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Antifouling sesquiterpene from the Indian soft coral, *Sinularia kavarattiensis* Alderslade and Prita

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A sesquiterpene, (l'*E*,5'*E*)-2-(2',6'-dimethylocta-l',5',7'-trienyl)-4-furoic acid [**1**], was isolated for the first time from the soft coral *Sinularia kavarattiensis* Alderslade & Prita collected from Gulf of Mannar (Tamil Nadu, India). This compound exhibited considerable larval settlement inhibition properties against the cosmopolitan biofouler, *Balanus amphitrite*. The observed mean EC_{50} value of 11.21 µg/ml is well within the biological potency cut off set for natural product antifoulants (NPA's). The low EC_{50} value and favourable therapeutic ratio (5.16) coupled with its known synthetic routes enhances the potential of **1** as a promising NPA.

[**Keywords:** Soft coral, *Sinularia kavarattiensis,* Sesquiterpene, Furoic acid; *Balanus amphitrite*]

Introduction

Biofouling is a cause of serious concern considering its long-ranging impacts on maritime structures such as ship hulls and platforms[1]. With the recent ban on antifouling coatings containing toxic agents like TBT, focus is on greener ways to combat biofouling[2]. In this regard, the chemistry behind the sessile, unfouled surfaces in the sea is largely being investigated. Marine organisms have been found to be storehouses of a variety of secondary metabolites[3,4]. In particular, soft corals are a rich source of several biologically active compounds with diverse structures including sesquiterpenes, diterpenoids, polyhydroxylated sterols etc[5,6]. Many of these compounds are known to possess strong antibacterial, anti-inflammatory, antioxidant, antitumour and cytotoxic properties[7-9,4].

Sinularia is one among the most abundant soft coral genera on many coral reefs and are abundant in Indian waters, especially along Gulf of Mannar and Lakshadweep Islands. However, the reports on the antifouling properties of soft corals of Indian waters, in general, are scanty and have been carried out only up to crude extract/partially purified level[10]. Continuing our studies on antifouling compounds from different marine organisms, we herein report isolation and identification of (l'*E*,5'*E*)-2-(2',6'-dimethylocta-l',5',7'-trienyl)-4-furoic acid [**1**] from soft coral *Sinularia kavarattiensis*. This compound has previously been reported from *S. capillosa*, collected from Australia as potent anti-inflammatory agent.

Materials and methods

Collection, Extraction and Purification

Sinularia kavarattiensis was collected from the Gulf of Mannar (Lat 9°5′ N; Long 79°5′ E) from a depth of 7 m by skin diving. 1.1 kg of the sample (wet weight) was washed with fresh water, soaked in methanol and transported to the laboratory. Crude extract so obtained was concentrated using a Rotary Vacuum Evaporator and fractionated into low polar Petroleum ether, PE (Fr. 1), medium polar Ethyl acetate, EA (Fr.2) and high polar Aqueous (Fr. 3) fractions. Fractions were then subjected to larval settlement inhibition assay against cyprids of the barnacle *Balanus amphitrite* and antibacterial assay against seven strains of fouling bacteria. Active Fr. 2 was subsequently purified on a Sephadex LH-20 column (30 × 5 cms, $CHCl_3$: MeOH 1:1), yielding 5 subfractions (Fr 2-1 to 2-5). Each fraction was again tested for larval settlement inhibition and antibacterial activity. Of these, Fr.2-4 exhibiting high activity was further purified on a silica gel column (55 cm × 2 cm, Gradient petroleum ether-ethyl acetate), yielding six subfractions (Fr. 2-4-1 to 2-4-6). Each of the fractions was subjected to antifouling activity testing as mentioned above. Among these, 2-4-4, the more active fraction was found to be pure on TLC and its structure elucidation was carried out with

the help of MS and NMR (^{1}H, ^{13}C, COSY, HMQC and HMBC) spectral data. MS and NMR (latter in $CDCl_3$ solution) spectra were recorded on Shimadzu GC-MS Model QP 2010 and BRUKER Avance 300 MHz NMR spectrometers respectively.

Larval Settlement Inhibition Assay and Determination of EC_{50}

Maintenance of Adult Barnacles—The adult barnacles were collected from their natural habitat at Vypeen beach, Kochi. They were maintained in the laboratory in 35 ppt aerated seawater on a diet of *Chaetoceros calcitrans* and *Artemia salina* nauplii. The barnacles were subjected to regular exposure periods to stimulate the release of nauplii[11].

Rearing of nauplii: The barnacle nauplii were reared in the laboratory at 30°C through the six different instars on a regular diet of *C. calcitrans* (12:12 hour Light: Dark cycle)[12]. Cyprids were collected on the sixth day and stored at 5°C for use in the settlement inhibition assay. Effective Concentration (EC_{50}) was determined by the serial dilution method for testing the larval settlement inhibition activity of the isolated compound against *B. amphitrite* cyprids[13]. The experiments were conducted in sterile 24-well polystyrene multiwell plates (Axigen). The isolated compound was dissolved in methanol and added to autoclaved 0.45 µ filtered sea water (FSW) to obtain concentrations ranging from 100 to 6.25 µg/ml. 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. 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 data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test at 95% confidence level[14]. One-way ANOVA was performed with the SPSS software package, Version 7.5. EC_{50} and LC_{50} values of the isolated compound were calculated using Probit programme[15]. The experiment was repeated twice with different batches of larvae.

Antibacterial assay

The antibacterial assay was conducted using the Disc Diffusion Method against seven different strains of fouling bacteria (*Bacillus cereus*, *B. pumilus*, *B. megaterium*, *Pseudoalteromonas haloplanktis*, *Pseudomonas chlororaphis*, *P. putida* and *P. aeruginosa*)[13].

Results

Identification of the Isolated Antifouling Compound

The ^{13}C NMR and DEPT.135 spectra of the purified compound (yield = 0.235 g, 0.021% based on wet weight) m/z = 246 $[M]^+$, indicated the presence of 15 carbons, distributed as 5 singlets, 5 doublets (CH), 3 triplets (CH_2), and 2 quartets (CH_3). Among these, the singlet at δ 167.7 was attributed to a carboxylic acid, which is also confirmed by the strong peak at m/z 202 $[M\text{-}CO2]^+$ in the EIMS spectrum. Similarly, the doublet at δ 110.9(2H) could be easily attributed to an exomethylene group. This was supported by the 1H NMR spectrum, which indicated presence of 7 vinyl protons: 3 singlets (1H each), one multiplet (1H) and the remaining 3 protons as a mutually coupled AMX pattern at δ 4.96, 5.12 and 6.38 (1H each). Detailed 2D NMR studies (1H-1H COSY, HMQC and HMBC) and comparison of these values with that of the synthetic product[16] confirmed its structure as (l'E,5'E)-2-(2',6'-dimethylocta-l',5',7'-trienyl)-4-furoic acid [**1**] (Table 1, Fig.1.).

Larval Settlement Inhibition Activity

When the competent larvae were exposed to concentrations ranging from 6.25 to 100 µg/ml of **1**, larval settlement was inhibited in a dose-dependent manner (Fig. 2). Bioassay results of both the batches of larvae tested showed similar trends. One-way ANOVA showed that **1** significantly affected the settlement of two different batches of *B. amphitrite* cyprids [p = 0.05; $F_{5,30}$ = 184.317 (batch 1) and 233.400 (batch 2)]. The EC_{50} was 11.17 µg/ml and 11.25 µg/ml for batch 1 and 2 respectively. The LC_{50} was calculated to be 58.59 µg/ml and 57.25 µg/ml for the two consecutive batches, thus yielding a mean therapeutic ratio (LC_{50}/EC_{50}) of 5.16.

Antibacterial activity

Compound **1** moderately inhibited the growth of six out of the seven fouling bacterial strains tested at a concentration of 100 µg/disc (Table 2).

Discussion

Soft corals, in general, are rich sources for bioactive compounds. Of the total 160 marine species from which potential NPAs have been isolated, 18% is contributed by this particular group of organisms[6]. The soft coral, *Sinularia kavarattiensis,* is an

Table 1 — NMR assignments of Compound **1***

C No	^{13}C NMR chem. shift δ	^{1}H NMR chem. shift δ	HMBC correlations
C-2	155.0(s) [155.1,s]		
C-3	106.7(d) [106.6,d]	6.54(1H,s) [6.52,1H,s]	119.8(s), 146.7(d), 155.0(s), 167.7(s)
C-4	119.8(s) [119.7,s]		
C-5	146.8(d) [(146.9,d]	8.01 (1H,s) [7.99,1H,s]	106.7, 119. 8, 155.0
C-1'	113.5(d) [13.5,d]	6.10(1H,s) [6.07,1H,brs]	18.7(q), 40.3(t), 106.7(d), 141.0(s)
C-2'	141.0(s) [141.1,s]		
C-3'	40.3(t) [40.3,t]	2.28(2H,m) [2.29,2H,m]	18.7(q), 26.7(t), 141.0(s)
C-4'	26.7(t) [26.7,t]	2.37(2H, m) [2.29,2H,m]	40.3(t), 141.0(s), 131.7(d)
C-5'	131.7(d) [131.7,d]	5.50(1H,m) [5.47,1H,brt,7Hz]	
C-6'	134.6(s) 134.6,s]		
C-7'	141.3(d) [141.3,d]	6.38 (1H,dd,16.4 & 11.4 Hz) [6.36, 1H,dd,17,11Hz]	11.7(q), 131.7(d), 134.6(s)
C-8'	110.9(t) [110.9,t]	4.96(1H, brd, 11.4 Hz) [4.94,1H,brd,11Hz] 5.12(1H, brd, 16.4 Hz) [5.10,1H,brd, 17Hz]	141.3(d), 134.6(s)
6'-Me	11.7(q) [11.7,q]	1.78(3H, s) [1.75,3H,brs]	131.7(d), 141.3(d)
2'-Me	18.7(q) [18.7,q]	2.00(3H,s) [1.98,3H,brs]	40.3(t), 113.5(d), 141.0(s)
4'-COOH	167.7(s) [168.5,s]		

*Values in the square brackets are from Williams and Faulkner (1996)

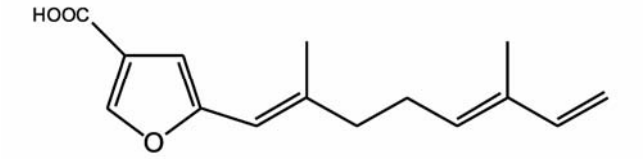

Fig. 1 — (l'E,5'E)-2-(2',6'-dimethylocta-l',5',7'-trienyl)-4-furoic acid

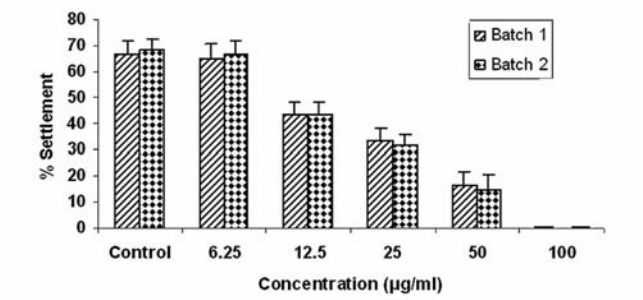

Fig. 2 — Effect of Compound **1** on the percentage of larval settlement of *Balanus amphitrite*. Data plotted are mean ± SD, n = 6.

abundant species in the Gulf of Mannar region and Lakshadweep islands. The only reported chemical examination of *S. kavarattiensis* is the isolation of methyl (1′*E*, 5′*Z*)-5-(2′,6′-dimethylocta-1′,5′,7′-trienyl)furan-3-carboxylate, methyl(5′*E*)-5-(2′,6′-dimethylocta-5′,7′-dienyl)furan-3-carboxylate, Δ9(15) africanene and spathulenol, a rare sesquiterpene by Goud *et al*. in 2002[7].

This is the first report on the occurrence of (l'*E*,5'*E*)-2-(2',6'-dimethylocta-l',5',7'-trienyl)-4-furoic acid [**1**] from *S. kavarattiensis.* Also, the antifouling property of **1** is reported here for the first time. It significantly inhibited the larval settlement of *B. amphitrite*. The observed mean EC_{50} value of 11.21 µg/ml is well within the biological potency cut off of 25 µg/ml[17-18] set for natural product antifoulants (NPA's). The calculated therapeutic ratio (5.16) confirmed the environmental acceptability of this compound[17], thereby complying well with the International Maritime Organisation (IMO) regulations. The moderate inhibition of fouling bacterial growth, as observed from the present study, further enhances the potential of **1** as a NPA (Table 2).

Table 2—Activity exhibited by Compound **1** (100 µg/disc) against fouling bacterial strains after 24 hours of incubation

Sl. No.	Bacterial strain	Zone of Inhibition (mm)*
1.	*Bacillus cereus*	4
2.	*Bacillus pumilus*	4
3.	*Bacillus megaterium*	3
4.	*Pseudoalteromonas haloplanktis*	2
5.	*Pseudomonas chlororaphis*	4
6.	*Pseudomonas putida*	4
7.	*Pseudomonas aeruginosa*	5

* [--, no activity; 1 - 2 mm, Low activity; 3 - 5 mm, Moderate activity; > 5 mm, High activity]

Compound **1** was earlier isolated from an Australian soft coral *S. capillosa*[19]. Realising its anti-inflammatory activity, Williams and Faulkner

(1996)[16] devised chemical routes for its synthesis. With successful synthetic routes already available, there is feasibility for large scale production of **1**. This is particularly important in view of its very poor yield (0.021%) observed in the present study.

Sesquiterpenes have long been isolated from soft corals of the genus *Sinularia*. Furano-sesquiterpenoid acid having anti-inflammatory activity was the first sesquiterpene isolated from *S. gonatodes*[20-21]. Mizobuchi *et al*. (1994)[22] isolated (9*E*)-4-(6,10-dimethylocta-9,11-dienyl)-furan-2-carboxylic acid from *Sinularia* sp. collected from Aulong Isand, Palau. This compound which has striking similarity to **1** was reported to have antifouling activity against mussels and barnacles.

Conclusion

The sesquiterpene (l'*E*,5′*E*)-2-(2′,6′-dimethylocta-l',5',7'-trienyl)-4-furoic acid [**1**] is isolated from *Sinularia kavarattiensis* for the first time. The low EC_{50} value and low toxicity coupled with the known synthetic route of **1** enhances its potential as a promising environmentally compatible NPA.

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References

1 Schultz M P, Effects of coating roughness and biofouling on ship resistance and powering, *Biofouling*, 23 (2007) 331-341.

2 Sjogren M, Dahlstrom M, Hedner E, Jonsson P R, Vik A, Gundersen L L & Bohlin L, Antifouling activity of the sponge metabolite agelasine D and synthesised analogs on *Balanus improvises*, *Biofouling*, 24 (2008) 251-258.

3 Paul V J, In: *Ecological Roles of Marine Natural Products*, Comstock Publishing Associates, Ithaca, New York, 1992, pp.245.

4 Blunt J W, Copp B R, Munro M H G, Northcote P T & Prinsep M R, Marine natural products, *Nat Prod Rep*, 23 (2006) 26-78.

5 Fusetani N, Biofouling and antifouling, *Nat Prod Rep*, 21 (2004) 91-104.

6 Chambers L D, Stokes K R, Walsh F C & Wood R J K, Modern approaches to marine antifouling coatings, *Surf Coat Technol*, 201 (2006) 3642-3652.

7 Goud T V, Reddy N S, Krishnaiah P & Venkateshwarlu Y, Spathulenol: A rare sesquiterpene from soft coral *Sinularia kavarattiensis*, *Biochem Systemat Ecol*, 30 (2002) 493-495.

8 Bhosale S H, Nagle V L & Jagtap T G, Antifouling potential of some marine organisms from India against species of *Bacillus* and *Pseudomoas, Mar Biotechnol*, 4 (2002) 111-118.

9 Kamel H N & Slattery M, Terpenoids from *Sinularia*: Chemistry and biomedical application, *Pharm Biol*, 43 (2005) 253-269.

10 Raveendran T V & Limna Mol V P, Natural Product Antifoulants – present scenario and future prospects, *Curr Sci*, (Accepted in June 2009).

11 Rittschof D, Branscomb E S & Costlow J D, Settlement and behavior in relation to flow and surface in larval barnacles, *Balanus amphitrite* Darwin, *J Exp Mar Biol Ecol*, 82 (1984)13-46.

12 Anil A C, Desai D & Khandeparker L, Larval development and metamorphosis in *Balanus amphitrite* Darwin (Cirripedia; Thoracica): Significance of food concentration, temperature and nucleic acids, *J Exp Mar Biol Ecol*, 263 (2001) 125-141.

13 Limna Mol V P, Raveendran T V & Parameswaran P S, Antifouling activity exhibited by secondary metabolites of the marine sponge, *Haliclona exigua* (Kirkpatrick), *Int biodeter biodegr*, 63 (2009) 67-72.

14 Conover W J & Iman L, Rank transformations as a bridge between parametric and non-parametric statistics, *Am Stat*, 35 (1981) 124-129.

15 Sakuma M, Probit analysis of preference data, *Appl Entomol Zool*, 33 (1998) 339-347.

16 Williams D H & Faulkner D J, Two practical syntheses of an anti-inflammatory sesquiterpene furoic acid from Sinularia spp. Tetrahedron, 52 (1996) 4245-4256.

17 Rittschof D, Lai C H, Kok L M & Teo S L M, Pharmaceuticals as antifoulants: Concept and principles, *Biofouling*, 19 (2003) 207-212.

18 Kwong T F N, Miao L, Li X & Qian P Y, Novel Antifouling and Antimicrobial Compound from a Marine-Derived Fungus *Ampelomyces* sp., *Mar Biotech*, 8 (2006) 634-640.

19 Bowden B F, Coll J C, Silva E D de & Costa M S L de, Studies of Australian soft corals. XXXI. Novel furanosesquiterpenes from several sinularian soft corals (Coelenterata, Octocorallia, Alcyonacea), *Aust J Chem*, 36 (1983) 371-376.

20 Coll J C, Mitchell S J & Stokie G J, Studies of Australian soft corals V. A novel furano-sesquiterpene acid from the soft coral *Sinularia gonatodes*, *Tetrahedron Lett*, 18 (1977) 1539-1542.

21 Grace K J S, Zavortink D & Jacobs R S, Inactivation of bee venom phospholipase A_2 by a sesquiterpene furanoic acid marine natural product, *Biochem Pharmacol*, 47 (1994) 1427-1434.

22 Mizobuchi S, Kon-ya K, Adachi K. & Sasa M, Antifouling substances from a Palauan octocoral *Sinularia* sp., *Fisher Sci*, 60 (1994) 345-346.