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Haliangicin, a Novel Antifungal Metabolite Produced by a Marine Myxobacterium

1. Fermentation and Biological Characteristics

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Haliangicin, a novel β-methoxyacrylate antibiotic with a conjugated tetraene moiety, was isolated from the culture broth of a marine myxobacterium. A bacterium tentatively named as *Haliangium luteum* required 2~3% NaCl for the growth and production of haliangicin. Haliangicin inhibits the growth of a wide spectrum of fungi but was inactive against bacteria. In mitochondrial respiratory chains, haliangicin interfered the electron flow within the cytochrome *b*−*c1* segment.

Myxobacteria have been regarded as a promissing source of secondary metabolites and more than 80 novel basic structures and more than 400 variants of these compounds have been isolated from this group[1~3]. Recently, we succeeded, for the first time, in isolating myxobacterial strains from marine environments and proved them to be adapted to marine environments[4].

In search for the bioactive agents from marine myxobacteria, we discovered the novel antifungal polyunsaturated antibiotic with a β-methoxyacrylate moiety and named it haliangicin[5] (Fig. 1). This paper describes the fermentation and biological properties of haliangicin. The isolation and structural elucidation of haliangin will be reported in the accompany paper[6].

Materials and Methods

Microorganisms

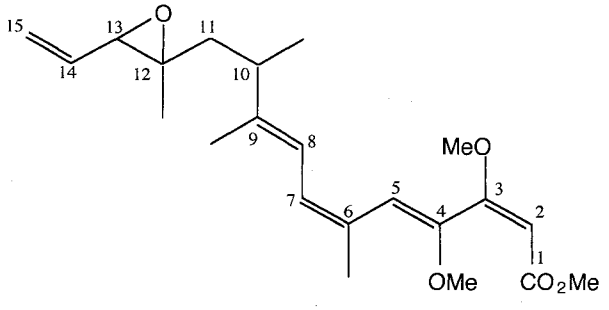
Myxobacterial strain AJ-13395 was isolated from a seaweed sample collected at a sandy beach in the Miura Peninsula, Kanagawa, Japan. From phylogenetic and physiological studies, this strain was assigned as a new genus in the myxobacterial clade[4], and was tentatively named as *Haliangium luteum*. The detailed description of the strain will be reported elsewhere.

Fermentation

Myxobacterial strain was cultivated at 28°C on the Vy2-ASW medium (seed medium)[4]. The Vy2-ASW medium contained (per liter of artificial seawater) Baker's yeast cake, 5 g; cyanocobalamin, 0.5 mg; agar, 15 g. After the diameter of the radiating colony reached around 5 cm, bacterial agar pieces were plucked with sterilized plastic straw (5 mm diameter) from the periphery of the colony.

Ten agar pieces were homogenized and inoculated into an Erlenmeyer flask containing 100 ml of a production

Fig. 1. Structure of haliangicin.



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medium which contained (per liter of artificial seawater) Bacto Casitone (Difco) 3 g, Yeast extract (Difco) 1 g, Hepes 2 g, NaCl 20 g, ferrous citrate 0.1 g, $MgSO_4 \cdot 7H_2O$ 8 g, $CaCl_2 \cdot 2H_2O$ 1 g, KCl 0.5 g, $NaHCO_3$ 0.16 g, H_3BO_3 0.02 g, KBr 0.08 g, $SrCl_2$ 0.03 g, glycerophosphate-2Na 0.01 g, trace element solution[4] 1 ml, (pH 7.0 by KOH). Adsorber resin SP207 (1%, Mitsubishi chemical Co.) was added to production medium on day 4 to enhance the productivity. Fermentation was conducted on a rotary shaker (180 rpm) at 28°C for 17 days.

Detection Methods

The productivity of haliangicin was measured by HPLC of an acetone extract of bacterial cells and adsorber resin. Separation was performed using a Capcel pak ODS column (250×4.6 mm) followed by elution with 70% acetonitrile solution. Detection was by UV absorption at 254 nm. Retention time of haliangicin was 16.5 minute.

The extraction and purification of haliangicin was also followed by monitoring the antifungal activity against *Aspergillus niger* (AJ 117374) using the paper disc diffusion method.

In Vitro Antimicrobial Assay

Minimal inhibitory concentrations (MICs) were determined by serial dilution method using Potato Dextrose Broth (Difco) and Mueller Hinton broth (Difco) for antifungal and antibacterial tests, respectively. About 10^4 freshly grown cells (or spores) of test strains were inoculated and grown at 25°C for fungi and 37°C for bacteria.

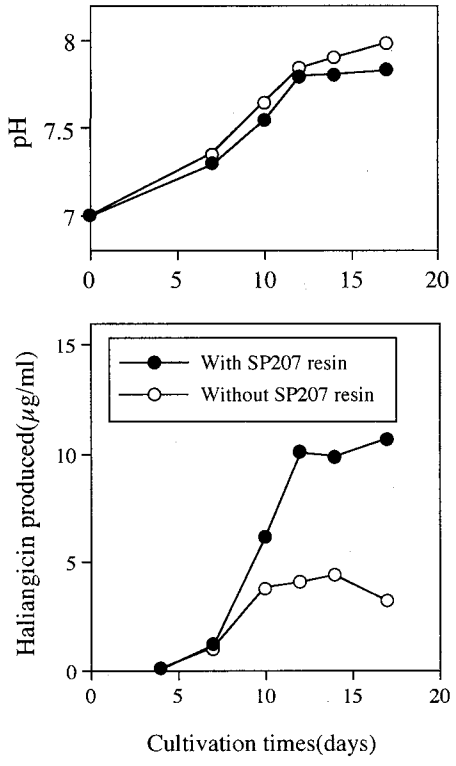
Inhibition Assay of the Submitochindrial Respiratory Chain

The inhibition rate of NADH oxidation of beef heart submitochondrial particles and the difference spectra of beef mitochondria (reduced state minus oxidized state) were determined principally according to the method of THIERBACH *et al.*[7]. In NADH oxidation assay, the submitochondrial membrane fractions were suspended with 10 mM Tris HCl buffer (pH 7.4) at a final concentration of 60 μg/ml. NADH was added to a final concentration of 0.16 mM.

General Experimental Procedures

UV-VIS absorption spectra were measured by Beckman DU 640 spectrophotometer. High performance liquid chromatography was performed on HPLC Waters996 with a photodiode array detector (Waters 997).

Fig. 2. Time course of haliangicin production.



Results

Fermentation

The typical time course of the haliangicin production is shown in Fig.2. Since the strain grew in clumps, pH was used as a growth parameter instead of the measurements of optical densities. Production started at about day 7 and reached a maximum at day 12. The productivity of haliangicin was greatly enhanced by the addition of 2% absorber resin SP207. Since the bacterium was originally isolated from marine environment, the effect of salt concentration on antibiotic production was investigated. As shown in Fig. 3, the bacterium required NaCl for the haliangicin production with optimal around 2~3%, which is roughly equivalent to the level in sea water.

Biological Properties

The antimicrobial activities of haliangicin is shown in Table 1. It had potent activities against filamentous fungi comparable to amphotericin B and nystatin. Interestingly, haliangicin also showed antimicrobial effect against

oomycetes fungi which are insensitive to other polyene-type compounds (amphotericin B and nystatin). Haliangicin did not show any inhibitory activity against bacteria.

Since several β-methoxyacrylate type compounds are known to inhibit respiration of fungi and other eukaryotes[8~12], we investigated the effect of haliangicin on the electron transport chains of mitochondria. Fig. 4 shows the inhibition of NADH oxidation by beef heart submitochondrial membranes with haliangicin along with myxothiazol A, cystothiazole A (as positive references) and amphotericin B (as negative control). Inhibition value of haliangicin was markedly higher than those of myxothiazol A and cystothiazole A. IC_{50} values of haliangicin, myxothiazol A and cystothiazole A were 2.5, 38 and 41 nM, respectively.

To elucidate the site of inhibition within the electron transport chain, the difference spectra of mitochondrial membranes (NADH-reduced minus air oxidized) were measured (Fig. 5). In the presence of haliangicin, only one peak (at 564 nm corresponding to α-band of cytochrome *b*) was observed indicating that cytochrome *b* of complex III was reduced whereas cytochrome *aa3* (α-band at 608 nm) and cytochrome *c+c1* (α-band at 553 nm) remained in the oxidized state. These results suggest that haliangicin blocked the electron flow within cytochrome *b−c1* segment of the respiratory chain.

Haliangicin was found to be toxic to the animal cell line; the cytotoxicity (IC_{50}) for mouse P388 cell was 0.21 μM.

Fig. 3. The effect of NaCl concentration on haliangicin production.

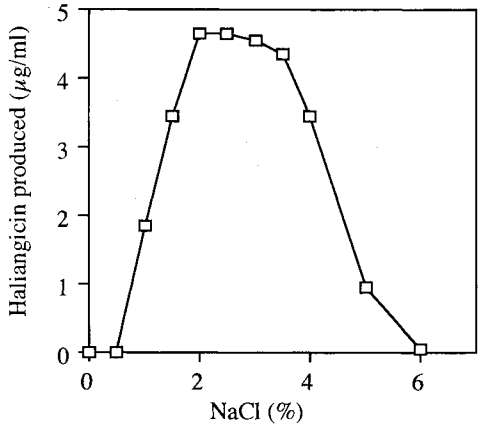


Fig. 4. The effects of haliangicin and other antifungal compounds on NADH oxidation in submitochondrial membranes.

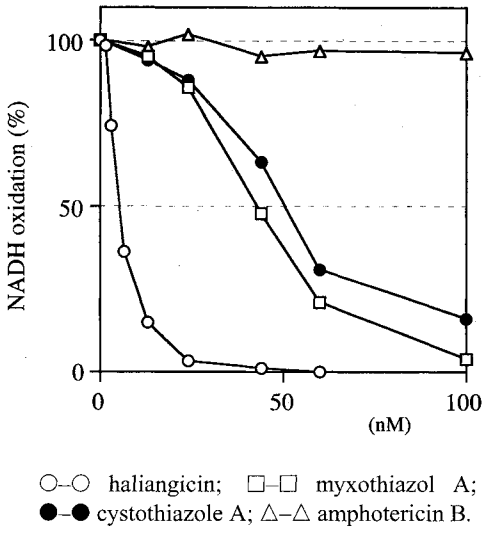
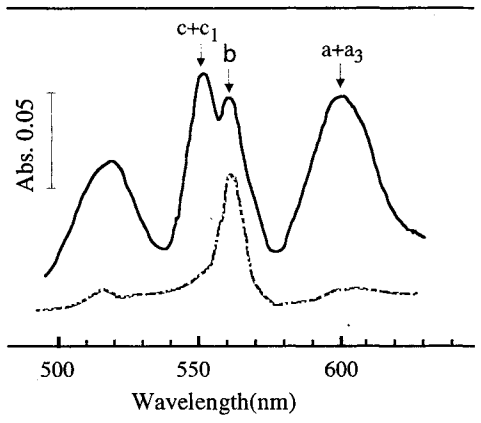


Table 1. Antimicrobial spectra of haliangicin and other polyene antibiotics.

Microorganisms tested	MIC(μg/ml)		
	Haliangicin	Amphotericin B	Nystatin
Aspergillus niger AJ 117374	12.5	3.1	3.1
Aspergillus fumigatus AJ-117190	6.3	6.3	3.1
Botrytis cinerea AJ 117140	3.1	1.6	1.6
Fusarium sp. AJ 117167	6.3	3.1	6.3
Mucor hiemalis AJ117396	12.5	12.5	12.5
Pythium ultimum IFO 32210	0.4	>100	100
Saprolegnia parasitica IFO 8978	0.1	50	100
Escherichia coli NIHJ	>100	>100	>100
Staphylococcus aureus AJ 12510	>100	>100	>100
Bacillus subtilis ATCC6051	>100	>100	>100

Fig. 5. The effect of haliangicin on the reduction of cytochromes by NADH.



Difference spectra (reduced minus oxidised) of beef mitochondrial membranes reduced with NADH.
—— without inhibitor; ------ in the presence of 2 μg/ml of haliangicin.

Discussions

Myxobacteria have been regarded as terrestrial bacteria and so far as we know, all the myxobacterial antibiotics are produced by isolates of terrestrial origin. In order to enhance the discovery of new metabolites, we explored the marine myxobacteria as a novel screening source[4]. Haliangicin is the first antibiotic isolated from a myxobacterial strain of marine origin.

Myxobacteria are known to be a rich source of antibiotics, especially those which act by inhibition of respiration. A series of respiratory inhibitors (nearly 20 compounds) have been found from myxobacteria[11]. Haliangicin is also a member of this class of compounds with β-methoxyacrylate moiety, which appear to confer its antifungal activity of haliangicin. This notion was supported by the fact that haliangicin specifically inhibits the electron transport within the complex III of respiratory chain, like other β-methoxyacrylate inhibitors such as myxothiazols[7], melithiazols[11] and cystothiazoles (our unpublished data). It is of special interest that haliangicin has the most potent *in vitro* activities among those substances with the IC_{50} of 2.5 nM ($\fallingdotseq$0.94 ng/ml), although the reason for that remains to be elucidated.

Acknowledgement

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