

Antifouling activity exhibited by secondary metabolites of the marine sponge, *Haliclona exigua* (Kirkpatrick)

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Scientific relevance: The isolation of environmentally-compatible Natural Product Antifoulants (NPAs) would help contain the problem of marine biofouling minimizing the hazardous consequences to the non-targeted marine biota. This will eventually wipe out the toxic and non-biodegradable antifouling agents such as TBT from the marine environment.

Abstract

Bioassay guided purification of the acetone extract of the marine sponge, *Haliclona exigua*, (Gulf of Mannar, India) yielded a fraction rich in bis-1-oxaquinolizidine alkaloids, active against seven strains of fouling bacteria as well as cyprids of the cosmopolitan barnacle, *Balanus amphitrite*. The major alkaloids in the mixture have been tentatively identified as nor-Araguspongine C (33.76%), Araguspongine C (6.49%), dihydroxy Araguspongine (36.36%) , methyl & dimethyl derivatives of the latter (12.98 & 10.38% respectively) from HRMS studies. The lower EC₅₀ (6.6 µg/ml as against the US Navy standard of 25 µg/ml for NPAs) and low toxicity (LC₅₀ 18 µg/ml as compared to 0.00001 µg/ml for TBT) values, coupled with it's favourable therapeutic ratio (2.7 as against the requirement of >1) makes these compounds ideal NPAs in environmentally compatible antifouling coatings.

Keywords: Antifouling, Sponges, *Haliclona exigua*, *Balanus amphitrite*, Araguspongines

1. Introduction

Marine biofouling is an extensive phenomenon causing large penalties to engineered structures such as ships and offshore platforms by way of increased use of manpower, fuel, material and dry-docking time (Chambers et al., 2006). Until recently, antifouling paints containing Tributyltin (TBT) and copper compounds were effectively used to combat fouling (Evans, 2001). However, these compounds, especially TBT, were reported to be highly toxic and persistent in the marine environment, and were proved to have adverse effects on non-target marine organisms (Alzieu et al., 1989; Claisse and Alzieu, 1993; Evans et al., 1995; Omae, 2003; Yebra et al., 2004). The alarming environmental pressure caused by TBT has led to a worldwide ban by the International Maritime Organisation (IMO) on the use of antifouling paints containing this biocide (Clare, 1998; Champ, 2000).

Under these circumstances, search for natural antifouling compounds as ecologically compatible substitutes for chemical biocides is progressing worldwide (Clare, 1998; Yebra et al., 2004; Sipkema et al., 2005; Raveendran et al., 2008). Natural products and their synthetic analogs exhibiting anaesthetic, repellent and settlement inhibition properties, but non-toxic to the non-target organisms, are preferred as potential antifouling agents (Omae, 2003). In this regard, sessile, soft-bodied marine organisms maintaining a clean surface were identified as possible sources of natural product antifoulants (NPAs). This was attributed mostly to the production of secondary metabolites, presumably as a means of protection from predation, colonization by epizoic organisms or to reduce competition for space (Wahl et al., 1994).

Sponges, with their rich chemical defence mechanisms, are one of the most studied organisms for the isolation of NPAs (Thakur and Anil, 2000). The constant threat from competitors, by way of overgrowth, poisoning, infection or predation has armed sponges with a storehouse of potent chemical defense agents (Bakus et al., 1986; Thakur and Muller, 2004). Sponges produce a wide array of secondary metabolites ranging from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids, aliphatic cyclic peroxides and sterols (Parameswaran et al., 1989; Sarma et al., 1993; Parameswaran et al., 1994; Faulkner, 1995; Scheuer, 1995; Parameswaran et al., 1997; Fontana et al., 2000; Tilvi et al., 2004). Possible

antifouling properties of compounds isolated from sponges was first recognized by Bakus et al. (1983). Further studies in this direction have revealed the tremendous antifouling potential of some of the bioactive metabolites inherent in sponges (Clare, 1996; Omae, 2003; Fusetani, 2004; Chambers et al. 2006). Notable among them is Poly-APS, a non-toxic NPA from the sponge *Reniera sarai* (Kaiser et al., 1998; Fusetani, 2004; Turk et al., 2007). Also, terpenoids isolated from *Acanthella cavernosa* and analogues of the sesquiterpene avaron from *Dysidea avara* are reported to be promising NPAs on account of the low EC₅₀ (< 1 µg/ml) values against barnacle cyprids (Fusetani, 2004, Tsoukatou et al. 2007).

Sponges belonging to the genus *Haliclona* have been a good source for several alkaloids exhibiting diverse pharmacological properties (Bokesch et al., 2002). These include the alkylpyridines from different *Haliclona* spp, (Orabi et al, 2002, Liu et al, 2004, Volk et al., 2004, Blunt et al., 2006), ceramides from *H. koremella* (Hattori et al., 1998), a hexapeptide from *Haliclona* sp. (Sera et al., 2003) etc. The latter two compounds were good inhibitors of the growth and settlement of the green algae *Ulva conglobata* and blue mussel *Mytilus edulis* respectively. In addition, several novel alkaloids have been reported from the species *Haliclona exigua* (Venkateswarlu et al. 1994; Rao et al., 1998; Orabi et al. 2002; Liu et al. 2004) and *H. cribricutis* (Parameswaran et al, 1998).

In the present study an attempt has been made to investigate the antifouling potential of *H. exigua* against micro- and macrofouling and also partially characterize the active metabolites.

2. Materials and methods

2.1. Collection, Extraction and Purification

H. exigua was collected from the Gulf of Mannar (Lat 9°5' N; Long 79°5' E) during March 2005 from a depth of 2 m by skin diving. The sponge was washed with fresh water, soaked in acetone and transported to the laboratory. The crude extract so obtained was concentrated using a Rotary Vacuum Evaporator and fractionated into Petroleum ether (Fr. 1), Ethyl acetate, (Fr.2) and Aqueous (Fr. 3) fractions in the usual way. Growth inhibition bioassay against fouling bacteria and larval settlement

inhibition assay against cyprids of the barnacle *Balanus amphitrite* indicated Fr. 2 to be more promising. The active compounds were finally purified from the above fraction using Gel Permeation Chromatography (Sephadex LH-20, 30 X 5 cm, CHCl₃: MeOH 1:1) followed by HPLC (ODS, MeOH: H₂O gradient elution, 2 ml/min). The structure elucidation of the active fraction was carried out using various spectroscopic techniques such as IR, UV-Vis, NMR and HRMS. NMR spectra were recorded in CDCl₃ solution in a BRUKER Avance 300 MHz NMR spectrometer, while HRMS analysis was performed in an Applied Biosystem instrument (QTOF). All solvents were procured from s.d. Fine Chem Limited (AR Grade) and used as such.

2.2. Antibacterial assay

Seven different strains of fouling bacteria (*Bacillus cereus*, *B. pumilus*, *B. megaterium*, *Pseudoalteromonas haloplanktis*, *Pseudomonas chlororaphis*, *P. putida* and *P. aeruginosa*) were isolated from fouling panels immersed at a depth of 1m at Fore Shore Road boat jetty, Kochi (Lat 9°9'N; Long 76°2' E). They were identified using Bergey's manual of systematic bacteriology (Bergey and Holt, 1994). The antibacterial activity was tested by the disc diffusion method (Becerro et al., 1994; Bhosale et al., 2002; Murugan and Santhana Ramasamy, 2003). 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 500, 250, 100 and 50 µg of Fr.2-3 dissolved in methanol, evaporated to dryness and placed onto the bacterial lawn. Discs containing Penicillin-G (10 units/disc each) and Streptomycin (10 µg/disc each) were used as positive controls and discs loaded with 25 µl of absolute methanol served as negative controls. The plates were incubated for 24 hours at 28°C and the resulting clear zones of bacterial growth inhibition were measured. The experiment was conducted in duplicate for each of the strains.

2.3. Larval Settlement Inhibition Assay and Determination of EC₅₀

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. Water and feed was changed every day, and the barnacles showing symptoms of stress (weak operculum) were immediately removed from the mass.

The barnacles were subjected to regular exposure periods to stimulate the release of nauplii (Rittschof et al., 1984; Hellio et al., 2004).

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* on a 12:12 hour Light: Dark cycle (Anil et al., 2001). Cyprids emerged on the sixth day and were collected and stored at 4 °C for use in the settlement inhibition assay.

Effective Concentration (EC₅₀) was determined by the serial dilution method for testing the larval settlement inhibition activity of Fr.2-3 against *B. amphitrite* cyprids (Rittschof et al., 1992; Marechal et al., 2004). The experiments were conducted in sterile 24-well polystyrene multiwell plates (Axigen). Fr.2-3 was dissolved in methanol and added to autoclaved 0.45 µ filtered sea water (FSW) to obtain concentrations ranging from 50 to 2.5 µg/ml. 10 competent cyprids were added to each of the 5 replicates of 2ml 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 (Conover and Iman, 1981). One-way ANOVA was performed with the SPSS software package, Version 7.5. EC₅₀ and LC₅₀ values were calculated using Probit analysis (Finney, 1971; Sakuma, 1998). The experiment was repeated twice with different batches of larvae.

3. Results

3.1. Identification of Secondary Metabolites

¹H and ¹³C NMR spectral data of the active Fr.2-3 indicated it to be related to bis-1-oxaquinolizidines, already reported from this species, while HRMS studies indicated it to be a mixture of at least 5 compounds. Thus, its ¹H NMR spectrum had strong signals in the region δ 1.0-2.3, indicative of several methylene groups (Fig.1). The signals in the region δ 3.0-4.0 were indicative of the presence of CHOH and CH-N moieties. Its ¹³C NMR and DEPT.135 spectra (Fig.2.) had multiple signals in the

region δ 20-45 (t, CH₂s), δ 50-55(t, CH₂s linked to N atoms), δ 70-95(d, CH linked to O and N atoms). In addition, the carbon signals at δ 96.4(d), 95.2(d), 95.0(d), 90.4(d) and 88.2(d) were typical of C-10 of bis-1-oxaquinolizidine ring systems (Venkateswarlu et al., 1994; Orabi et al., 2002; Liu et al., 2004). The relatively down field shift of C-10 signals is due to its linkage to both nitrogen and oxygen atoms and is characteristic of oxaquinolizidines. The strong signals at δ 53.4 (t) and 53.2 (t) could be due to the C-4 and C-6. The peaks at δ 77.3(d), 76.8(d), 76.6(d) and 75.9(d) are expected to be from the C-2 (of the 1-oxaquinolizidine rings) and other oxygenated carbons. HRMS data revealed the presence of 5 major compounds (Table 1, Fig 3), i.e. nor-Araguspongine C (m/z: 465.3686, C₂₇H₄₉N₂O₄); Araguspongine C (m/z: 479.3912, C₂₈H₅₁N₂O₄; Fig.4); dihydroxy Araguspongine (533.3535, C₂₈H₅₀N₂O₆Na) and mono & dimethyl derivatives of the latter (547.3781, C₂₉H₅₂N₂O₆Na & 561.3960, C₃₀H₅₄N₂O₆Na). Absence of vinyl proton signals in the ¹H NMR spectrum and the observed double bond equivalence of five, as revealed by the elemental compositions of all above compounds also confirmed the araguspongine-type molecular structures.

3.2. Antibacterial activity

Fr.2-3 considerably inhibited the growth of all the seven fouling bacterial strains at a concentration of 100 μ g/disc (Table 2). The negative solvent control (MeOH) had no effect on bacterial growth, while all bacteria were susceptible to the positive controls (Streptomycin and Penicillin-G).

3.3. Larval settlement Inhibition Activity

Fr.2-3 noticeably inhibited the settlement of *B. amphitrite* cyprids. When the competent larvae were exposed to concentrations ranging from 2.5 to 50 μ g/ml, larval settlement was inhibited in a dose-dependent manner (Fig.5). Bioassay results of both the batches of larvae tested showed similar trends. One-way ANOVA showed that Fr.2-3 significantly affected the settlement of two different batches of *B. amphitrite* cyprids [$p = 0.05$; $F_{4,20} = 181.545$ (batch 1) and 197.500 (batch 2)]. The EC₅₀ was 6.55 μ g/ml and 6.57 μ g/ml for batch 1 and 2 respectively. The LC₅₀ was calculated to be 18.06 μ g/ml and 18.07 μ g/ml for the two batches respectively, thus yielding a therapeutic ratio (LC₅₀ / EC₅₀) of 2.7.

4. Discussion

The sponge *H. exigua* is a rich source for several bis-1-oxaquinolizidine alkaloids, exhibiting diverse biological properties such as cytotoxic, antifungal, antimalarial, antituberculosis and anti-rat brain nitric oxide synthase activities (Venkateswarlu et al., 1994; Rao et al., 1998; Orabi et al., 2002; Liu et al., 2004). Araguspongine C, belonging to the bis-1-oxaquinolizidine type of alkaloids was first isolated from *H. exigua* (Orabi et al., 2002). A number of pharmacological activities have been reported for this compound. However, no reports are available on antifouling activity by this class of compounds. In the present study, a fraction rich in bis-1-oxaquinolizidine alkaloids was found to be active against fouling bacterial strains as well as barnacle cyprids, for the first time. As several araguspongine analogs have already been successfully synthesized, (Hoye and North, 1990; Ahn and Lee, 1992), their use in antifouling paint industry may not be a distant dream.

Marine biofouling is a complex assemblage of organisms on artificial structures comprising micro- as well as macro-foulers and often it has been reported that microfouling facilitates macrofouling process (Callow and Callow, 2002). Therefore, NPAs that inhibit macrofoulers alone may not be an effective anti-foulant. Rather, it would be desirable to have NPAs, capable of inhibiting both micro as well as macrofoulers. In view of this, the sponge fraction was assayed against the fouling bacterial strains as well as larvae of the cosmopolitan macrofouler, *B. amphitrite*. It is obvious from the present studies that the bis-1-oxaquinolizidines in Fr.2-3 significantly inhibited the growth of all the seven strains of fouling bacteria and also inhibited the settlement of barnacle cyprids at low concentrations. In the case of cyprids, the EC₅₀ value is much lower than the standard set by the US Navy program (25 µg/ml) as an efficiency bar for natural antifoulants (Kwong et al., 2006).

Environmental compatibility is one of the major criterions to be satisfied by the NPAs as per the IMO regulation. The bis-1-oxaquinolizidines isolated from *H. exigua* is found to be satisfying this requirement. The LC₅₀ value of this fraction against cyprids of *B. amphitrite* was 18 µg/ml and a therapeutic value of 2.7 was obtained. This is in accordance with the recommended target ratio i.e, > 1, for the potential use of NPA's in environmentally compatible antifouling coatings (Rittschof et al., 2003). It may be remembered here that the LC₅₀ of TBT is less than 0.00001 µg/ml.

To summarize, Fr.2-3 rich in bis-1-oxaquinolizidine alkaloids isolated from the marine sponge, *H.exigua*, showed high inhibitory activity against the growth of fouling bacteria as well as settlement inhibition activity against barnacle cyprids. The low toxicity of Fr.2-3 to *B. amphitrite* cyprids ($LC_{50}=18 \mu\text{g/ml}$) as well as its relatively high therapeutic ratio highlights the potential use of bis-1-oxaquinolizidine alkaloids as environmentally compatible antifoulants and or antimicrobial agents. This study is, in fact, the first report on the antifouling property of bis-1-oxaquinolizidine class of compounds. With further characterization of each of these metabolites, and taking into account the possibility of analogue synthesis, there are high prospects for bis-1-oxaquinolizidines from *H. exigua* as potential environmentally compatible antifouling agents.

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TABLE 1. Elemental composition of the 5 major compounds from Fr.2-3 based on HRMS data

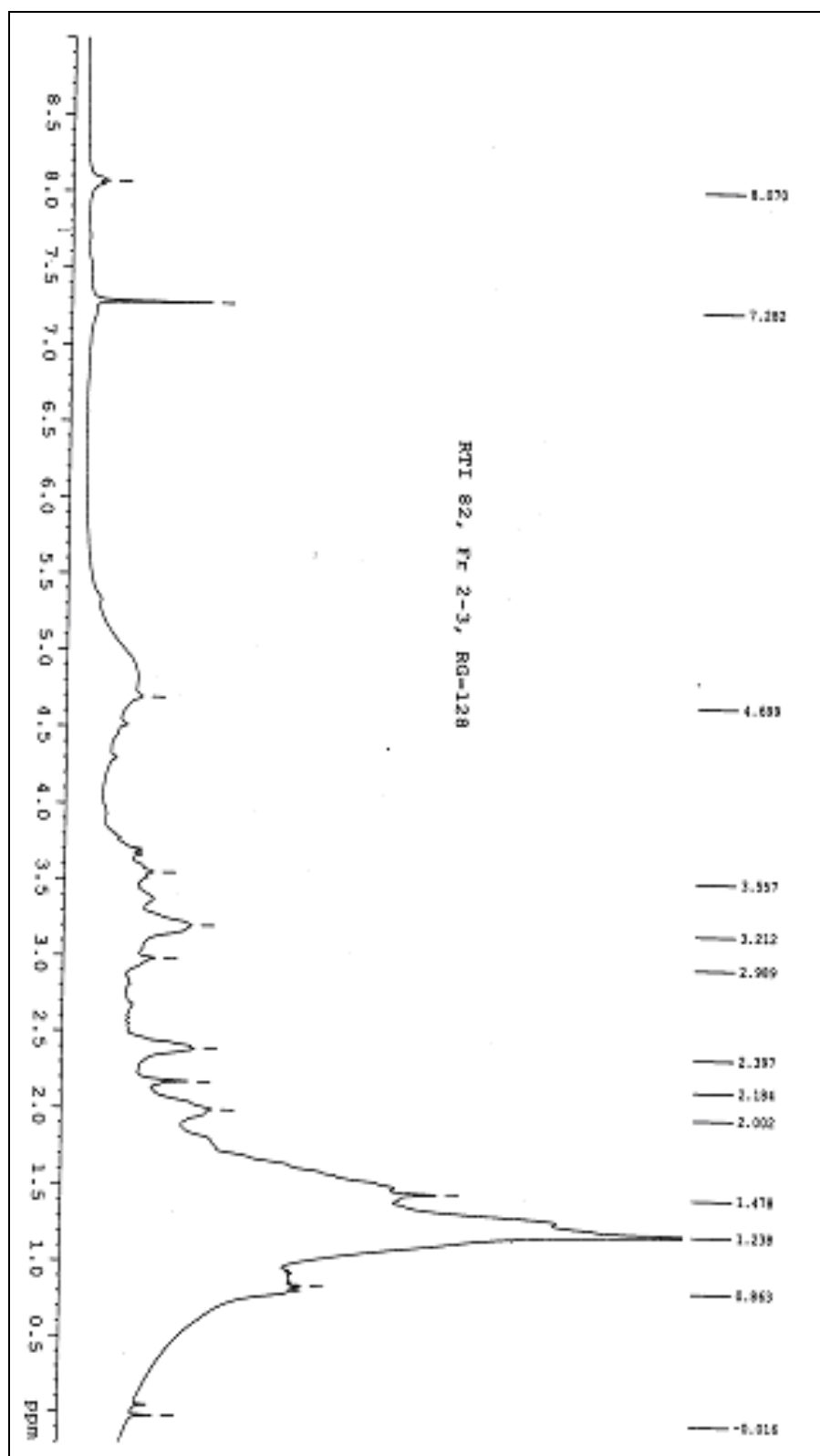
Sl. No.	m/z	Elemental Composition	Calculated Mass	ppm Error	DBE
1.	561.3960 (10.38%)	C ₃₀ H ₅₄ N ₂ O ₆ Na	561.3960	15.3026	4.5
2	547.3781 (12.98%)	C ₂₉ H ₅₂ N ₂ O ₆ Na	547.3717	11.5842	4.5
3.	533.3535 (36.36%)	C ₂₈ H ₅₀ N ₂ O ₆ Na	533.3561	-04.8916	4.5
4.	479.3912 (6.49%)	C ₂₈ H ₅₁ N ₂ O ₄	479.3843	14.3201	4.5
5.	465.3686 (33.76%)	C ₂₇ H ₄₉ N ₂ O ₄	465.3686	-00.1800	4.5

TABLE 2. Activity exhibited by Fr.2-3 at four different concentrations against fouling bacterial strains after 24 hours of incubation

Sl. No.	Conc. (µg)	Zone of Inhibition (mm) *						
		BC	BP	BM	PH	PC	PP	PA
1.	500	+++	+++	+++	+++	+++	+++	+++
2.	250	+++	+++	+++	+++	+++	+++	+++
3.	100	+++	+++	+++	+++	+++	+++	+++
4.	50	++	++	++	+	+	++	+

* [+, Low activity; ++, Moderate activity; +++, High activity]

BC, *Bacillus cereus*; BP, *B. pumilus*; BM, *B. megaterium*; PH, *Pseudoalteromonas haloplanktis*; PC, *Pseudomonas chlororaphis*; PP, *P. putida*; PA, *P. aeruginosa*.

Fig.1. ^1H NMR of Fr. 2-3

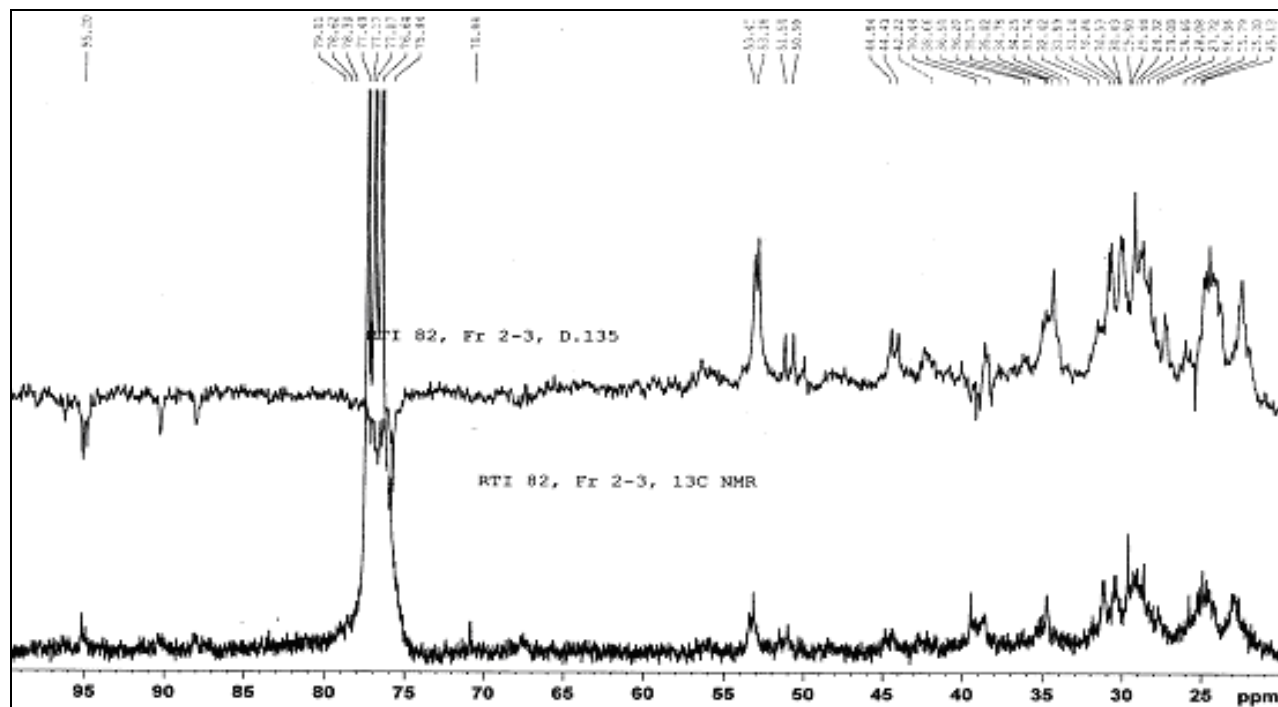


Fig.2. ^{13}C NMR and DEPT. 135 spectra of Fr. 2-3

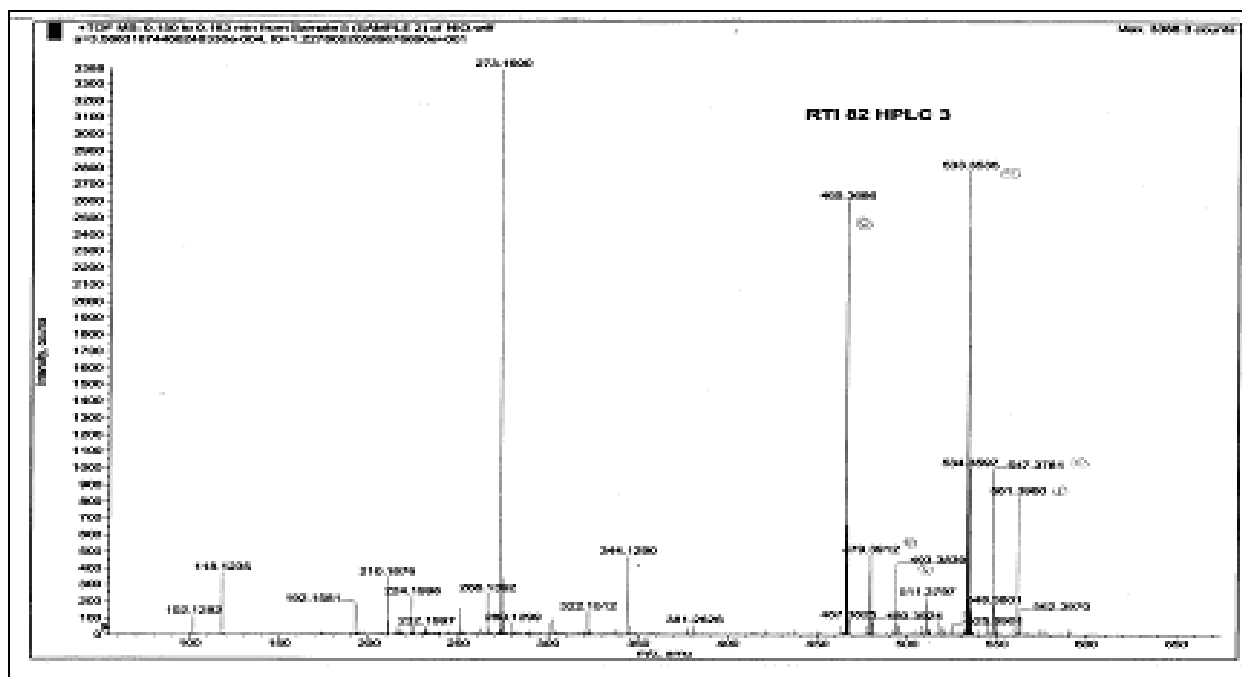


Fig.3. HRMS spectra of Fr. 2-3

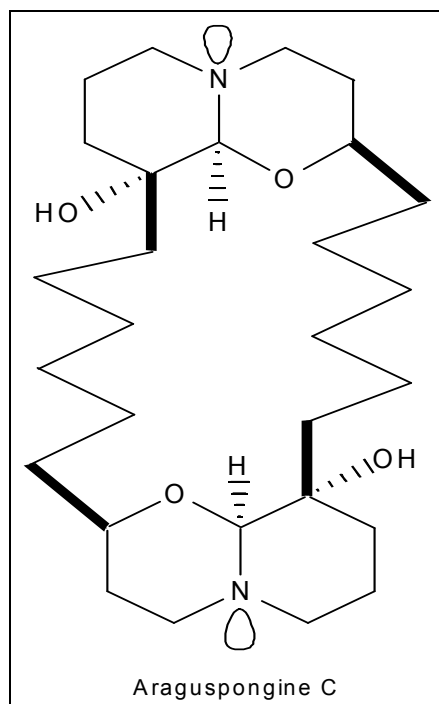


Fig.4. Araguspongine C

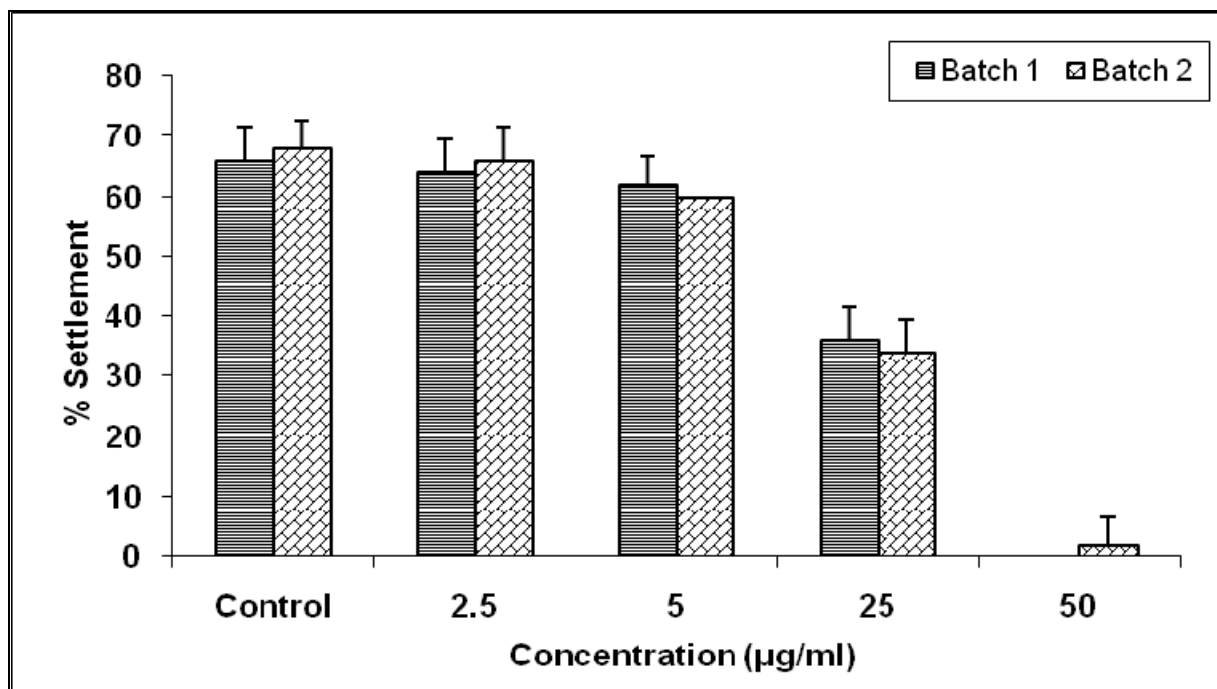


Fig.5. Effect of Bis-1-oxaquinolizidines on the percentage of larval settlement of *Balanus amphitrite*. Data plotted are means \pm SD (n=5)