A Potent Bioactive Substance Obtained from the Marine Sponge, *Cribrochalina* sp, Showing Some Biological Activities

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Abstract

Extract of the marine sponge, ***Cribrochalina*** *sp., was tested for antimicrobial and antimitotic activities. Results show that the extracts exhibited strong growth inhibitions against* ***Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Saccharomyces cerevisciae and Aspergillus niger,*** *but not against a polychaete embryo (****Hydroides ezoensis****) having caused lysis at one - cell stage few minutes after fertilization. Extracts at a diluted concentration did not induce lysis, however, it has inhibited further embryonic development.*

Isolation and purification of the active component was done using chromatographic methods. Employing nuclear magnetic resonance (NMR) spectrometry, a pure substance was chemically identified as halistanol sulfate, a steroid-derivative antimicrobial constituent previously isolated from an Okinawan sponge, ***Halichondria*** *cf.* ***moorie*** *Bergquist.*

Introduction

Until recently, marine sponges continue to be fruitful source of novel bioactive compounds with several bioactivities most of which are very useful as *materia medica.* In fact, a lot of known substances against fungi are derived or are synthesized from marine sponges. Several pharmacologically active compounds were discovered or studied from this group of organisms in the marine environment. Previous studies revealed that some species belonging to the genus *Cribrochalina* have active substances. Duryne, a cytotoxic metabolite which inhibits the growth of both mouse and human tumor cell lines *in vitro* was isolated from *Cribrochalina dura* (Wright *et al.* 1987). Another substance, petrosterol, was studied by Doss *et al.* (1990) and was shown to be synthesized by the same sponge. This compound was first isolated from species of marine sponges such as *Petrosia ficiformis* (Sica & Zollo, 1978), *Petrosia hebes* (Cho & Djerassi, 1987) and

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Halichondria sp. (Ravi *et al.*, 1978). Their works suggest that the derivatives of the active compound were of exogenous origin and modified metabolically by the sponge itself.

This present paper describes some bioactivities of extracts of the marine sponge, *Cribrochalina* sp. This work is part of the continuing efforts in searching for some enzyme inhibitors from marine natural products. moreover, the project aimed at finding some useful compounds that may be harnessed for therapeutic purposes.

Materials and Methods

Collection of Sponge Sample

The sample *Crichalina* sp. was collected off the coast of Oshima Island, Miyazaki, Japan, located at 31"20.59^0 N latitude and 131"27.65^{0}E longitude during the Field Expedition last April 14, 1991 on board the Hiroshima University Research Vessel, *Toyoshio-maru*. The sample was taken at depths ranging from 15 to 20 meters.

Extract Preparation

One gram of the sample was homogenized in mortar and pestle after which, 3 mL of 95% methanol was added. The mixture was allowed to stand for 24 hours then filtered through Whatman filter paper no. 1. The filtrate was ready for assay.

Antimicrobial Bioassay

To determine the spectrum of antimicrobial properties of the extract, representative groups of test organisms were used namely: *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Saccharomyces cerevisciae* and *Aspergillus niger.*

Nutrient agar (NA) plates were used for bacteria while yeast peptone dextrose (YPD) plates for fungi. All necessary materials were autoclaved at 121^{0}C for 15 minutes. To ensure uniformity of inoculum in each assay plate, top agar technique was employed. This was prepared by dissolving 0.5% Difco agar in 0.6% saline solution. For every 100 ml of this medium, 2ml of bacterial of fungal suspensions were added and throughly mixed to facilitate even distribution. The seeded top agar was pipetted into each of the respective agar plate at 2.5 mL per plate. The inocula were spread over by slight rotation of the plate.

Filter paper disc diffusion technique was used for the evaluation of antimicrobial activities (Raymundo *et al.*, 1981). For every filter paper disc (with 8 mm in diameter), 30 ul of extract was impregnated. All discs were dried before they were applied to the assay plates.

Polychaete Embryo Assay

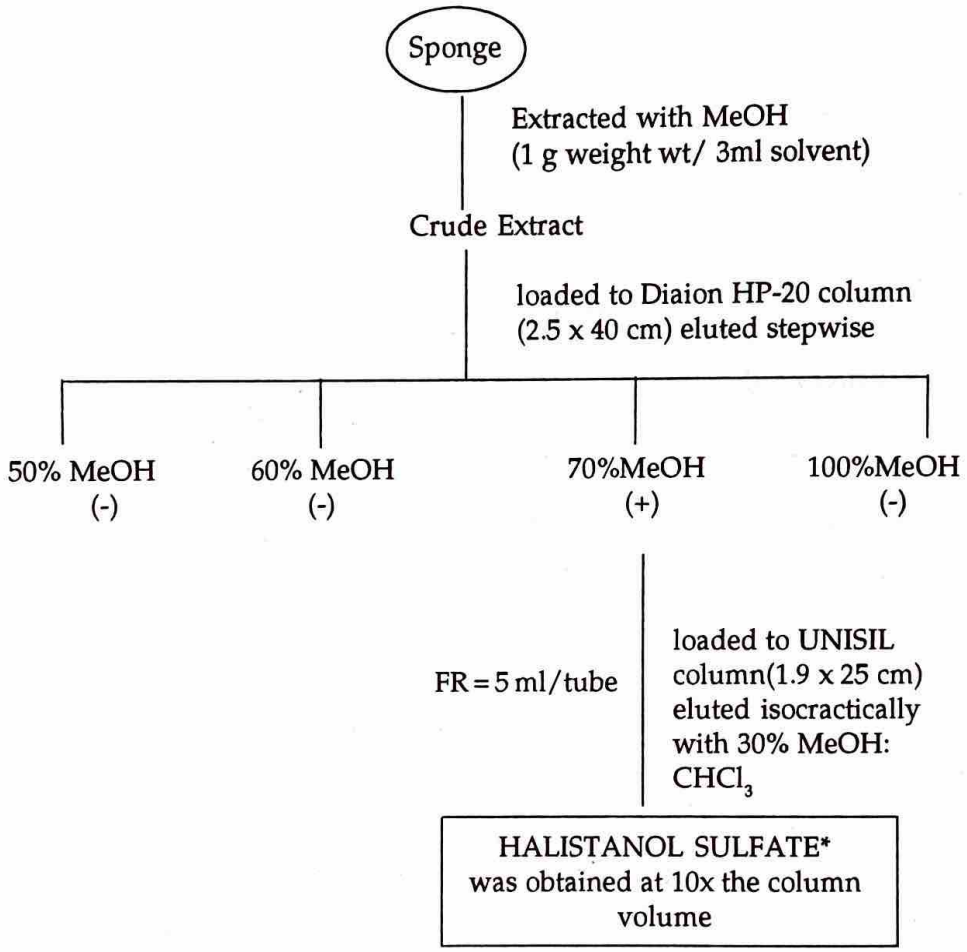
The method described by Wisely (1958) was employed in obtaining the embryos of polychaete, *Hydroides ezoensis.* The eggs and sperms were selected thoroughly according to the normal morphology and viability. Fertilization was initiated by adding few drops of sperm suspensions to the selected batch of eggs.

The assay was executed using tissue culture plates (48-wells,Sumilon MS-80480). Each well was filled with 250 ul of FSW. Fifty microliter of previously prepared sponge extract was evaporated to dryness in a Rotavapor and after being dried, 15 ul of 100% methanol was added to reconstitute the solution. This was then dissolved in 500 ul of 100% methanol was added to reconstitute the solution. This was then dissolved in 500 ul of FSW and poured into the well, followed by serial dilutions (1:1 ratio) into the blanks to obtain logarythmic concentrations. Approximately, 100-200 newly fertilized embryos were placed into the tissue culture wells. The control set up is consist of 15 ul methanol serially diluted in the same manner as the sponge extract. Observations on the effects to embryonic development were done through an inverted microscope attached to a TV monitor for wide and proper viewing. Embryonic stage observation is limited up to one-day trocophore formation only.

Isolation, Purification and Identification of the Active Constituent (assay guided)

The crude methanolic extract of *Cribrochalina* sp. was loaded to a Diaion HP-20 column (2.5 x 40 cm) eluted stepwise by an aqueous methanol solution of 50%, 60%, 70% and finally washed with 100% methanol. The volume applied for each eluent is five times the column volume. Active fraction was recovered in 70% methanol in chloroform. After this, it was loaded to Unisil column (1.9 x 25 cm) and eluted isocratically with 30% methanol in chloroform at a flow rate of 5 ml/tube. Finally, the active compound was recovered at 10 times the column volume (see Figure 1). High resolution mass spectrometry and nuclear magnetic resonance (NMR) spectrometry was employed to identify the molecule.

Figure 1. Procedure for the Isolation and Purification of Active Substance from *Cribrochalina* sp. (Antimicrobial Bioassay Guided)



*Analyzed by Nuclear Magnetic Resonance Spectrometry High Resolution Fast Atom Bombardment Mass Spectrometry

Note: (+), denotes positive for antimicrobial activity; (-), denotes negative for activity

Results and Discussion

Antimicrobial activity of the sponge extract

Methanolic extract of *Cribrochalina* sp. clearly showed broad-spectrum antimicrobial substance because all test organisms used were inhibited except that of a Gram-negative bacteria, *Escherichia coli.* (Table 1)

Table 1. Antimicrobial activity of extracts of *Cribrochalina* sp.

Test Organisms	Zone of inhibition (mm)	Remarks
Escherichia coli	-	-
Pseudomanas aeruginosa	13.3	++
Staphylococcus aureus	12.3	++
Bacillus subtilis	18.0	+++
Saccharomyces cerevisciae	14.3	++
Aspergilus niger	11.6	++

Zone of inhibitions are as follows: (-), no inhibition; (+), 8-11 mm; (++), 12-15 mm; (+++), 16-20 mm, in diameter.

Diameter inhibition was greater in *Bacillus subtilis* than all other test organisms. This result is consistent with the previous work done in the Philippine by Oclarit (1987). The antimicrobial activity of the extracts from *Cribrochalina* sp. could be attributed to its cytolytic activity as shown in the polychaete embryo assay. However, *Escherichia coli* does not seem to be affected by the lytic action of the extracts.

Effort was done to isolate microorganisms associated with the sponge in order to find out the possibility that the active component might have been produced by the symbiotic organisms as suggested by Bergquist (1978). At least three bacterial isolates were obtained from the body of the sponge and four viable isolates in the surrounding water. Each of this isolate was cultured in broth and the filtrates were tested for antibiosis. Additionally, a methanolic extract was also obtained from fresh culture and tested in the same manner as that of the broth filtrates and all results were negative. Based on this finding, it could be inferred that the active antimicrobial compound is synthesized metabolically by the sponge and not from microorganisms associated with the sponge.

Gunasekera and Faircloth (1990), Carballeria and Reyes (1990), Doss *et al.*, (1990) and Wright *et al.* (1987) have done studies on *Cribrochalina* sp., however, not one among them reported for any antifungal activity of this sponge.

Effects on Polychaete Embryos

Prior to assay, a study on the various stages of development was done and noted in Table 2.

Table 2. Stages of development in the polychaete, *Hyrroides ezoensis*

Stages of development	Time
Germinal vesicle breakdown	15-20 min.
First polar body	-
Second polar body	-
First cleavage	45-60 min.
Second cleavage	1 hr., 30 min.
Third cleavage	1 hr., 50 min
Rotating stage	3 hrs., 30 min.
Swimming balstula	4 - 5 hrs.
Gastrula	5 - 6 hrs.
Trocophore	1 - 3 days
Metamorphosis	-

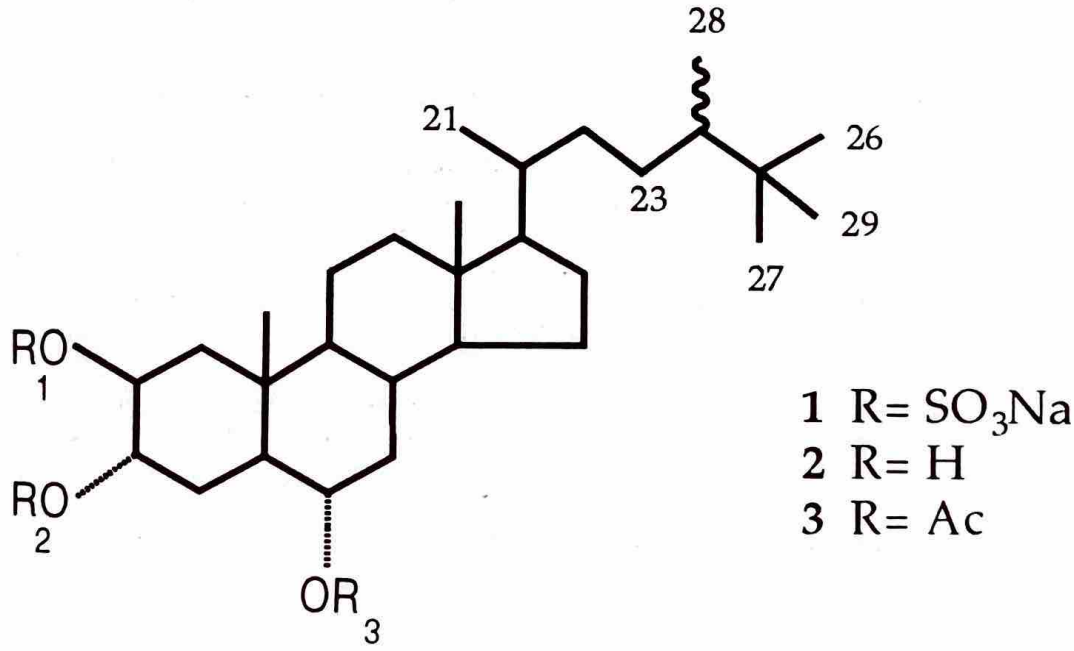
Note: (-) not determined

For the purpose of assay, the data were gathered according to strategic stages of development namely: cleavage, rotating/swimming blastula, and gastrula/trocophore stages. Assay plates were prepared by serial dilutions on 1:1 ratio starting from the crude extract with a concentration of 33 mg/ml. Eight different concentrations were made. The least concentration at which lysis occured was at 4 mg/ml. On the other hand, at concentration of 2 mg/ml, the embryos died. At concentration of 1 mg/ml, 50% of embryos developed normally to trocophore larvae. The cytotoxicity of the substance is due to its lytic action on the cell membrane of the fertilized eggs, however, at a diluted concentration the action of the substance ranged from arrest of cleavage to complete formation of trocophore. It should be noted that crude sample was used in the assay since the amount of purified sample is insufficient conduct the antimitotic activity.

Identification of the Active Compound

After several flash open column chromatographies, the active principle was isolated and purified according to the procedures described above. The purified compound appeared as colorless needles and has a melting point of 161°C. It is higly soluble in methanol and water. Mass spectral analysis suggested a chemical formula of $C_{29}H_{52}O_{12}S_3Na_3$. Nuclear magnetic resonance spectrometry revealed that the compound is identical to halistanol sulfate (Figure 2), an active antimicrobial constituent previously isolated from a marine sponge, *Halichondria* cf. *moorie* Bergquist (Fusetani *et al.*, 1981).

Figure 2. Structure of Halistanol Sulfate



Conclusion

The above preliminary bioactivities revealed that the extracts from marine sponge *Cribrochalina* sp. definitely contained cytolytic substance identified as halistanol sulfate, a steroid derivative compound. Lower concentrations of the extract may not be toxic, however, further embryonic development is either retarded or prevented. Therefore, this compound could be useful in preventing the growth of tumor or cancer cells.

Acknowledgment

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