

## Microfouling inhibitors from the Indian gorgonian *Subergorgia reticulata* (Ellis and Solander, 1786)

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Antifouling research currently focuses on greener ways of biofouling control in view of the International Maritime Organisation (IMO) imposed regulations on toxic biocides. Microfouling, the initial phase of fouling, is an important target to prevent further macrofouling assemblage. In this perspective, the antimicrofouling potential of three calamenene derivatives (2-methoxy calamenene, **1**; 2,5-dimethoxy calamenene, **2** and 2-methoxy, 5-acetoxy calamenene, **3** isolated from the Indian gorgonian *Subergorgia reticulata* was investigated. These compounds inhibited growth of five fouling bacterial strains (*Bacillus megaterium*, *Vibrio furnissii*, *V. fischeri*, *V. gazogenes* and *V. parahaemolyticus*) as well as the two species of diatoms (*Amphora coffeaeformis* and *Navicula transitans*). Of these, the highest activity was exhibited by compound **3** against *B. megaterium* (MIC, 12.5µg) and *N. transitans* (EC<sub>50</sub>, 14µg/ml). The environmental-compatibility of this compound was evidenced from the non-toxicity to *B. amphitrite* nauplii (IC<sub>50</sub> 1µg/ml) and *Artemia* sp. nauplii (IC<sub>50</sub> 0.6µg/ml). Analysis of structure-activity relationship suggested that the acetoxy group present in **3** may be responsible for the comparatively high antifouling activity exhibited by this compound.

[**Keywords:** Biofouling; microfouling; antifouling; *Subergorgia reticulata*; calamenenes]

### Introduction

Surfaces submerged in seawater are subjected to intricate conditioning processes, facilitating the colonization by pioneering bacteria and diatoms followed by the attachment and growth of macrofouling organisms. With our increasing dependence on the oceans not only for the exploration and exploitation of oil, gas, minerals and living resources but also for the transport and recreation purposes, the biofouling prone areas are increasing day-by-day. This assemblage of flora and fauna, adversely affecting the intended performance of surfaces such as ship hulls, offshore platforms, Ocean Thermal Energy Conversion (OTEC) plants etc. is a cause of serious concern to all maritime nations<sup>1-10</sup>.

Strategies for containing fouling have probably been initiated since man's first venture into the sea. However, the development of environmentally compatible antifouling procedure remains an unresolved problem till date. The imposition of global ban on the most efficient antifouling agent, Tri butyl tin (TBT), due to its environmental toxicity, triggered the search for

eco-friendly alternatives. This resulted in several potential antifouling agents like Avaron from the sponge *Dysidea avara*, Furanone from the red seaweed *Delisea pulchra*, Juncellin from the octocoral *Juncella juncea* etc. The analog synthesis, field-testing and commercialisation of these compounds are under various stages of progress<sup>10</sup>.

Among the different marine organisms found in coral reefs, the gorgonian corals constitute an important group, secreting an array of secondary metabolites to protect themselves from fish and invertebrate predators<sup>11-14</sup>, bacterial pathogens<sup>15</sup> and surface fouling assemblages<sup>16-17</sup>. During our earlier investigations, we could successfully isolate three antifouling calamenene derivatives from the Indian gorgonian *Subergorgia reticulata* (Fig.1,<sup>17</sup>). Several calamenene analogs have been reported in recent years, from both terrestrial and marine organisms<sup>18-19</sup>. Many of these compounds show promising antifungal, insecticidal, antifouling and ichthyotoxic properties<sup>20-21,17</sup>. However, their antibacterial properties are not reported till date.

Here we present the antimicrofouling potential of the calamenene derivatives against fouling bacteria and diatoms. The environmental compatibility of the derivatives was tested using barnacle and artemia nauplii.

## Materials and Methods

### Antibacterial bioassay

Five different strains of fouling bacteria (*Bacillus megaterium*, *Vibrio fischeri*, *V. furnissii*, *V. gazogenes* and *V. parahaemolyticus*) were isolated from fouling panels immersed at Fore Shore Road boat jetty, Kochi (Lat 9°9'N; Long 76°2' E). The isolates were identified using taxonomic, physiological and biochemical methods based on Bergey's manual of systematic bacteriology<sup>22</sup>. Identification was further authenticated using gas chromatographic analysis of whole cell fatty acid methyl ester (FAME). Fatty acids of the bacteria were extracted according to the protocol of MIS operating manual by MIDI Corporation, USA and compared with the library of Sherlock MIS System (Agilent GC-6950). The antibacterial activity was tested by the disc diffusion method<sup>23-25</sup>. 18-hour-old cultures of the selected fouling bacterial strains were spread onto Zobell marine agar. Sterile 6-mm Whatman (GF/C) filter paper discs were impregnated with 50 µg of compounds **1**, **2** and **3** dissolved in methanol, evaporated to dryness and placed onto the bacterial lawn. Discs containing Streptomycin (10 µg/disc each) were used as reference standard and discs loaded with 25 µl of absolute methanol served as control. The plates were incubated for 24 hours at 28±1°C and the resulting clear zones of bacterial growth inhibition were measured. The experiment was conducted in duplicate for each of the strains.

### Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of compound **3**, exhibiting the highest activity, was determined using the **broth micro-dilution susceptibility assay**<sup>26-27</sup>. The tests were conducted using 18 hour-old bacterial cultures in Zobell Marine Broath (ZMB). The test samples were prepared in a 24-well multiwell plate (Axigen) with 50 µl of 20 to 1 mg/ml dilutions. Subsequently, 400 µl of ZMB were added to each well, followed by inoculation of 50 µl of 10<sup>8</sup> colony forming units (cfu/ml) of the standardized

bacterial suspensions to make a final test volume of 500 µl. The plates were incubated at 28°C for 24 hours. Penicillin-G was used as the reference compound for the antibacterial activity. After 24 hours, 50 µl of the test solution in each of the wells was spread onto Zobell Marine Agar (ZMA) plates and incubated at 28°C for 24 hours. The plate showing complete absence of growth was recorded and the highest dilution pertaining to this was considered as the MIC.

### Antidiatom bioassay

The fouling diatoms, *Amphora coffeaeformis* and *Navicula transitans* were procured from the National Institute of Oceanography (NIO), Goa. Prior to bioassay the test diatoms were subcultured in f/2 medium. To about 250ml of medium, 10ml of inoculum was added. It was then incubated at a constant temperature of 25°C providing 1000 lux light for 7-8 days. Test solution was prepared by dissolving 0.005g of compounds **1**, **2** and **3** in 1ml dimethylsulfoxide (DMSO), which was added to 50 ml of sterilized f/2 medium to get a concentration of 100µg/ml. The bioassay was done in triplicate. The test solutions (50 ml) were taken in 100ml conical flasks (Riviera) each and the control consisting of f/2 medium only was inoculated with 500 µl of 7-day-old *A. coffeaeformis* and *N. transitans* culture. The experimental flasks were incubated in the environmental chamber at a constant temperature of 25°C providing 1000lux light for 7-8 days. After incubation, the diatom cells were separated from media by suction filtering through Whatman GF/F filter paper, using 0.5 HP motor. The filter paper containing cells was transferred to 10ml of 90% acetone and kept under dark conditions in a refrigerator at 4°C for 16hours. The intensity of green color developed due to chlorophyll *a* extraction was measured using fluorimeter (Turner Designs, Trilogy). The inhibition percentage was calculated using the formula:

$$\text{Inhibition \%} = \left\{ \frac{\text{Conc. of Chlorophyll } a \text{ in the control} - \text{Conc. of Chlorophyll } a \text{ in the test solution}}{\text{Conc. of Chlorophyll } a \text{ in the control}} \right\} * 100$$

Table 1. Activity exhibited by Calamenene-derivatives at 50µg/disc concentration against fouling bacterial strains after 24 hours of incubation

Test Solution	Zone of Inhibition (mm) *				
	B.m.	V.fi.	V.fu.	V.g.	V.p.
Compound 1	13.50 ± 0.71	15.50 ± 0.71	07.25 ± 0.35	11.75 ± 0.35	08.75 ± 0.35
Compound 2	10.00 ± 0.71	11.00 ± 0.00	05.00 ± 0.00	05.50 ± 0.71	06.00 ± 0.00
Compound 3	20.25 ± 0.35	17.75 ± 1.06	13.00 ± 0.00	14.25 ± 0.35	10.50 ± 0.71

B.m., *Bacillus megaterium*; V.fi., *Vibrio fischeri*; V.fu., *V. furnissii*; V.g., *V. gazogenes*; V.p., *V. parahaemolyticus*; 1-2 mm = Low activity; 2-5 mm = Moderate activity; >5 mm = High activity

#### Determination of EC<sub>50</sub>

Compound 3, exhibiting the highest activity against both the diatom species, was tested at concentrations ranging from 100 to 1µg/ml and the EC<sub>50</sub> was determined by Probit analysis.

#### Toxicity assays

The nauplii of *Balanus amphitrite* and *Artemia* sp. were used for the toxicity assays. *B. amphitrite* nauplii were collected from the barnacle culture system in the laboratory<sup>25</sup>, while the artemia nauplii were obtained by hatching cysts procured from O.S.I., USA. For *B. amphitrite* nauplii, the assay was conducted in sterile 6-well polystyrene multiwell plates (Axigen). Ten nauplii (stage II) were added to the 6 replicates of 5ml test solutions of compound 3 (ranging from 100 to 0.01µg/ml). Observations were made under a stereomicroscope for 90min. The inability of *B. amphitrite* nauplii to stay in the water column and the loss of phototactic reaction were scored as toxic responses<sup>28</sup>. From these observations, the concentration required for inhibitory activity against 50% of *B. amphitrite* nauplii within the given time period (IC<sub>50</sub>) was determined using Probit analysis<sup>29-30</sup>.

The toxicity assay against *Artemia* sp. nauplii was also conducted in sterile 6-well polystyrene multiwell plates<sup>31</sup>. Ten competent nauplii (one-day old) were introduced into each of the 6 replicates of 5ml test solutions of compound 3 (ranging from 100 to 0.01µg/ml). The numbers of active, feeble and dead nauplii were recorded after 90 minutes of observation and expressed as a proportion of the total number of nauplii in the well. From the data obtained, IC<sub>50</sub> value of the compound against *Artemia* sp. nauplii was determined using Probit analysis.

## Results

#### Antibacterial assay

All the three compounds inhibited the growth of bacteria isolated from fouling panels. Compound 3 exhibited high activity against all the tested bacterial strains, with maximum inhibition of *Bacillus megaterium* (Table 1). Compound 1 also exhibited relatively high activity against all the test bacteria, while compound 2 exhibited high activity against *B. megaterium*, *Vibrio fischeri* and *V. parahaemolyticus*, and was only moderately active against the remaining two strains of *Vibrio* (Table 1).

#### Minimum Inhibitory Concentration

Compound 3 exhibited an MIC of 25 µg against majority of the strains (Table 2). The lowest MIC recorded was 12.5 µg against *B. megaterium*, only five times greater than the MIC of the reference standard, Penicillin G against this strain (2.5 µg).

Table 2. Minimum Inhibitory Concentration of compound 3 against fouling bacterial strains after 24 hours of incubation

Sl. No.	Test Bacteria	MIC (µg)	
		Compound 3	Penicillin-G
1.	<i>Bacillus megaterium</i>	12.5	2.5
2.	<i>Vibrio fischeri</i>	25	2.5
3.	<i>V. furnissii</i>	25	7.5
4.	<i>V. parahaemolyticus</i>	50	5.0
5.	<i>V. gazogenes</i>	25	5.0

#### Antidiatom bioassay

All the three compounds inhibited the diatom species, with the highest activity (97%) being exhibited by compound 3 against *N. transitans* (Table 3). This compound also caused an inhibition of 86% in *A. coffeaeformis*. It had an EC<sub>50</sub> of 16 and 14µg/ml against *A. coffeaeformis* and *N. transitans*, respectively (Fig.2).

Table 3. Activity of calamenene derivatives against fouling diatoms

Sl. No.	Test Solution (50 µg/ml)	% Activity	
		<i>Amphora coffeaeformis</i>	<i>Navicula transitans</i>
1.	Compound 1	77	81
2.	Compound 2	63	68
3.	Compound 3	86	97

*Toxicity assays*

The concentration required for inhibitory activity against 50% of *B. amphitrite* nauplii within the given time period ( $IC_{50}$ ) was 1µg/ml, while for *Artemia* sp. nauplii it was 0.6µg/ml. In both the cases, no mortality was observed at concentrations of <5µg/ml (Fig.3).

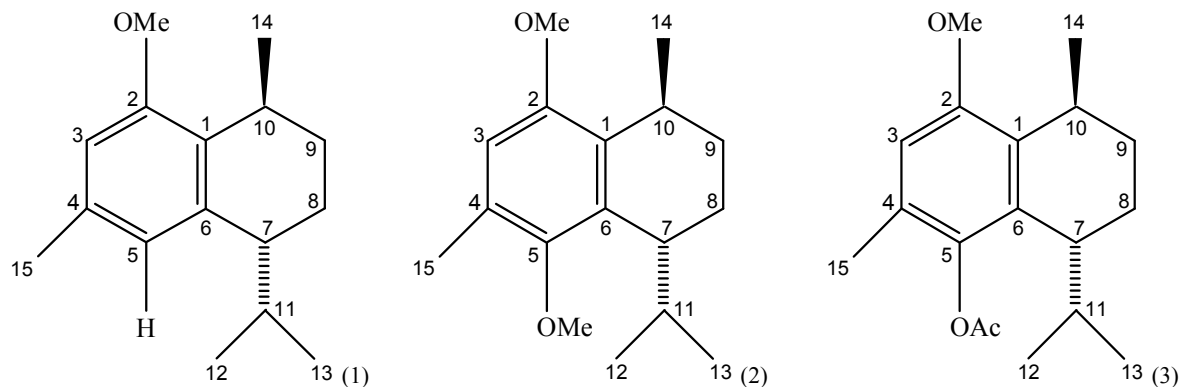


Fig.1 - Calamenenes isolated from *Subergorgia reticulata* (1) 2 - methoxy calamenene; (2) 2, 5 - dimethoxy calamenene; (3) 2 methoxy, 5 acetoxy - calamenene<sup>17</sup>

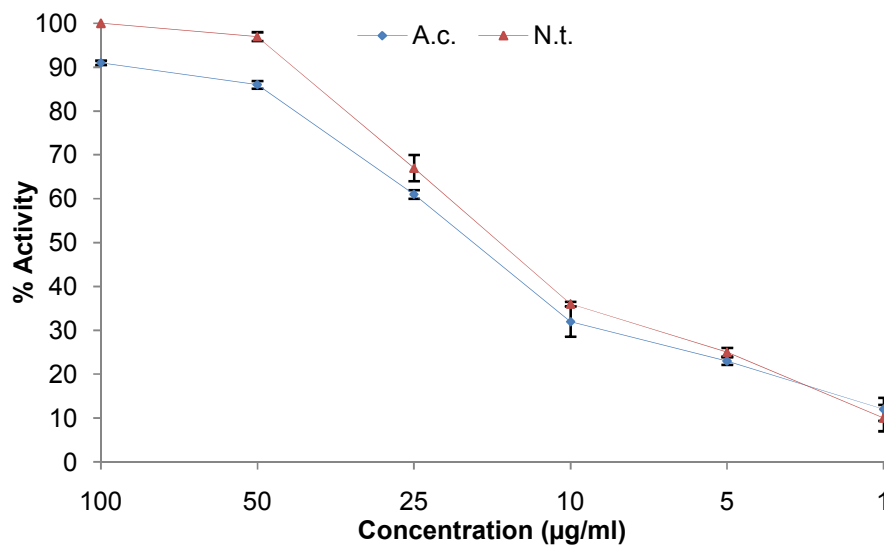


Fig.2 - Inhibition of *A. coffeaeformis* and *N. transitans* by compound 3

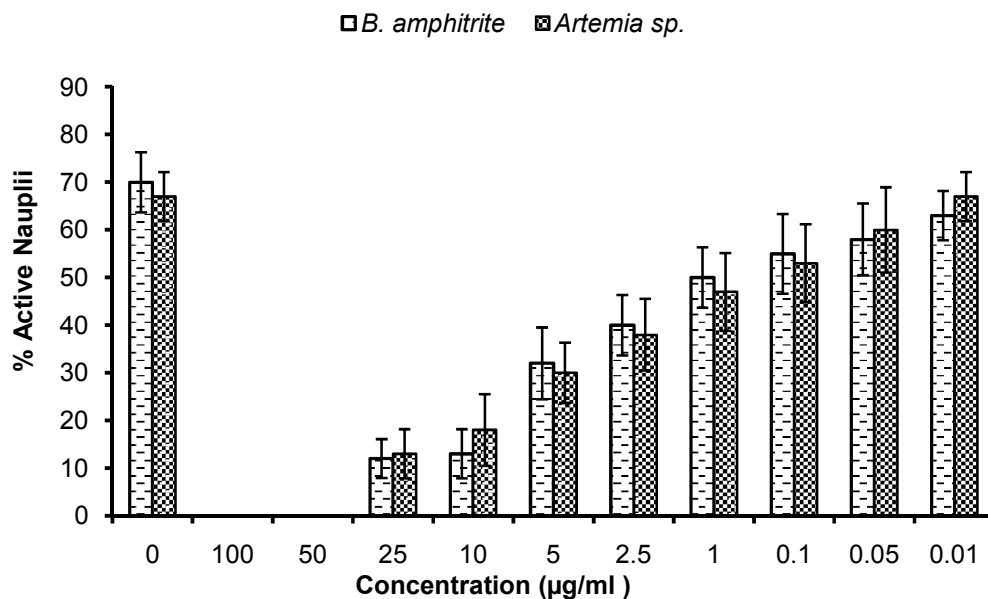


Fig.3 - Percentage of active *Balanus amphitrite* nauplii and *Artemia sp.* nauplii after 90 minutes exposure to varying concentrations of compound 3.

## Discussion

Calamenenes, the aromatic sesquiterpene compounds, have seldom been explored for their biological properties. Although a few reports indicate their antimicrofouling properties<sup>32,17</sup>, there are no studies pertaining to their antimicrofouling potential. Microfouling organisms like bacteria colonise a surface within hours of its immersion into water. Soon after adhering to a surface, they multiply and secrete exopolymeric substances resulting in the formation of a slime layer (biofilm). The deleterious impact of biofilms on marine industry is manifold, including lowering of filtration efficiency in desalination and water purification plants (friction drag increases of 10% to 20% in ships, reduced heat transfer performance of heat exchanger tubes etc. Moreover, biofilm is also known to facilitate macrofouling, which is an economically and environmentally important problem for all maritime nations<sup>33-34,25</sup>. Considering the possibility that microfouling facilitates macrofouling process, it would be desirable to have NPAs capable of preventing microfouling. Considering the dearth of information on antimicrofouling potential of calamenene derivatives, the present study forms a pioneering attempt in this direction.

As evident from the results, compound 3 was the most active among the three derivatives, and *Bacillus megaterium* isolated from the fouling panels, exhibited the highest susceptibility (Table 1). The other strains were also susceptible to compound 3 (Table 1). Since the test bacterial strains were directly isolated from fouling panels, the observations can be accounted on par with the natural conditions.

The bacteria are not the only organisms which can produce initial colonization; diatoms with their sticky mucopolysaccharide secretions may also be involved in early film formation, and when they are abundant can promote bio-corrosion of the surface<sup>33</sup>. Therefore, the high activity exhibited by 2-methoxy, 5-acetoxy calamenene 3 against the fouling diatoms, *Amphora coffeaeformis* and *Navicula transitans*, indicates its bright potential as an antimicrofouling agent.

The fact that we have already explored the rich antimicrofouling potential of this compound against the cosmopolitan biofouler, *Balanus amphitrite* (EC<sub>50</sub> 33.50ng/ml, therapeutic ratio 799.25<sup>17</sup>, further highlights its potential as a broad-spectrum antifouling agent. Formulation of such natural product antifoulants capable of inhibiting both micro as well as macrofoulers are

highly desirable, because marine biofouling is a complex process, involving interactive assemblage of micro- as well as macrofoulers<sup>33,35</sup>.

Realisation of compound **3** as a promising antifoulant encouraged us to pursue further its ecological compatibility and acceptability in the context of IMO ban on toxic antifouling agents. According to Qian *et al.* 2010, a compound with a therapeutic ratio ( $LC_{50}/EC_{50}$ ) greater than 15 is often considered as a non-toxic AF compound and a much higher therapeutic ratio is highly recommended when selecting candidate compounds. In view of this, compound **3**, having a therapeutic ratio of 799<sup>17</sup>, is a highly prospective non-toxic candidate molecule. The non-toxic nature of this compound has been further highlighted in the present study using the toxicity assays against barnacle and artemia nauplii. The  $IC_{50}$  value of the compound against both, *B. amphitrite* nauplii (1 $\mu$ g/ml) and *Artemia* sp. nauplii (0.6 $\mu$ g/ml) is much higher than the  $EC_{50}$  against *B. amphitrite* cyprids (33.50ng/ml)<sup>17</sup>. This implies that the non-targeted organisms will not be adversely affected by the active concentration of the compound.

The present study also provides an insight into the structure-activity relationship of the candidate molecule. All the three tested compounds had a bicyclic cadinane skeleton with methoxy group as a common functional unit. The only notable difference of compound **3** was the presence of an acetoxy group (Fig.1). Therefore, the surge in activity of this compound can be attributed to the presence of acetoxy group in this molecule. Earlier studies also suggest that the entire molecule is not necessary for the activity. Usually only a small active site within the molecule such as a particular functional group, halogen etc. may be responsible for the antifouling potential of the molecule<sup>36</sup>. The fact that synthetic routes for the calamenenes are already known<sup>19</sup> provides optimism towards the possibility for synthesis of simpler analogues of this compound, a prerequisite for ensuring supply commensurate with the needs of the antifouling paint industry. Considering the rich antifouling potential of the isolated calamenene derivatives in laboratory-scale experiments, the compounds would be subjected to field-testing as a future initiative.

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