Production of an Antibacterial Agent, *0*-Aminophenol, by a Bacterium Isolated from a Marine Sponge, *Adocia* sp.

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Abstract

A number of associated bacteria and cyanobacteria in sponges were found to be sources of antibiotics and other bioactive compounds in the marine environment. A purple-colored bacterium was isolated from the body of a marine sponge ***Adocia*** *sp. The culture of this bacterial isolate produced a substance which inhibited the growth of* ***Staphylococcus aureus*** *and* ***Bacillus subtilis****. The substance was purified and consequently identified by various spectrometric methods as o-aminophenol. Moreover, the extract of the host sponge did not contain any antimicrobial activity against those two Gram-positive bacteria.*

Introduction

Marine sponges are filter feeders and strainers of microorganisms, cyanobacteria, and microplankton. These microorganisms form as part of their diet (Reiswig, 1971; 1975). Such kind of adaptation may, in their evolution, develop resistance to the action of organisms that are found in their canal systems and porous bodies. This adaptive mechanism may bring about the production of substances that will enable them to fight against invading microorganisms. Therefore, bacteria associated with sponges are considered to be a source of antimicrobial substances. However, there are very few reports on the isolation of antimicrobial substances from the culture of bacteria thriving in marine sponges (Fenical, 1993).

Here, I described that isolation of *o*-aminophenol as an antimicrobial substance from the culture broth of a bacterium which was isolated from a marine sponge, *Adocia* sp.

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Materials and Methods

Sponge Sample

A sponge, *Adocia* sp. was collected at depths of 15-20 m off Nichinan-Ooshima Island, Miyazaki, Japan, in April 1991, on board the R/V *Toyoshiomaru* of the Hiroshima University. Sponge sample was kept alive in an aquarium supplied with running seawater until arrival at the laboratory.

Isolation of Bacteria

Live sponge specimens were washed thoroughly with sterile artificial seawater (ASW) to remove contaminants. After this, they were cut into cubes of 0.5 cm and homogenized with sterile ASW. The homogenate was serially diluted with the same solution and inoculated immediately into marine agar (Difco 2216) plates. The plates were incubated for 4-6 days at 20° C, and distinct colonies were picked up separately for single colony isolation. This process was repeated 2-3 times to ensure homegeneity of each colony.

Bacterial Characterization and Identification

Taxonomic identification was carried out on a strain that showed an antimicrobial activity against any of the test microorganisms used. This strain was then subjected to an established taxonomic tests such as Gram-stain characteristics, motility, flagellar arrangement and location, growth in the presence or absence of oxygen, and oxidative cleavage process and fermentation of glucose (Hendrie & Shewan, 1979).

Assay for Antimicrobial Activity

The antimicrobial activity of the substance present in the methanolic extract of *Adocia* sp. and the marine culture broth of the bacterial strain were determined using six test microorganisms, namely: *Escherichia coli* (NIHJ), *Pseudomonas aeruginosa* (ATCC 6633), *Saccharomyces cerevisciae* (A364A), and *Aspergillus Niger* (ATCC 9642). Bouillon's agar (consists of meat extract, 5.0 g; peptone, 150 g NaCl, 5.0 g; K_2HPO_4, 5.0 g; agar, 15.0 g; distilled water, 1 liter, pH adjusted to 7.0) plates were used for bacteria while Yeast-Peptone-Glucose agar (consists of yeast extract, 4.0 g; peptone, 5.0 g; glucose, 20.0g; KH_2PO_4, 1.0 g; $MgSO_4$, 0.5 g; and distilled water, 1 liter) plates for fungi. All

necessary materials were autoclaved at 121 °C for 15 minutes. To ensure uniformity of inoculum in each assay plate, top agar technique described by Raymundo *et al.*(1981), was employed.

Filter paper disc-diffusion technique was used for the evaluation of antimicrobial activities (Collins, 1967). For every filter paper disc (8mm in diameter), 30 of the extract was impregnated. All discs were air dried in sterile chamber before they were applied to respective assay plates.

Extraction and Purification of the Active Compound

Freshly prepared culture of the bacterial strain was inoculated into 10L of marine broth. This was then incubated in large Erlenmeyer-flasks fastened into a rotating flat form for 3 days at 20°C. The cell-free culture supernatant was obtained by centrifugation of the culture broth (8,000 xg, for 10 min). The resulting supernatants were extracted 2-3 times with an equal volume of ethyl acetate. The ethyl acetate layer was concentrated under reduced pressure and dissolved in chloroform. The solution was loaded unto an Unisil (Clarkson Chem. Co., 0 1.9 x 15 cm) column and eluted stepwise with 0-100% acetone in chloroform. The active fraction was subjected to a high performance liquid chromatographed (HPLC) using a Wakosil 5C18 AR column (07.5 X 250 mm) which was eluted with 70% aqueous methanol. Finally, the purified compound was obtained after it was re-chromatrographed by HPLC using a Wakosil 5Sil column (07:5 X 250 mm) with 10% methanol in chloroform as eluent (Fig. 1).

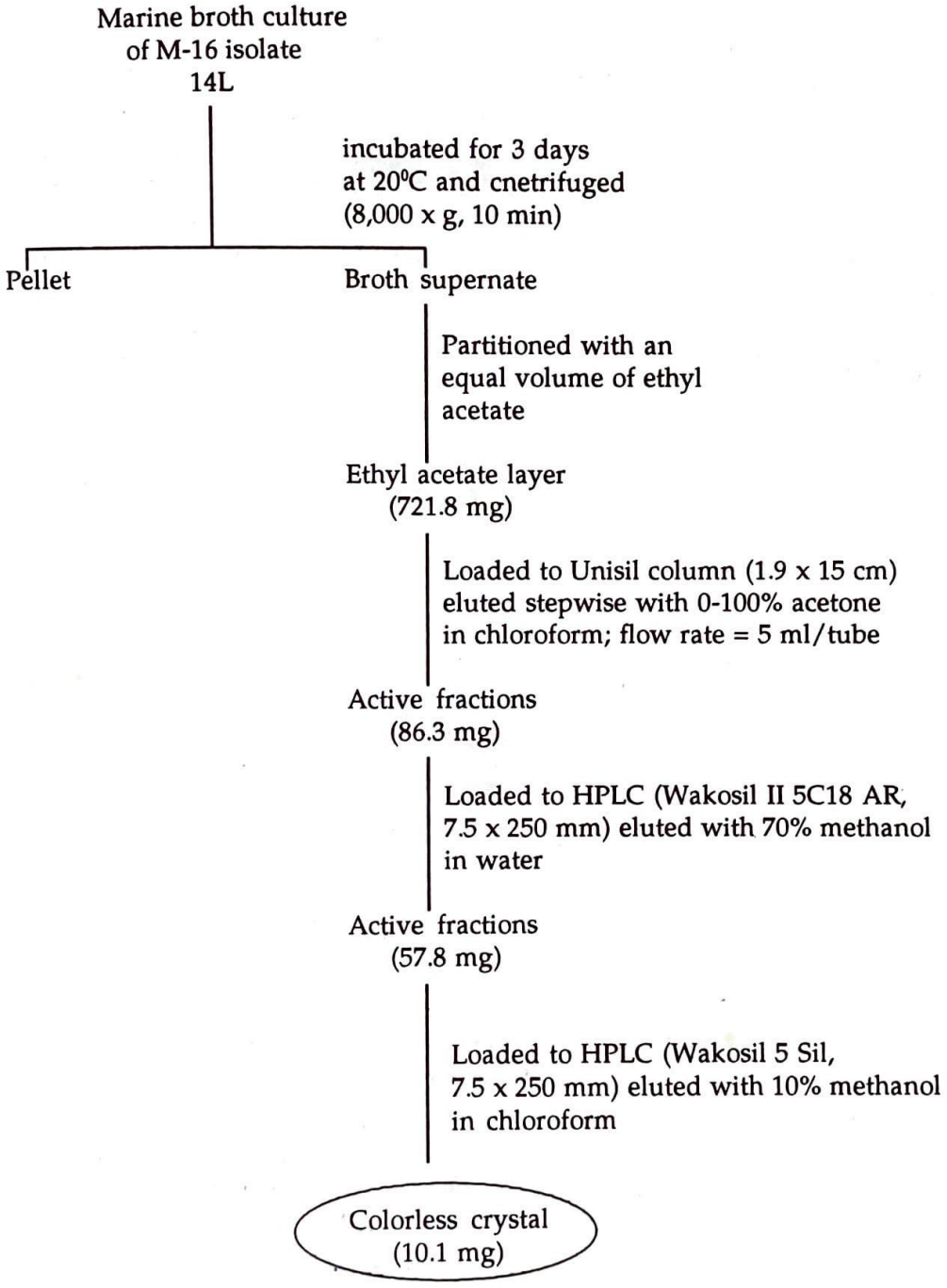
Analytical Methods

Secondary ion mass spectra (SIMS) were obtained by detecting positive ions with a Hitachi M-80B double focussing spectrometer equipped with an M-8086 Xenon beam generating system. Analytical conditions were as follows: matrix, glycerol; accelerating voltage, 3 keV. Assignment of mass number was carried out by comparing the spectra with the mass spectrum of CsI.

^{1}H and ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained at 500 and 125 Mhz, respectively, on a JEOL GSX-500 spectrometer operating in the Fourier transform mode. Tetramethysilane (TMS) was used as the internal standard. Chemical shifts of all other signals were expressed in p.p.m. downfield of TMS.

Thin layer chromatography (TLC) was carried out on GF_{254} silica gel plates (Merk; thickness: 0.25 mm).

Figure 1. Purification scheme of the active compound obtained from the bacterial isolate M16-2 which is associated with the marine sponge, *Adocia* sp.



Results and Discussion

Antimicrobial Activity of the Sponge and the Bacterial Isolate

The host sponge, *Adocia* sp., has specific inhibition against Pseudomonas *aeruginosa* but not against *Esherichia coli, Staphylococcus aureus, Bacillus subtilis, Saccharomyces cerevisciae and Aspergillus niger* (Table 1). On the other hand, the associated bacterial strain (M16-2) had demonstrated strong inhibition against *Staphylococcus aureaus and Bacillus subtilis* with minimum inhibitory concentrations (MIC) of 150 ug/mL and 350 ug/mL, respectively. Apparently, the MIC value of chloramphenicol against *Staphylococcus aureus* is 6 ug/mL while it is 8 ug/mL against *Bacillus subtilis.*

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.

Characteristics of the Bacterial Isolate (M16-2)

The strain M16-2 is a purple-pigmented bacterium, Gram-negative, strictly aerobic, and motile. Biochemical characterizations of the strain revealed the following characteristics: Oxidase and catalase tests were negative and acid production from glucose was also negative. Nitrate reduction is positive, gelatin and esculin were hydrolyzed which then implied that the bacterium is capable of producing gelatinase and B-glucosidase enzymes, respectively. The NaCI requirement for growth is 2-3%. There are at least three known genera, namely: *Chromobacterium,*

Janthinobacterium and *Alteromonas* which produced purple pigment. Hamilton and Austin (1967) cited a number of purple or violet - pigmented Gram - negative rods of marine origin which requires at least 1% Nacl but their relation to *Janthinobacterium* and *Chromobacterium* are still uncertain. In many cases *Janthinobacterium* is misidentified as *Chromobacterium* because such genus also possessed a purple-pigment, violacein. However, de Ley *et al.*, (1991). have established several criteria that placed a strong demarcation line between these two genera, namely: (a) production of acid from *n*-acetyl glucose amine is not observed among *Janthinobacterium* but it is observed among *Chromobacterium;* (b) nitrate reduction is positive for *Janthinobacterium,* but it is negative for Chromobacterium; (c) esculin hydrolysis for *Janthinobacterium* is positive, while it is negative for *Chromabacterium;* and (4) fermentation of glucose (O/F test) in *Janthinobacterium* is negative, while it is positive for *Chromobacterium.* The G + C contents of the isolated bacterium (M16-2) was shown to be higher than that of *Alteromonas* (data not shown), therefore, based on the characteristics described above and in Table 2, it is suggested that the strain M16-2 belongs to the genus *Janthinobacterium.* However, the occurrence of *Janthinobacterium* which required NaCI for growth has never been reported yet, therefore a further study is highly recommended to definitive taxonomic identification.

Isolation, Purification, and Identification of the Active Compound

The active substance was purified according to the scheme shown in Figure 1.

The final purification was carried out in HPLC column, and the active substance was obtained as a single peak. The purified substance appeared as colorless crystals. When subjected to TLC performed at room temperature with 10% methanol in chloroform as the developing solvent, it was found that a single spot with Rf value of 0.50 as detected under an UV (254 nm) lamp.

The SIMS showed the molecular ion at m/z 110. The ^{1}H and ^{13}C NMR spectra (Table 3) and the molecular structure (Figure 2) were identical with those of authentic o-aminophenol (Wako Pure Chemicals). The admixture of the purified material and the authentic o-aminophenol was ran by HPLC using a Wakosil 5Sil column which was eluted with 10% methanol in chloroform. It was found that they were inseparable from each other. These results clearly demonstrated that the active substance is *0*-aminophenol. While previous studies reported the presence of violacein from purple bacteria as antibiotic substance (Gauthier, 1976), the present study showed that *0*-aminophenol as antibacterial substance is produced by strain M16-2. The antimicrobial activity in the culture broth of the *Janthinobacterium* sp.

Table 2. Characteristics of the strain (M16-2)

Criteria	Characteristics
Colonial morphology	round, smooth, purple
Gram stain	negative
Cell morphology	slightly curved rods with rounded ends
Motility	+
NaCl requirement	2-3 %
Hugh-Leifson (H.L.) test	- (strict aerobe)
Nitrate reduction	+
Indole production	-
Gelatin hydrolysis	+
Esculin hydrolysis	+
Arginine dehydrase	-
Urease	-
B-galactosidase	-
Utilization of :	
arabinose	-
mannose	-
manitol	-
n-acetylglucosamine	-
maltose	-
potassium gluconate	-
caprinate	-
malate	-
sodium citrate	-
phenyl acetate	-

Table 3. ^{1}H and ^{13}CNMR Spectral Data of *0*-aminopheno[a]

Carbon No.	*delta* C	*delta*H (multiplicity)
1	145.3	
2	115.1	6.74 (dd,*J* = 1.8 and 7.3 Hz)
3	119.8	6.65 (dt, *J*=1.8 and 7.3 Hz)
4	120.5	6.70 (dt, *J* = 1.8 and 7.3 Hz)
5	116.8	6.75 (dd, *j* = 1.8 and 7.3 Hz)
6	134.6	

[a] ^{1}H and ^{13}C NMR spectra were measured in $CDCI_3$ + CD_3OD (1:1, v/v) at 500 + 125 Mhz respectively.

was different from that of the host sponge. Hence, it is possible that the bacterium has produced different substances under different growth conditions or that strain M16-2 is not involved in the production of antimicrobial substance present in the extracts of sponge, *Adocia* sp. Although, Berquist (1978) suggested that the antimicrobial property of marine sponge is due to symbiotic microorganisms that harbor them, the present study presents very little evidence on whether these thriving microorganisms are the sources of active substances or the sponge itself synthesizes these compounds.

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