

Evaluation of single and joint effect of metabolites isolated from marine sponges, *Fasciospongia cavernosa* and *Axinella donnani* on antimicrobial properties

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ABSTRACT

The biochemical mechanisms that marine sponges have developed as a chemical defense to protect themselves against micro and subsequent macrobiofouling process might comprise a potential alternative for the preventing attack of biofilm forming bacteria. The present study investigated the antimicrobial activity of a series of major secondary metabolites isolated from the sponges *Fasciospongia cavernosa* and *Axinella donnani* against fouling bacteria. Secomanoalide (**1**), dehydromanoalide (**2**) and cavernosine (**3**) have been isolated from *F. cavernosa*. Their structures were determined by MS, ¹H NMR spectra analyses and by comparison with those reported in the literature. The most promising activity was exhibited by the metabolites from *A. donnani* i.e. cerebroside (**5**) against three strains *Aeromonas hydrophila* subsp. *salmonicida* A449 and *Erythrobacter litoralis*. Our investigation revealed that combined metabolites **1**, **2** and **3** retained strong activity but individual metabolite had moderate activity indicating that activity probably results from synergistic interactions between multiple compounds. The antibacterial screening of compounds **3**, **5** and synergistic effect of **1-3** against fouling bacteria has been studied for the first time. Further, isolation of manoolide related compounds and their synergistic screening can be accelerated for the development of new biofilm inhibitors.

Keywords: Biofilm, *Fasciospongia cavernosa*, Marine bacteria, Marine natural products, Synergistic effect.

Sponges an important member of the marine ecosystem are the soft bodies of sessile or slow-moving invertebrates often live exposed and exhibit conspicuous colours, despite the lack of protecting shells or other physical defence structures. Many of these organisms compensate for their lack of a physical defence by producing chemicals. As consequences, these organisms have for long been the focus of attention concerning their chemical defence mechanisms as well as source of bioactive molecules.¹ Many studies showed that they are bank of different types of metabolites such as terpenoids, alkaloids and steroids, indicating their ecological significance as antipredation, competition for space and control of epibiont overgrowth.^{2,3} In a marine environment, antimicrobial and antifouling metabolites are energetic for many sessile organisms to insure that they do not host hazardous biofilms on their exposed surfaces.^{4,5} The interference with bacterial quorum sensing (QS) has been proposed as one of potential approach for controlling biofilms and subsequently many QS inhibiting marine natural products are also exhibiting antifouling activity.⁶

The sponges of the genus *Fasciospongia* found in marine ecosystems are most of the times free of epibionts and have at the

same time proven to be rich sources of interesting bioactive metabolites, such as linear or cyclic lactones and sesterterpenoids related to manoolide.⁷ Some *Fasciospongia* metabolites have exhibited antimicrobial and cytotoxic effects. Additionally, several constituents in particular manoolide type of compounds have been reported to show fish deterrent properties.⁸ Another sponge used in present study belongs to *Axinellidae* family which has been known as common source of structurally unique and biologically active metabolites, such as terpenoids,⁹ sterols,¹⁰ peptides, alkaloids¹¹ and macrolides.¹² Few of these metabolites possessed anti-tumor, anti-inflammatory, and neuroprotective properties. Additionally, the crude extract from *Axinellidae* family has been utilized in fish feeding studies demonstrating the potential of its chemical defense against predatory fishes.¹³ Still there is need to explore these two sponges for their novel metabolite as well as for new biological applications as therapeutic agents. In view of this observations and our research interest in marine natural products,¹⁴ herein we are reporting isolation and antimicrobial activity of metabolites secomanoalide (**1**), 4E,6E-dehydromanoalide (**2**) & cavernosine (**3**)

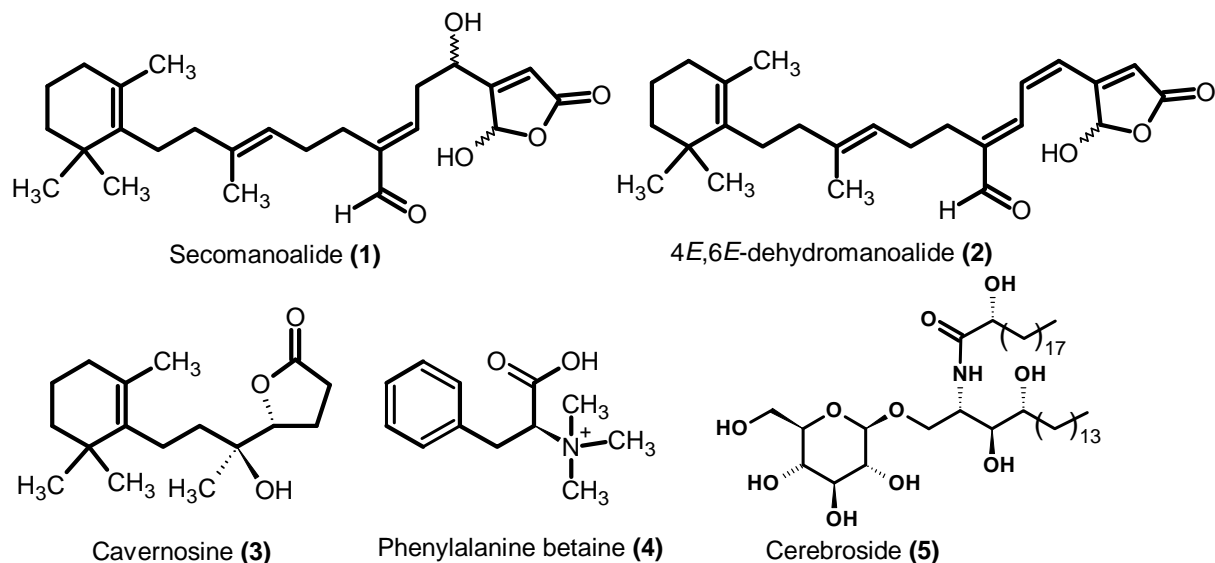


Figure 1. Structure of metabolites isolated from marine sponges *F. cavernosa* and *A. donnani* in the present work

from the marine sponge *Fasciospongia cavernosa*, as well as phenylalanine betaine (4) & cerebroside (5) from *Axinella donnani*. *In vitro* screening of these compounds against 10 biofilm forming bacteria has been also highlighted. Moreover, work has been extended to evaluate the synergistic effect of these compounds for antimicrobial activity against fouling bacteria indicating the importance of combined metabolites effects produced by the individual marine organism.

Towards this end, the purification of the 1-butanol fraction of sponge *F. cavernosa* resulted in the isolation of sesterterpenes: secomanoalide (1), 4E,6E-dehydromanoalide (2) and terpenoid lactone: cavernosine (3) (Figure 1). Examination of ^1H NMR data of the fraction provided evidences for the presence of terpene molecules. The isolation and purification of these molecule required two or three chromatographic separations on silica gel. Fractions were combined and distinguished by their ^1H NMR spectra. The combined fractions were again chromatographed on HPLC on C18 X-bridge and eluted with water and acetonitrile gradient elution to obtain pure compounds 1, 2 and 3 as viscous light yellow oil. The structure of active compounds secomanoalide (1), 4E,6E-dehydromanoalide (2) and cavernosine (3) were readily identified by comparison of their spectral data with those reported in literature.¹⁵ Manoalide was first reported in 1980 from the marine sponge *Luffariella variabilis* by De Silva and Scheuer as an antibacterial agent.¹⁶ The related compounds have been isolated from marine sponge genera of *Diacarnus*, *Fascaplysinopsis*, *Hyrtios*, *Luffariella*, and *Thorectandra*.¹⁷ They possess potent anti-inflammatory properties inactivating phospholipase A2 (PLA2). Also, manoalide, secomanoalide, manoalide 25-acetate and 4E,6E-dehydromanoalide has been reported for their antifouling activity against cypris larvae of the barnacle *Balanus amphirite* with EC_{50} value 0.24, 0.80, 0.53 and 2.7 $\mu\text{g}/\text{ml}$, respectively.¹⁸ Although manoalide skeleton is well explored for their antifouling activity, still it is valuable to understand the combined effects of these metabolites on broad spectrum of fouling organisms. With this intension in mind, we had undertaken the biological studies of these isolated molecules of sponge.

The biofilm forming bacteria was developed on the copper plates exposed in the Arabian Sea. 10 strains were purified and identified as Gram positive bacteria (*Planococcus donghaensis*)

& Gram negative bacteria (*Alcanivorax sp.*, *Aeromonas hydrophila* subsp *hydrophila* ATCC 7966, *Aeromonas hydrophila* subsp. *salmonicida* A449, *Erythrobacter litoralis*, *Pseudomonas mendocina*, *Alcanivorax borkumensis*, *Allivibrio salmonicida*, *Pseudoalteromonas sp.*, *Vibrio furnisii*).¹⁹ Compounds 1-3 were tested to determine the anti-microbial capacity against these 10 strains of fouling bacteria [Table 1].^{19c} As evident from the table 1, secomanoalide (1) expressed broad spectrum of activity against all the tested strains except *Pseudoalteromonas sp.* Among these, it showed considerable good activity against *Alcanivorax sp.* and *Erythrobacter litoralis*. Dehydromanoalide (2) (a dehydrated metabolite of secomanoalide) exhibited weak activity against seven strains except *Allivibrio salmonicida*, *Pseudoalteromonas sp.* and *Vibrio furnisii*. It is interesting to note that, the antimicrobial efficacy of dehydrated product *i.e.* dehydromanoalide (2) is reduced compared to secomanoalide (1). This result reveals the importance of allylic hydroxyl functionality at lactone ring for enhancing the activity of this class of terpenes. Another terpene lactone *i.e.* cavernosine (3), showed weak activity against 4 strains (*Alcanivorax sp.*, *Aeromonas hydrophila* subsp. *salmonicida* A449, *Erythrobacter litoralis* and *Pseudomonas mendocina*). So far, cavernosine 3 was reported to exhibit only ichthyotoxic activity. This is the first report of cavernosine to show antimicrobial activity against fouling bacteria. Thus, we concluded that although manoalide derivatives possess a common structural framework, their biological activity varied. Since these major metabolites are produced by sponge at same time, it is fascinating to study the combined effects of metabolites on fouling bacteria. Hence, the mixture of compounds 1, 2 and 3 (1:1:1) were used for further studies. Interestingly, we observed that the compound 1-3 synergistically improved the activity against 8 strains out of 10 tested organisms (Figure 2). In fact, the commixture of metabolites (1+2+3) retained strong activity and found to be comparatively better than each individual metabolite of this sponge. Probably, this enhanced activity results from synergistic interactions between multiple compounds. In terms of ecological standpoint of view, the synergism in biological activity of these major metabolites produced by *Fasciospongia cavernosa* could develop chemical defense by delivering the combined effect on fouling bacteria in natural environment.

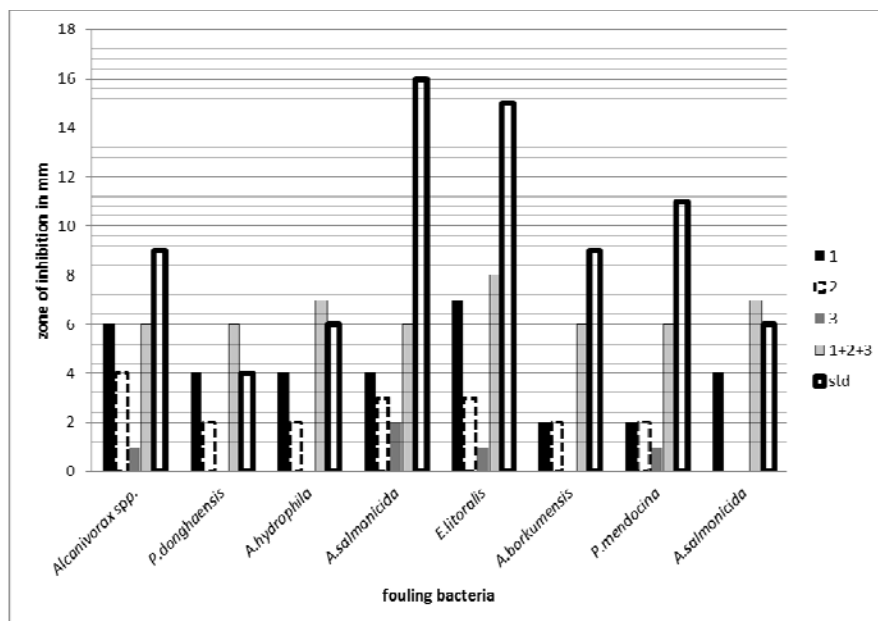


Figure 2. Synergistic effects of metabolites isolated from marine sponge *Fasciospongia cavernosa* (Std is Streptomycin)

Table 1. Antimicrobial activities of compound 1-5 against fouling bacteria at concentration of 50 µg/disc

Strain Fouling bacteria	Metabolites of <i>F. cavernosa</i>				<i>A. donnani</i>		Standard
	1	2	3	1+2+3	4	5	Streptomycin
<i>Alcanivorax sp.</i>	++	+	+	++	-	++	+++
<i>Planococcus donghaensis</i> ^a	+	+	-	++	-	-	+++
<i>Aeromonas hydrophila subsp hydrophila</i> ATCC 7966	+	+	-	++	-	-	+++
<i>Aeromonas hydrophila subsp. salmonicida</i> A449	+	+	+	++	-	+++	++
<i>Erythrobacter litoralis</i>	++	+	+	++	-	+++	+++
<i>Alcanivorax borkumensis</i>	+	+	-	++	-	++	++
<i>Pseudomonas mendocina</i>	+	+	+	++	-	-	+++
<i>Allivibrio salmonicida</i>	+	-	-	++	-	-	+++
<i>Pseudoalteromonas sp.</i>	-	-	-	-	-	-	+
<i>Vibrio furnisii</i>	+	-	-	-	-	-	+++

Zone of inhibition in mm; 0 mm: No activity (-); 1-5 mm: weak activity (+), 6-10 mm: good activity (++); 11-16 mm: significant activity (+++); data given are the mean of three replicates; ^aGram positive bacteria (Standard: Penicillin).

The chemical investigation of the marine sponge *Axinella donnani* led to the isolation of the zwitterionic compound, phenylalanine betaine (**4**) and cerebrosides (**5**). The structure of the phenylalanine betaine was interpreted based on the ¹H and ¹³C NMR spectra recorded in methanol-d₄. The characteristic proton and carbon signals were observed at δ_H 7.2-7.3 (m), 7.9 (s) and 126.9, 128.5, 129.0, 130.0, 135.9, for the presence of monosubstituted aromatic ring. Signal at δ_H 3.36 (s) and δ_C 56.1 is attributed to trimethyl amine. The carbonyl group showed peak

at δ_C 172.3. The ESI-MS spectrum gave molecular ion peak at *m/z* 208 [M]⁺. Another feature of the ¹H NMR spectrum of phenylalanine betaine was two signals at δ 3.77 and 3.02 due to a methylene AB system (δ_C 36.8). Signals at δ_H 4.6 and δ_C 78.0 were attributed to deshielded methine. This was exactly matching with the standard compound. Fascinatingly, trimethylammonium amino acid betaines have been reported from natural sources including plants and sponges.²⁰ Modified amino acids betaines, axiphenylalaninium, axityrosinonium and pyridinium derivative,

polyaxibetaine, have been isolated from the sponge *Axinella polypoids*.²¹ In biological systems, betaines are known to serve as an osmoprotectants which can stabilize proteins and membranes when salt levels or temperatures are unfavorable. Therefore, they could play important roles in the adaption of cells of marine invertebrates to various adverse environmental conditions.²²

Cerebroside (**5**) was obtained as a white, amorphous solid. It showed characteristic signals for a sugar (an anomeric proton at δ_{H} 4.30), an amide linkage (a nitrogenated methine proton at δ_{H} 4.27, and a carbonyl carbon at δ_{C} 176.9), and a long acyl chain (terminal methyl protons at δ_{H} 0.88 and methylene protons at δ_{H} 1.3) were observed, indicating its nature as a glycosphingolipid. In the ¹³C-NMR data, the carbon resonances appeared at 102.3 (C-1''), 73.3 (C-2''), 77.5 (C-3''), 71.5 (C-4''), 76.2 (C-5''), and 61.4 (C-6''), revealing the presence of a β -glucopyranoside.²³ The coupling constant of the anomeric proton at δ_{H} 4.30 (d, $J=8.0$ Hz), and ¹³C chemical shift value (δ_{C} 102.3) further confirmed the β configuration of the glucose unit (in the case of α -glucopyranoside: $J=3.7$ Hz, δ_{C} 98.5).²⁴ It closely resembled with halicerebroside and astraicerebrosides without double bond peaks in NMR.²⁵ ESI-MS spectrum gave molecular ion peak at m/z 788. Detailed investigation on ESI-tandem mass spectra data revealed that it is number of methylene groups with d18:0 and d20:0 side chain. Metabolites isolated from sponge *Axinella donnani* was tested for antimicrobial activity. Phenylalanine betaine (**4**) did not exhibit any activity whereas, sphingolipid cerebroside (**5**) showed significant activity against three strains, *Aeromonas hydrophila* subsp. *salmonicida* A449 and *Erythrobacter litoralis* (table 1). It also expressed good activity against *Alcanivorax* sp. and *Alcanivorax borkumensis*. The combined effect of metabolite **4** and **5** (1:1) does not alter the antimicrobial potential of cerebroside (**5**). Thus, phenylalanine betaine (**4**) is inactive against fouling organisms and does not produce any effects on activity of other metabolites. This contributes, the first report of cerebroside showing antimicrobial activity against biofilm forming bacteria. Overall, our results reveal that; metabolites from two sponges *Fasciospongia cavernosa* and *Axinella donnani* shows broad spectrum of activity against fouling bacteria. This suggests that the compounds have great scope as antibiofilm agent in particular the combined effects of metabolites (synergistic effect) and should be taken for further development.

In summary, marine organisms have developed chemical defense systems that keep their body surfaces free of fouling, and their metabolites are an excellent source of antimicrobial compounds. In the present study, several marine biofilm bacteria were isolated from the copper panels exposed in the sea. We have screened 5 secondary metabolites isolated from the marine sponges *F. cavernosa* and *A. donnani* against these fouling bacteria. Of the active compounds identified in the study reported herein, 3 terpenoids and one sphingolipid isolated from sponges exhibited potent activity. Intriguingly, the presence of allylic hydroxyl in manoalide skeleton was found to influence antimicrobial efficacy. The commixture of metabolite (**1+2+3**) from sponge showing synergistic effect in biological activity is a highlight of this study. Additionally, this stands the first report of phenylalanine betaine and cerebroside from the sponge *A. donnani* wherein cerebroside showed significant antimicrobial potential against fouling bacteria. As evident from the literature that the initial step in marine fouling is the formation of biofilm. So application of these compounds synergistically can be the one of the earliest methods of solving the problem of biofilm formation as well as marine biofouling. This study opens further scope of synergistic screening of manoalide related compounds to give new anti-biofilm agent in the future.

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Supplementary Material

Supplementary data associated with this article can be found, in the online version.

Graphical Abstract

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