored at −80°C. A portion of the CFE was incubated with 50 μCi [1-^{3}H] GGPP and 1 mM $MgCl_2$. The reaction mixture was lyophilized and extracted with ethyl acetate. Partitioning between hexane and methanol/water (9:1) yielded a hexane fraction (21.86 μCi) which was injected on normal phase HPLC with a gradient from 100% hexane to hexane/ethyl acetate (1:1). Throughout the HPLC run, fractions were collected every minute for 30 min. The radioactivity of these fractions was monitored using a liquid scintillation counter (Fig. 1). Two nonpolar fractions with significant radioactivity (each ~200,000 dpm) were observed at 5.0 and 6.0 min. There were also numerous more polar fractions with significant radioactivity. The most active fraction at 16 min was found to be due to geranylgeraniol, indicating significant phosphatase activity. The identity of other radioactive peaks has not yet been addressed.

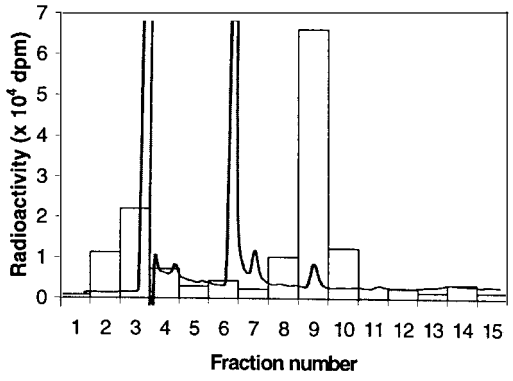


Figure 2. Reversed phase HPLC trace with radioactivity profile of hexane fraction from incubation of CFE with ^{3}H-GGPP.

The 5.0–7.0 min radioactive fraction, was then injected on reversed phase HPLC using methanol as the mobile phase and fractions were collected at 2 min intervals. Fig. 2 displays a radioactivity profile superimposed on the refractive index trace, and indicates significant radioactivity (66,500 dpm) corresponding to fraction 9.[8] Other fractions that were collected before and after fraction 9 were not radioactive.[9]

An additional 10 mg of this compound, which we have termed elisabethatriene (**9**), was obtained by subjecting an organic extract (6.6 g) to the same purification procedure. The high-resolution electron impact mass spectrum of compound **9** indicated a molecular formula of $C_{20}H_{32}$ suggesting either a bicyclic diterpene skeleton with 3 degrees of unsaturation or a tricyclic diterpene skeleton with 2 degrees of unsaturation. The ^{1}H NMR spectrum (500 MHz, C_6D_6)[10] (Table 1) showed four distinct olefinic protons [δ 6.12 (1H, d, J=2.0 Hz), 5.19 (1H, m), 4.85 (1H, s), and 4.76 (1H, dd, J=1.5, 1.0 Hz)] with the protons at δ 6.12 and 5.19 being characteristic of the C-5 and C-14 protons in the *seco*-pseudopterosin skeleton.[2] Allylic coupling of the C-14 proton with the methyl proton at C-16 was evident. The two resonances at δ 4.85 and 4.76 suggested an exocyclic methylene was present in the molecule. Two methyl doublets at δ 0.85 (3H, d, J=7.5 Hz) and 0.89 (3H, d, J=6.5 Hz) were assigned to the C-18 and C-20 methyl protons. In addition, the other methyl protons for the two olefinic methyl resonances were observed at δ 1.57 (3H, s) and 1.63 (3H, d, J=4.5 Hz). The COSY-45° experiment was recorded to establish connectivities and assign other proton resonances. The olefinic proton at C-5 was coupled with one of the exocyclic C-19 methylene protons and a C-7 methylene proton. The C-14 olefinic proton exhibited coupling with the C-13 methylene protons

Table 1. ^{1}H and ^{13}C NMR Assignments for elisabethatriene (**9**). (The ^{1}H NMR spectrum was recorded at 500 MHz in C_6D_6. Assignments were made with the help of 2D homonuclear and heteronuclear experiments. Chemical shifts are presented in δ units; The ^{13}C NMR spectrum was recorded at 125 MHz in C_6D_6)

Carbon number	^{1}H NMR assignments	^{13}C NMR assignments	Multiplicity[a]
1	1.70 (1H, m)	31.9	CH
2	1.20 (1H, m), 1.60 (1H, m)	23.2	CH_2
3	1.45 (1H, m), 1.58 (1H, m)	27.5	CH_2
4	2.24 (1H, m)	37.1	CH
5	6.12 (1H, d, J=2 Hz)	127.5	CH
6		144.0	–C–
7	2.15 (1H, m), 2.34 (1H, m)	29.8	CH_2
8	1.02 (1H, m), 1.59 (1H, m)	35.2	CH_2
9	1.80 (1H, m)	49.9	CH
10		143.9	–C–
11	1.77 (1H, m)	35.5	CH
12	1.20 (1H, m), 1.63 (1H, m)	29.6	CH_2
13	1.96 (1H, m), 2.15 (1H, m)	25.9	CH_2
14	5.19 (1H, m)	125.6	CH
15		130.9	–C–
16	1.63 (3H, d, J=4.5 Hz)	25.9	CH_3
17	1.57 (3H, s)	17.7	CH_3
18	0.85 (3H, d, J=7.5 Hz)	15.1	CH_3
19	4.76 (1H, dd, J=1.5, 1.0 Hz), 4.85 (1H, s)	109.0	CH_2
20	0.89 (3H, d, J=6.5)	17.6	CH_3

[a] The multiplicities were determined using DEPT.

as well as the C-16 methyl protons. Other couplings served to establish the identities of the upfield signals, which could not be assigned by ^{1}H NMR.

The ^{13}C NMR spectrum (125 MHz, C_6D_6) showed resonances for all twenty carbon atoms. The DEPT spectrum was recorded for the establishment of multiplicities of each carbon signal appearing in the broad band spectrum and revealed the presence of four $-CH_3$, seven CH_2, and six –CH carbons. The subtraction of the DEPT spectra from the broad band spectrum indicated the presence of three quaternary carbon atoms in the compound. There were six olefinic carbons [δ 144.0, 143.9, 130.9, 125.6, 127.5, 109.0] confirming the presence of three double bonds and therefore a bicyclic structure for **9**. The resonance at δ 109.0 is characteristic of an exocyclic methylene carbon and appeared as a CH_2 in the DEPT spectrum. The other two methyl signals in the molecule were observed at δ 15.1 and δ 17.7. Furthermore, the HMQC spectrum of **9** established the direct $^{1}H/^{13}C$ one-bond shift correlation of each protonated carbon and aided in the assignments presented in Table 1. The HMBC spectrum was helpful for the verification of the structure of **9** as well as for the determination of ^{13}C NMR chemical shift assignments of quaternary carbon atoms. The C-4 methine proton (δ 2.24) showed HMBC interactions with C-3 (δ 27.5) and C-10 (δ 143.9). Cross peaks were also apparent between the C-18 methyl protons (δ 0.85) and C-3 (δ 27.5), C-4 (δ 37.1), C-11 (δ 35.5) and C-12 (δ 29.6). The C-20 methyl protons (δ 0.89) showed long-range heteronuclear multiple bond connectivities with C-1 (δ 31.9), C-2 (δ 23.2), C-8 (δ 35.2) and C-9 (δ 49.9). The complete HMBC interactions of compound **9** are presented in Fig. 3. The relative stereochemistry at C-1, C-4, C-9 and C-11 was established with the aid of a NOESY spectrum. A correlation between the methyl protons at C-18 and C-20 with the protons at C-4 and C-9, respectively, established a *cis* relationship between these methine protons and the methyl groups. The NOESY spectrum also revealed the positions of the C-19 methylene protons by establishing the connectivity of the C-7 protons with the C-19 proton at δ 4.76.

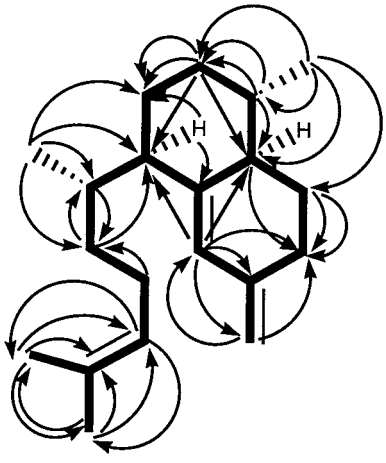
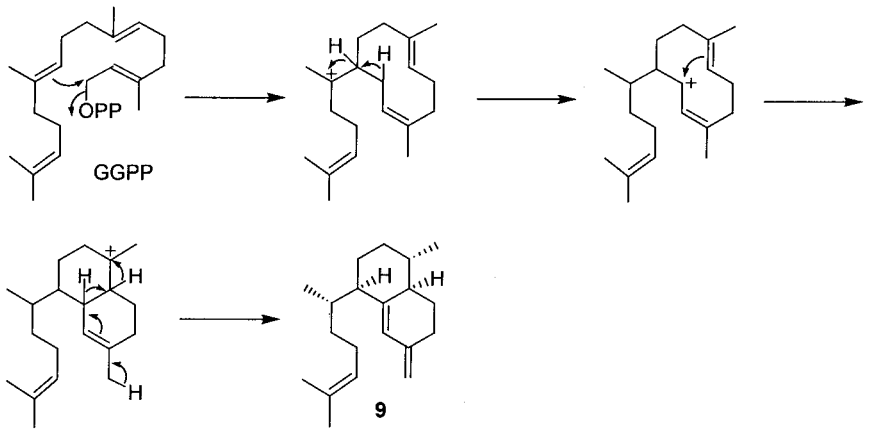


Figure 3. HMBC Correlations for elisabethatriene (**9**).

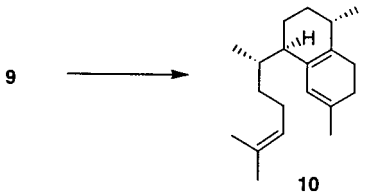
Based on these spectral data, structure **9** was established for this metabolite. The structure of elisabethatriene (**9**) suggests the cyclization of GGPP as described in Scheme 1. Presumably, the initially formed tertiary carbocation undergoes a rearrangement to generate an allylic cation. A second ring closure generates a second tertiary carbocation, which upon proton loss generates the bicyclic triene **9**.

To ensure that an impurity was not responsible for this observed radioactivity, a portion of radioactive **9** was transformed to derivative **10** and purified to constant specific activity (Eq. (1)). Due to the small amounts of **9** available from extraction of the coral tissue, we sought a simple transformation to produce a stable derivative that could be rigorously purified. We found that treatment of **9** with



Scheme 1.

selenium dioxide in the presence of trimethylsilyl polyphosphate (PPSE)[11] afforded **10** in fair to good yield depending on the scale. Reversed phase HPLC afforded base-line resolution of **9** and **10**. The ^{1}H NMR spectrum of **10** was similar to that of **9** with the exception that the two signals for the exocyclic methylene protons (4.85 (1H, s), and 4.76 (1H, dd, J=1.5, 1.0 Hz) were absent and a new methyl signal was present at δ 1.73 (3H, s). Further, there was a downfield shift of the signal at δ 6.12 (1H, d, J=2.0 Hz) to δ 6.47 (1H, s). A cross peak in the COSY-45° experiment between the C-19 methyl protons and the C-5 proton confirmed the location of the unsaturation. This indicated that there was an isomerization of the double bonds to form a conjugated endocyclic system as would be expected in this transformation. Following the incubation with GGPP, radioactive **9** (3.8×10^4 dpm/mg following RP HPLC) was treated with selenium dioxide/PPSE and isomeric **10** was purified by reversed phase HPLC (7.9×10^4 dpm/mg). Importantly, the specific activity did not decrease significantly from the initial purification of elisabethatriene (**9**) through its conversion to **10** and the subsequent purification of this derivative. The apparent increase in specific activity could be explained by errors associated with measuring very small weights of compounds **9** and **10**.



(1)

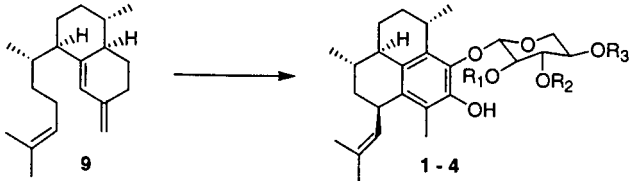
Table 2. Radioactivity of pseudopterosins A–D following incubation with ^{3}H-elisabethatriene

Compound	Radioactivity[a] (dpm)	Radioactive yield (%)
Pseudopterosin A	370	0.5
Pseudopterosin B	150	0.2
Pseudopterosin C	760	1.1
Pseudopterosin D	405	0.6

[a] The radioactivity of all samples was measured three times. The reported values represent a typical data set.

In vitro conversion of elisabethatriene to pseudopterosins A–D

To establish the intermediacy of **9** in pseudopterosin biosynthesis, ^{3}H-elisabethatriene (66,500 dpm) was incubated with a cell-free extract of *P. elisabethae*. The ^{3}H-elisabethatriene used in this incubation was collected as described above from the incubation of ^{3}H-GGPP with a CFE of *P. elisabethae*, and was re-incubated with a cell-free extract. The quenched incubation mixture was extracted with ethyl acetate and pseudopterosins A–D were purified by normal phase HPLC. Subsequent liquid scintillation counting indicated a transformation of **9** to pseudopterosins A–D (Table 2). The overall radiochemical yield for the four pseudopterosins was 2.5%, which is consistent with our radiochemical yields obtained with similar systems. Moreover, since the ^{1}H NMR spectrum of **9** indicated a purity of at least 99%, and the derivative **10** was rigorously purified without loss of specific activity, we can conclude that the radioactivity observed in the pseudopterosins is due to the transformation of elisabethatriene (Eq. (2)).



(2)

This account represents the first radioactivity-guided isolation to establish the structure of an intermediate in the biosynthesis of a marine-derived natural product. This technique proved to be a rapid method for such purposes and should be applicable to other marine systems. Our data suggests that geranylgeranyl diphosphate is transformed to elisabethatriene (**9**) through a series of carbocation rearrangements typical of terpene metabolism (Scheme 1). The bicyclic nature of the cyclization product is somewhat surprising given the structure of the pseudopterosins. The conversion of **9** to the pseudopterosins confirms the intermediacy of elisabethatriene in the biosynthesis of this valuable family of natural products (Eq. (2)) and for the

first time suggests a common biosynthetic origin of the pseudopterosins and *seco*-pseudopterosins.

Experimental

General

NMR spectra were recorded on a 500 MHz spectrometer in C_6D_6; mass spectra were obtained under EI conditions.

Collection

P. elisabethae was collected in May 1999 at Sweetings Cay in the Bahamas at a depth of 35 feet. Animals were flash frozen with liquid nitrogen and stored at −80°C.

Preparation of the cell-free extract

Frozen *P. elisabethae* (322 g) was homogenized in a commercial blender with liquid nitrogen and 600 mL of 0.1 M phosphate buffer (pH=7.7) containing 3.0 mM EDTA and 0.035% β-mercaptoethanol and the homogenate centrifuged at 9700 g for 15 min. The pellet was discarded and the supernatant centrifuged at 39,000 g for 2.75 h. The supernatant (CFE) was stored in 40 mL aliquots at −80°C.

Incubation of CFE with ^{3}H-GGPP and purification of ^{3}H-elisabethatriene

Two 40 mL aliquots of the CFE were incubated with 50 μCi [1-^{3}H] GGPP and 1 mM $MgCl_2$ for 24 h at 29°C. The reaction mixtures were then combined, lyophilized and extracted four times with 5 mL ethyl acetate. Partitioning between hexane and methanol/water (9:1) yielded a hexane fraction which was injected on normal phase HPLC with a gradient from 100% hexane to hexane/ethyl acetate (1:1) over 20 min and maintained at hexane/ethyl acetate (1:1) for 10 min. Throughout the HPLC run, fractions were collected every minute for 30 min. The radioactivity of these fractions was monitored using a liquid scintillation counter. The elisabethatriene (5–7 min fraction) was subjected to reversed phase HPLC (methanol) and again, the radioactivity measured.

Conversion of elisabethatriene (9) to 10. Selenium dioxide (2.35 mg, 21 μmol) and 84 μL of trimethylsilyl polyphosphate (PPSE) were added to 5 mL of CCl_4 and stirred for 15 min at 76°C. Purified **9** (4 mg) was added, and the mixture refluxed for 16 h. The reaction mixture was concentrated under a stream of N_2 and purified by reverse phase HPLC with 100% methanol to afford **10** (2 mg) as a colorless oil. Compound **10**: UV (MeOH) λ_{max} 245 nm; HRMS calcd for $C_{20}H_{32}$ (M^+) 272.2504, found 272.2509; ^{1}H NMR (500 MHz, C_6D_6) δ 6.47 (1H, br s), 5.24 (1H, m), 3.05 (1H, m), 2.02 (1H, m), 1.92 (1H, m), 1.73 (3H, s), 1.69 (3H, s), 1.67 (1H, m), 1.59 (3H, s), 1.48 (1H, m), 1.41 (1H, m), 1.24 (1H, m), 1.21 (1H, m), 1.06 (1H, m), 1.02 (3H, d, *J*=6 Hz), 0.99 (3H, d, *J*=6.5 Hz).

Synthesis and purification of radioactive 10. The radioactive elisabethatriene (117 μg, 1 μmol, 4500 dpm) produced above was treated with selenium dioxide (69 μg, 0.62 μmol) and PPSE (2.48 μl) in refluxing CCl_4 for 16 h as described previously. The reaction mixture was worked up by reverse phase HPLC to afford 14 μg (12%) of **10** and the radioactivity measured (1100 dpm). Weights were obtained from integration of the HPLC peaks.

Incubation of CFE with ^{3}H-elisabethatriene and purification of ^{3}H-pseudopterosins A–D

^{3}H-Elisabethatriene was collected as described above from the incubation of ^{3}H-GGPP with a CFE of *P. elisabethae*. The ^{3}H-elisabethatriene (66,500 dpm) was transferred to a 50 mL centrifuge tube using ethyl acetate, and the solvent evaporated. To the centrifuge tube, 1 mL of phosphate buffer, 1 mM $MgCl_2$, and 0.5% of Tween 20 were added and the mixture vortexed for 10 min. One 40 mL aliquot of cell-free extract was then added and the mixture incubated at room temperature and 200 rpm for 24 h at 29°C. Flash freezing and lyophilization quenched the reaction. The lyophilized incubation mixture was extracted four times with 5 mL of 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 over 15 min). Radioactivity was monitored using a liquid scintillation counter.

Purification and characterization of elisabethatriene (9). *P. elisabethae* (54 g) was extracted by blending with ethyl acetate (500 mL) and subsequently with methylene chloride (500 mL). The extract (6.60 g) was then partitioned between hexanes and methanol/water (9:1) in a 500 mL separatory funnel to yield 4.38 g of the hexane layer. Flash column chromatography of the hexane layer over silica with 100% hexanes yielded a colorless oil (107 mg). Elisabethatriene (10 mg, 0.15% of crude extract) was purified as a pale yellow oil from the 100% hexane fraction by reversed phase HPLC with 100% methanol. Compound **9**: $[\alpha]_D^{20}=+31.5°$ (c 2.857×10^{-4}, MeOH); UV (MeOH) λ_{max} 240 nm (ϵ 10,360); EI MS m/z (relative intensity) 272 (M^+, 40), 257 (M^+-CH_3, 8), 187 ($C_{14}H_{19}$, 47) 159 ($C_{12}H_{15}$, 60), 119 (C_9H_{11}, 90), 105 (C_8H_9, 100); HREIMS calcd for $C_{20}H_{32}$ (M^+) 272.2504 (2.7), found 272.2496.

Acknowledgements

Financial support of this research has been provided by the Camille and Henry Dreyfus Foundation and the American Cancer Society and is gratefully acknowledged. We also wish to thank the Florida Institute of Oceanography for providing ship time aboard the R/V Bellows. Low resolution and high resolution mass spectra were recorded at the University of Florida and the University of Nebraska Center for Mass Spectrometry respectively. In addition we would like to acknowledge Brian Killday at Harbor Branch Oceanographic Institute and Dr A. Ata, FAU, for assistance with the NMR experiments.

References

1. (a) Look, S.; Fenical, W.; Matsumoto, G.; Clardy, J. *J. Org.*

Chem. **1986**, *51*, 5140–5145. (b) Roussis, V.; Wu, Z.; Fenical, W. *J. Org. Chem.* **1990**, *55*, 4916–4922.
2. Look, S.; Fenical, W. *Tetrahedron* **1987**, *43*, 3363–3370.
3. Ettouati, W.; Jacobs, R. *Mol. Pharmacol.* **1987**, *31*, 500–505.
4. Look, S.; Fenical, W.; Jacobs, R.; Clardy, J. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 6238–6240.
5. Haimes, H.; Glasson, S.; Harlan, P.; Jacobs, R.; Fenical, W.; Jimenez, P. *Inflammation Res.* **1995**, *44* (3W13/17).
6. Rouhi, M. *CE News* **1995**, *20*, 42–44.
7. Coleman, A. C.; Mydlarz, L. D.; Kerr, R. G. *Org. Lett.* **1999**, *1*, 2173–2175.
8. Following the initial NP HPLC purification, an attempt was made to assess the purity of compound **9** using RP HPLC with acetonitrile/water as eluent. This resulted in partial decomposition of elisabethatriene and thus only 66,500 dpm of purified **9** was available for the second incubation experiment.
9. The ^{1}H NMR of this fraction from the parallel purification of the non-radioactive extract indicated a purity of approximately 99%.
10. Elisabethatriene was found to be unstable in chloroform as determined by NMR.
11. Lee, J. G.; Kim, K. C. *Tetrahedron Lett.* **1992**, *42*, 6363–6366.