

Author version: *Nat. Prod. Res.*, vol.25(2); 2011; 169-174

Calamenenes – aromatic bicyclic sesquiterpenes – from the Indian gorgonian *Subergorgia reticulata* (Ellis and Solander, 1786)

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Abstract

Three aromatic sesquiterpene derivatives, (+)-(7*R*, 10*S*)-2-methoxy calamenenes (**1**), (+)-(7*R*, 10*S*)-2,5-dimethoxy calamenene (**2**) and (+)-(7*R*, 10*S*)-2-methoxy-5-acetoxy calamenene (**3**) were isolated from the methanol extract of the Indian gorgonian *Subergorgia reticulata*. Compound **2** was not described before in the literature. Compound **3** has not been isolated previously from a natural source.

Compounds **1**, **2** and **3** showed settlement inhibition activity against cyprids of *Balanus amphitrite* with EC₅₀ of 4.4, 7.8 and 0.03 μg/mL, respectively. These compounds exhibited, also, considerable activity against artemia nauplii at 50 μg/mL, indicative of their potential use as a cytotoxic agent.

Keywords: Calamenenes; bioactivity; *Subergorgia reticulata*; *Balanus amphitrite*; Artemia nauplii

1. Introduction

Calamenenes are aromatic bicyclic sesquiterpenes of cadinane skeleton, found in several terrestrial and marine organisms (Bunko, Ghisalberti, & Jefferies, 1981; Kashman, 1979; Kelman, Benayahu, & Kashman, 2000; Nakashima et al., 2002; Nishizawa, Inoue, Sastrapradja, & Hayashi, 1983; Salmoun et al., 2007). Thus, (+)-2-hydroxy calamenene, a potent fish toxin was obtained from the seeds of *Dysoxylum acutangulum* and *D. alliaceum* (Nishizawa et al., 1983). The more polar 2, 5-dioxygenated calamenenes were isolated from gorgonians (*Pseudoplexaura porosa* and *Subergorgia hicksoni*) and soft corals (*Parerythropodium fulvum fulvum* and *Lemnalia cervicornis*) (Bunko et al., 1981; Kashman, 1979; Kelman et al., 2000). The absolute stereochemistry of the natural (+) calamenene and (+)-2-hydroxy calamenene was confirmed by synthesis of both the optical isomers using ring-closing metathesis reaction followed by comparison of their ^{13}C NMR spectra and optical rotation values (Nakashima et al., 2002). Continuing our search for bioactive compounds of marine origin, we hereby report isolation and structure elucidation of calamenene analogues **1-3** from the gorgonian coral *Subergorgia reticulata* collected from Kavaratti, Lakshadweep islands, India. Compound **2** was not described before in the literature. Compound **3** has not been isolated previously from a natural source.

2. Results and Discussion

The crude methanolic extract of *Subergorgia reticulata* was fractionated using different organic solvents of varying polarity. Purification of the active petroleum ether fraction on sephadex LH-20 (CHCl_3 – MeOH 1:1) and silica gel columns (Hexane-EtOAc gradient) yielded compounds **1**, **2** and **3** (Fig.1).

Compound **1**, $[\alpha]_{\text{D}}^{28} = +42.3$ (c 0.1, CHCl_3), displayed prominent IR absorptions at 2932, 1605, 1464, 1402, 1090 cm^{-1} . GC-MS indicated its molecular weight to be 232, corresponding to the probable molecular formula $\text{C}_{16}\text{H}_{24}\text{O}$, having five degrees of unsaturations. In agreement with this, the ^{13}C NMR and DEPT 135 spectra indicated presence of 16 carbon signals (5 CH_3s , 2 CH_2s , 5 CH and 4 quaternary carbons). The four quaternary carbon signals between 108 and 160 ppm as well as the two aromatic ^1H signals [δ 6.57 (1H,s), δ 6.67 (1H,s)] indicated a tetrasubstituted six membered aromatic ring system. The methyl signals at δ 2.37 (Ar-Me) and δ 3.87 (OMe) could be two of the above substitutions, while the bicyclic nature of the compound would account for the remaining two substitutions. The ^1H and ^{13}C NMR values as well as the optical rotation of **1** are in agreement with those reported previously for the same compound (Bunko et al., 1981; Kashman, 1979). The structure was further confirmed to be (+)-(7*R*, 10*S*)-2 methoxy calamenene (**1**) using 2D-NMR studies such as COSY and HMBC.

Compound **2**, $[\alpha]_{\text{D}}^{28} = +60.6$ (c 0.1, CHCl_3), $M^+ = 262$, IR (Neat): 2955, 2838, 1612, 1597, 1462, 1454, 1416, 1383, 1356, 1100, 1049, 1003, and 831 cm^{-1} . The molecular weight indicated possible molecular formula to be $\text{C}_{17}\text{H}_{26}\text{O}_2$, indicating an OMe derivative of compound **1**. The absence of aromatic proton signal of compound **1** at δ 6.67 (1H, s, H-5) and appearance of an additional OMe peak at δ 3.67 (3H, s) further supported this view. The presence of only one aromatic ^1H signal at δ

6.54(1H, s) indicating possible replacement of one proton with OMe group also supported the above formula. Structure of this compound was assigned as (+)-(7*R*, 10*S*)-2, 5-dimethoxy calamenene (**2**) from a comparison of its ¹H NMR and optical rotation data with those of similar compounds.

Compound **3**, $[\alpha]_D^{28} = +28.53$ (c 0.1, CHCl₃), $M^+ = 290$, indicated the molecular formula to be C₁₈H₂₆O₃. Its IR (Neat) spectrum had prominent bands at 2960, 1615, 1580 and 1759 cm⁻¹, indicative of a phenolic ester group. Its ¹H and ¹³C NMR spectra (Table 1) had 18 carbon signals distributed as one carbonyl, six aromatic (one CH, rest quaternary), six CH₃s, two CH₂s and three CHs, indicating it to be a methoxy, acetoxy calamenene derivative. The structure was finally assigned as (+)-(7*R*, 10*S*)-2-methoxy-5-acetoxy calamenene (**3**), from a comparison of its optical rotation, ¹H and ¹³C NMR data (Table 1). The ¹H NMR values are in agreement with those reported previously for the same compound (Bunko et al., 1981; Kashman, 1979). The *trans* stereochemistry at C-7 and C-10 was confirmed by a comparison of the chemical shifts of C-5, C-8, C-11 and C-12 carbons of both *cis* and *trans* isomers (Table 1). The structure was further confirmed by 2D NMR studies such as COSY and HMBC.

Compounds **1-3** displayed promising activity against artemia nauplii, with compound **3** exhibiting 100% activity at 50µg/mL, indicative of possible cytotoxicity (Carballo, Hernandez-Inda, Perez, & Garcia-Gravalos, 2002). This concentration is comparable to the cytotoxic activity (14-30µg/mL) of Argentinean essential oils (Oliva, Gallucci, Zygadlo, & Demo, 2007). Compounds **1-3** also inhibited settlement of cyprids of the fouling barnacle, *Balanus amphitrite* (EC₅₀ of 4.4, 7.8 and 0.03µg/mL, respectively) with therapeutic ratios (T.R.) of 7.3, 4.6 and 799, respectively. Of these, compound **3** exhibits a very high T.R. compared to the reference standard, copper sulphate solution (1.9).

Gorgonians of the genus *Subergorgia* are known to be rich in cyclic sesquiterpenes. Previously we had reported triquinane sesquiterpene subergorgic acid and analogues from a related species, *S. suberosa* (Parameswaran et al., 1998). Another species *S. hicksoni* is reported to be a rich source for dioxygenated calamenene derivatives (Kashman, 1979). In this regard, the present study, isolating different calamenene derivatives from *S. reticulata* is in agreement with the earlier results. Also, several polyoxygenated sterols and briarane-type diterpenoids have already been reported from this gorgonian (Yang, Qi, Zhang, & Li, 2006; Yang et al., 2007).

3. Materials and methods

3.1. General

NMR spectra were recorded in CDCl₃ solution in BRUKER Avance 300 MHz NMR spectrometer, using a standard 5mm probe while EIMS analysis was performed in a GCMS instrument (Shimadzu). For column chromatography, silica gel (60-120 mesh size) from s.d. Fine Chem Limited was used. All solvents were procured from s.d. Fine Chem Limited (AR Grade) and used as such.

3.2. Animal Material

The Indian gorgonian *Subergorgia reticulata* (Ellis and Solander, 1786) was collected from Kavaratti, Lakshadweep islands, India (Lat 10°33'0 N; Long 72°38'0 E) during November 2006 from a depth of 25 m. Voucher specimen (No. NIO/RCK/194) has been deposited at the National Institute of Oceanography, Kochi, India.

3.3. Extraction and Isolation

The specimen was washed thoroughly with fresh water and soaked in methanol before transporting to the laboratory. The crude extract so obtained was concentrated using roteva and partitioned into petroleum ether (Fr.1), ethyl acetate (Fr.2), and aqueous (Fr.3) fractions. Of these, the active Fr.1 was purified on a sephadex LH-20 column with (CHCl₃-MeOH, 1:1) as the eluant. The 53 fractions so obtained were recombined into 5 fractions (Fr. 1-1 to 1-5) based on their TLC profile. Of these, Fr.1-5 was further purified on a silica gel column using (hexane-EtOAc) gradient elution, yielding compounds **1**, **2** and **3**.

3.3.1. 2-methoxy calamenene

(+)-(7*R*, 10*S*)-2-methoxy calamenene (**1**): ¹H NMR δ 0.89 (3H, d, 6.6 Hz, H-12), 1.10 (3H, d, 6.9 Hz, H-13), 1.20 (3H, d, 6.9 Hz, H-14), 1.60 (1H, m, H-9), 1.90 (1H, m, H-9), 1.8-1.9 (2H, m, H-8), 2.10 (1H, m, 6.9 Hz, H-11), 2.40 (3H, s, H-15), 2.51 (1H, m, H-7), 3.20 (1H, m, H-10), 3.87 (3H, s, Me-2), 6.57 (1H, s, H-3), 6.67 (1H, s, H-5). The prominent HMBC cross peaks were: δ 0.89/ 33.3, 43.1; 1.10/ 33.3, 43.1; 1.20/ 26.6, 27.2, 22.2, 128.5; 1.60-1.90/ 26.6, 43.1, 128.5; 1.84-1.92/ 19.7; 2.10/ 19.7, 22.6; 2.40/ 21.6, 108.5, 122.6, 128.5, 134.6; 2.51/ 122.6, 27.2, 33.3, 128.5, 140.7, (26.6 or 27.2), 33.3; 3.20/ 19.1, 19.9, 26.6, 128.5, 140.7; 3.87/ 108.5, 159.1; 6.57/ 22.2, 122.6, 128.5, 140.7, 134.6, 159.1; 6.67/ 21.5 or 21.6, 43.1, 108.5, 128.5, 140.7, 159.5. The prominent COSY cross peaks were: δ 0.89/ 2.10; 1.10/ 2.10, 3.90; 1.20/ 3.20; 2.10/ 0.90, 1.10, 2.50; 2.40/ 6.57, 6.67; 3.20/ 2.40, 2.10, 1.60, 1.20, 1.80; 3.87/ 1.60, 6.57; 6.57/2.53; 6.67/ 2.53.

3.3.2. 2, 5-dimethoxy calamenene

(+)-(7*R*, 10*S*)-2,5-dimethoxy calamenene (**2**): ¹H NMR δ 0.89 (3H, d, 7.2 Hz, H-12), 0.91 (3H, d, 7.2 Hz, H-13), 1.18 (3H, d, 6.9 Hz, H-14), 1.48-1.98 (1H, m, 2H-9), 1.86 (2H, m, H-8), 2.03 (1H, m, H-11), 2.31 (3H, s, H-15), 2.79 (1H, m, H-7), 3.18 (1H, m, H-10), 3.82 (3H, s, Me-2), 3.67 (3H, s, Me-5), 6.54 (1H, s, H-3).

3.3.3. 2-methoxy-5-acetoxy calamenene

(+)-(7*R*, 10*S*)-2-methoxy-5-acetoxy calamenene (**3**): ¹H NMR δ 0.91 (3H, d, 6.9 Hz, H-12), 0.95 (3H, d, 6.9 Hz, H-13), 1.16 (3H, d, 6.6 Hz, H-14), 1.50-1.99 (1H, m, 2H-9), 1.83 (2H, m, H-8), 2.02 (1H, m, H-11), 2.16 (3H, s, H-15), 2.34 (3H, s, Ac-5), 2.49 (1H, m, H-7), 3.21 (1H, m, H-10), 3.84 (3H, s, Me-2), 6.60 (1H, s, H-3).

3.4. Biological Assays

The antifouling activity against *B. amphitrite* cyprids was determined using the settlement-inhibition method (Limna Mol, Raveendran, & Parameswaran, 2009). Effective concentrations (EC₅₀) for settlement inhibition of *B. amphitrite* cyprids by the compounds **1** and **2** were determined by serial

dilution method at concentrations ranging from 100 to 1 µg/mL and for compound **3** at concentrations ranging from 100 to 0.01 µg/mL. The experiments were conducted in sterile 24-well polystyrene multiwell plates (Axigen). The compounds, dissolved in methanol, were added to autoclaved 0.45 µ filtered sea water (FSW) to obtain the desired concentrations. 10 competent cyprids were added to each of the 6 replicates of 2 mL of the test solution. Wells containing only FSW with MeOH served as control and copper sulphate solution was used as the reference standard. The plates were incubated for a 24 hour period under similar conditions at which the nauplii were reared. After the incubation period, the numbers of settled and metamorphosed cyprids were counted under a stereomicroscope and expressed as a proportion of the total number of larvae in the well. The experiment was repeated twice with different batches of larvae.

The cytotoxicity assay against *Artemia* sp. nauplii was conducted in sterile 24-well polystyrene multiwell plates (Carballo et al., 2002). 10 competent nauplii (1-day old) were introduced into each of the 3 replicates of 5 mL of 50 µg/mL test solution. The numbers of active and dead nauplii were recorded after 24 hours of incubation and expressed as a proportion of the total number of nauplii in the well.

Acknowledgements Our sincere thanks are due to CMLRE, Ministry of Earth Sciences, New Delhi for the partial financial support and the Director, NIO for the facilities provided and encouragements. We also gratefully acknowledge the help rendered by Dept. of Science and Technology, Lakshadweep Islands for the collection of samples and Dr. P.A. Thomas for identification of the specimen. One of the authors (VPL) is thankful to CSIR, Govt. of India for the grant of Senior Research Fellowship.

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Table 1. Comparison of ^{13}C NMR values of compounds **1** and **3** with those of similar compounds [5]

C. No.	Cmpd 1	Cmpd 3	(+)-(7 <i>R</i> , 10 <i>S</i>)- 2-hydroxy calamenene	(+)-(7 <i>R</i> , 10 <i>R</i>)- 2-hydroxy calamenene
1	128.5 s	126.9 s	126.1	126.7
2	159.1 s	154.7 s	153.2	153.0
3	108.5 d	109.8 d	113.4	112.8
4	134.6 s	132.7 s	135.1	135.7
5	122.6 d	141.5 s	123.0	120.8
6	140.7 s	130.0 s	141.3	141.3
7	43.1 d	38.5 d	43.0	43.3
8	19.3 t	19.2 t	19.1	16.4
9	27.2 t	26.5 t	27.1	28.8
10	26.6 d	25.8 d	26.5	26.5
11	33.3 d	32.6 d	33.1	30.9
12	19.7 q	20.9 q	19.6	17.3
13	22.2 q	21.9 q	21.0	20.5
14	21.6 q	21.7 q	22.1	21.2
15	21.5 q	16.8 q	21.1	21.1
OMe	55.1 q	55.2 q		
OAc		20.8 q		
		169.3		

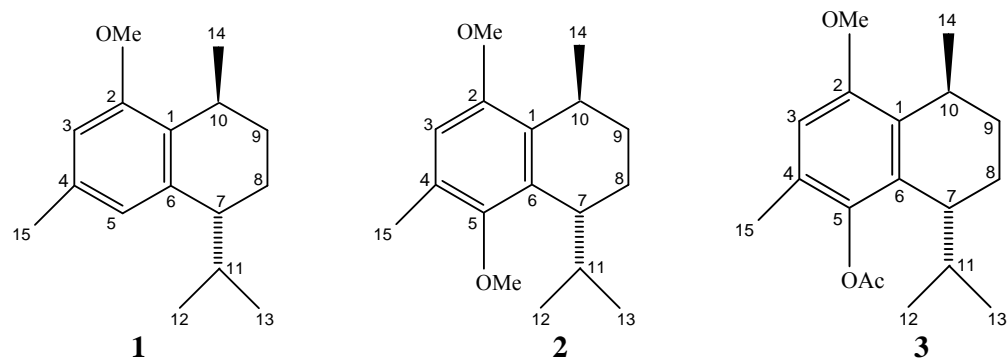


Fig.1. Calamenenes isolated from *S. reticulata*