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Byssochlamysol, a New Antitumor Steroid against IGF-1-dependent Cells from *Byssochlamys nivea*

I. Taxonomy, Fermentation, Isolation and Biological Activity

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A new antitumor steroid, byssochlamysol, was isolated from the mycelium of *Byssochlamys nivea* M#5187. Byssochlamysol inhibited IGF-1-dependent growth of MCF-7 human breast cancer cells with an IC_{50} of 20 ng/ml, whereas serum-dependent cell growth was not inhibited by less than 10 μg/ml of byssochlamysol. This substance induced apoptosis in IGF-1-dependent Colo320DM human colon cancer cells.

Insulin-like growth factor 1 (IGF-1) is a potent mitogen and survival factor for many cell types including breast cancer cells[1]. IGF receptors are overexpressed in most breast cancer cell lines and high circulating IGF-1 concentrations are associated with an increased risk of breast cancer or colon cancer[2,3]. There is substantial experimental evidence to suggest that the IGF-1 receptor (IGF-1R) plays an important role in cellular transformation, mitogenesis and the inhibition of apoptosis[4]. Treatment of murine breast cancer cells with antisense RNA to IGF-1R inhibits cell growth *in vitro* and reduces tumorigenesis *in vivo*[5]. Simian virus 40 large T antigen is unable to transform mouse embryonic fibroblasts lacking IGF-1R[6]. Thus, IGF signaling is expected to be a potential target for anticancer agents against IGF-dependent tumor cells.

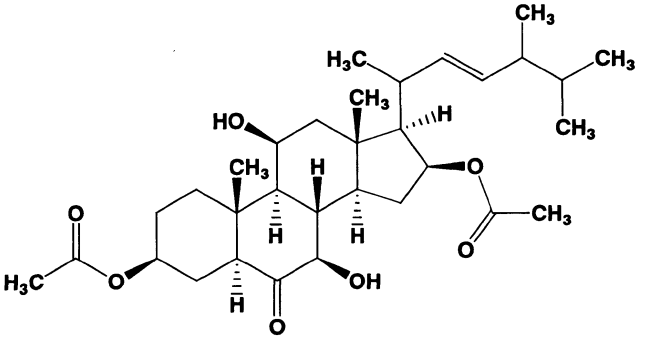
IGF-1 stimulates the growth of MCF-7 human breast cancer cells and protects the cells from apoptosis in a serum-free medium[7]. In the course of our screening for inhibitors of IGF-1-dependent cell growth or survival using MCF-7 cells, a fungal strain identified as *Byssochlamys nivea* was found to produce a new active substance, byssochlamysol (Fig. 1). This paper describes the fermentation, isolation and biological activity of byssochlamysol as well as the taxonomy of the producing organism. The physico-chemical properties and structure elucidation of byssochlamysol are described in the accompanying paper[8].

Materials and Methods

Cells and Cell Culture

Colo320DM, Saos-2, KATO-III, MKN-45 and 3Y1 cell

Fig. 1. Structure of byssochlamysol.



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lines were obtained from the Japanese Cancer Research Resources Bank (JCRB). MCF-7, MDA-MB-231 and HBC-4 cell lines were provided by Dr. TAKASHI TSURUO (Institute of Molecular and Cellular Biosciences, The University of Tokyo). HBC-4 cells and MDA-MB-231 cells were maintained in RPMI-1640 medium and the other cell lines were cultured in DULBECCO's modified EAGLE's medium (DMEM). These culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Serum-free media were supplemented with 0.1% bovine serum albumin.

Cell Growth Assay

Adherent cells were plated in each well of 96-well plates at the density of 2×10^4 to 2×10^5 cells/ml. After incubation with various concentrations of a sample at 37°C for 72 hours, the cells were treated with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37°C for 1 or 2 hours. Relative cell number was measured with formazan formation at 570 nm using a multilabel counter (Wallac 1420 ARVOsx, Perkin Elmer, Inc.).

Nonadherent KATO-III cells were plated in each well of 96-well plates at the density of 5×10^4 cells/ml. After incubation with various concentrations of a sample at 37°C for 72 hours, the cells were treated with 0.5 mM of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8, Dojindo Co.) at 37°C for 1 hour. Relative cell number was measured with formazan formation at 450 nm using a multilabel counter (Wallac 1420 ARVOsx, Perkin Elmer, Inc.).

Apoptosis Assay

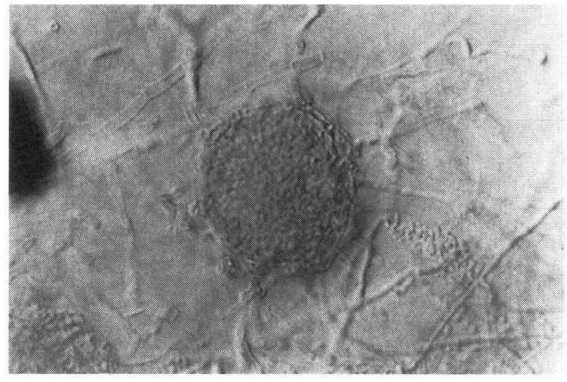
Cells were treated with a sample for 72 hours and adherent and nonadherent cells were pooled. The cells were fixed in 100 μl of 1% glutaraldehyde-phosphate-buffered saline (PBS) at room temperature for 30 minutes. The cells were collected by centrifugation and suspended in 20 μl of PBS. Chromatin structure was visualized by fluorescence microscopy (Axiovert 135, Carl Zeiss Co. Ltd.) after staining the cells (1×10^6) with 4 μl of Hoechst Dye 33258 (1 mM).

Results and Discussion

Taxonomy

The producing strain M#5187 was isolated from decayed canned peach. The colonies on potato-dextrose agar grew fast, reaching a diameter of 75 mm after incubation at 30°C for 2 weeks. The surface was cream to white in color and partly floccose. The reverse side was pale brown to pale yellow. No soluble pigment was produced. From the microscopic findings, the strain formed ascocarps that were white in color and globose to subglobose with a diameter of 200 to 350 μm (Fig. 2). The asci were colorless and globose with a diameter of 8 to 12 μm. The ascospores were single, colorless and subglobose to ellipsoid with a size of 4~5×2.5~3 μm. This strain showed anamorph stage. The conidia were subglobose with a size of 3~5×2.5~4.0 μm. The chlamydospores (6~8 μm) were particularly abundant on malt-yeast agar.

Fig. 2. Ascocarps of *Byssochlamys nivea* M#5187.

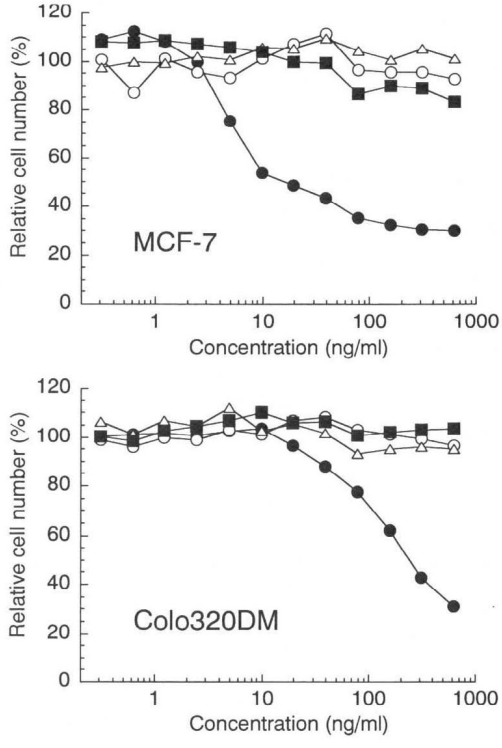


From the colonial characteristics and these morphological features (shapes and sizes) of ascospores and conidia, it was clearly identified as *Byssochlamys nivea* given by SAMSON and HOEKSTRA[7]. Strain M#5187 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the name of *Byssochlamys nivea* M#5187 under accession No. FERM BP-7722.

Fermentation

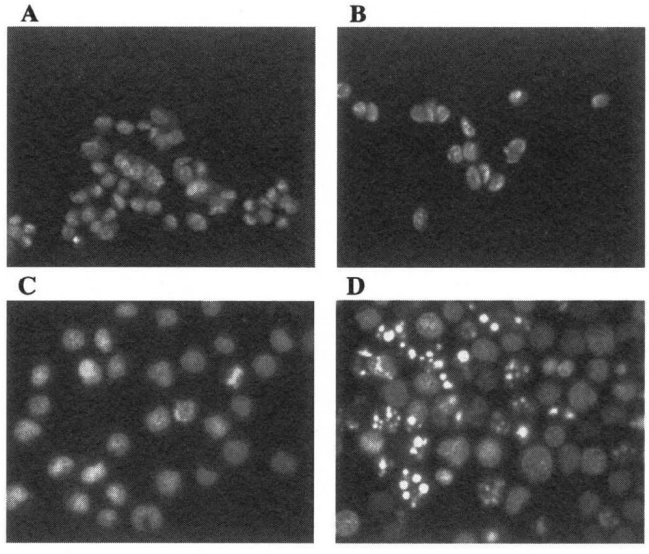
A seed medium consisted of glucose 0.4%, malt extract 1.0% and yeast extract 0.4% (pH 7.3). *Byssochlamys nivea* M#5187 was cultured in flasks containing the seed medium on a rotary shaker at 27°C for 5 days. The resultant seed culture was transferred into 500-ml Erlenmeyer flasks containing a solid medium consisting of 9 g of brown rice, 18 mg of yeast extract, 9 mg of sodium tartarate, 9 mg of

Fig. 3. Effect of byssochlamysol on the growth of MCF-7 and Colo320DM cells.



Cells were cultured for 72 hours with various concentrations of byssochlamysol in a serum-free medium with (●) or without (■) IGF-1 (MCF-7: 30 ng/ml, Colo320DM: 50 ng/ml), a 0.5% FBS-containing medium (○) or a 10% FBS-containing medium (△). The relative cell number was measured by the MTT method.

Fig. 4. Fluorescence micrographs of MCF-7 and Colo320DM cells stained with Hoechst Dye 33258.



MCF-7 cells (A, B) and Colo320DM cells (C, D) were cultured with (B, D) or without (A, C) 2 μg/ml of byssochlamysol for 72 hours in a serum-free medium containing IGF-1 (MCF-7: 30 ng/ml, Colo320DM: 50 ng/ml).

potassium dihydrogenphosphate and 27 ml of water. The fermentation was carried out at 27°C for 14 days.

Isolation

The 80% acetone extract of the culture (6 flasks) was evaporated to an aqueous concentrate and then partitioned between ethyl acetate and water. The organic layer was subjected to silica gel column chromatography with chloroform - methanol (85 : 1). The active eluate was chromatographed on a Sephadex LH-20 column with methanol. The active fraction was purified by HPLC using a Senshu Pak PEGASIL ODS column (particle size: 7 μm, 20 i.d.×250 mm) with 70% acetonitrile. Further purification was carried out on a Senshu Pak PEGASIL ODS column (particle size: 5 μm, 20 i.d.×250 mm) with 80% methanol to give a colorless powder of byssochlamysol (1.2 mg).

Biological Activity

Byssochlamysol inhibited the growth of MCF-7 cells in a serum-free medium containing 30 ng/ml of IGF-1 with an IC_{50} of 20 ng/ml (Fig. 3). Such growth inhibition was not observed in FBS-containing media. This compound exhibited cytotoxicity against Colo320DM human colon cancer cells in a serum-free medium containing 50 ng/ml of IGF-1 (IC_{50} 240 ng/ml) but not in FBS-containing media. To analyze whether FBS inactivated byssochlamysol under the assay condition, byssochlamysol was incubated with 0.5% FBS at 37°C for 72 hours. The FBS-treated sample retained the activity against MCF-7 cells (IC_{50} 38 ng/ml).

IGF-1 has been shown to suppress apoptosis in many types of cells[4]. To determine whether growth inhibition by byssochlamysol resulted from apoptosis, we observed chromatin structure in MCF-7 and Colo320DM cells treated with byssochlamysol for 72 hours in a serum-free medium containing IGF-1. Condensed chromatin and fragmented nuclei were found in byssochlamysol-treated

Table 1. IC_{50} values (ng/ml) of byssochlamysol against various cell lines.

Cell line	Cell type	Medium				IGF-1 dependency*
		IGF-1	0.5% FBS	10% FBS	Serum-free	
MCF-7	Breast cancer	20	>10,000	>10,000	>10,000	+
Colo320DM	Colon cancer	240	>10,000	>10,000	>10,000	+
MDA-MB-231	Breast cancer	4,100	>10,000	>10,000	>10,000	+
Saos-2	Osteosarcoma	>10,000	>10,000	>10,000	>10,000	+
HBC-4	Breast cancer	410	>10,000	>10,000	420	-
HeLa	Cervical cancer	240	>10,000	>10,000	510	-
KATO-III	Gastric cancer	320	370	>10,000	340	-
MKN-45	Gastric cancer	>10,000	>10,000	>10,000	>10,000	-
3Y1	Fibroblast	>10,000	>10,000	>10,000	>10,000	-

*Cell line whose growth was stimulated by IGF-1 in a serum-free medium.

Colo320DM cells but not in MCF-7 cells after staining Hoechst Dye 33258 (Fig. 4). These findings indicate that byssochlamysol induced apoptosis in Colo320DM cells and inhibited the growth of MCF-7 cells without apoptosis.

The cytotoxic and cytostatic effects of byssochlamysol were investigated using various cell lines. As shown in Table 1, byssochlamysol was active against several IGF-1-independent cell lines as well as IGF-1-dependent cell lines. In all the cell lines, the activity of byssochlamysol was reduced by addition of FBS. These results suggest that byssochlamysol induced apoptosis or growth arrest by inhibiting an IGF-1-independent pathway, which cooperates with IGF-1 signaling to stimulate cell growth and survival. Further biological studies are in progress.

Acknowledgements

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