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Urukthapelstatin A, a Novel Cytotoxic Substance from Marine-derived *Mechercharimyces asporophorigenens* YM11-542

I. Fermentation, Isolation and Biological Activities

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Abstract Urukthapelstatin A, a novel cyclic peptide, was isolated from the cultured mycelia of marine-derived *Thermoactinomycetaceae* bacterium *Mechercharimyces asporophorigenens* YM11-542. The peptide was purified by solvent extraction, silica gel chromatography, ODS flash chromatography, and finally by preparative HPLC. Urukthapelstatin A dose-dependently inhibited the growth of human lung cancer A549 cells with an IC_{50} value of 12 nM. Urukthapelstatin A also showed potent cytotoxic activity against a human cancer cell line panel.

Keywords urukthapelstatin A, cyclic peptide, cytotoxic, *Thermoactinomyces*

Introduction

In the course of screening for new antibiotic compounds from marine-derived isolates, *Mechercharimyces asporophorigenens* YM11-542 was found to produce a novel anticancer compound, urukthapelstatin A (**1**, Fig. 1), which was determined by a spectral analysis and chemical degradation to be a unique cyclic peptide. This peptide possessed potent cytotoxicity against human cancer cell

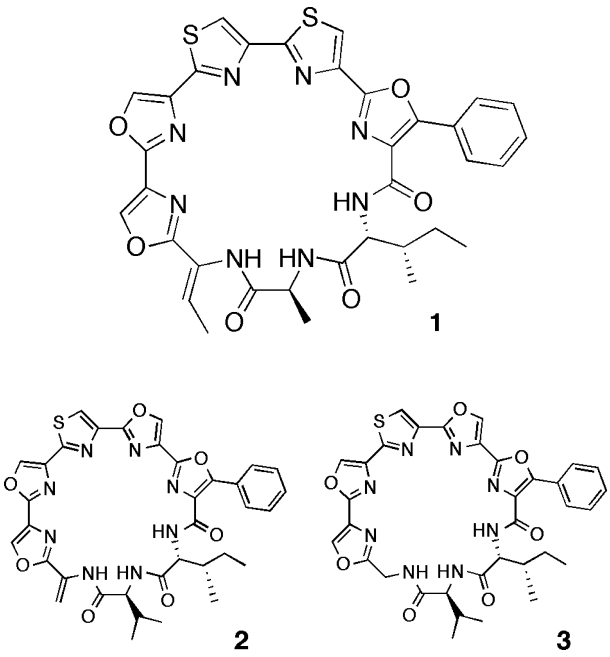

Fig. 1 Structures of urukthapelstatin A (**1**), mechercharstatin (**2**; former name, mechercharmycin) and YM-216391 (**3**).

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lines *in vitro*, and is structurally related to mechercharstatin (**2**; former name, mechercharmycin), [1, 2] and YM-216391 (**3**), [3, 4] which are also potent cytotoxic compounds respectively isolated from the *Thermoactinomycetaceae* bacterium and *Streptomyces*. We describe in this paper the fermentation, isolation, and biological activities of **1**. To evaluate the profile of its anticancer activity *in vitro*, we used a human cancer cell line panel in combination with a database analysis [5]. The physico-chemical properties and structural determination of **1** are described in the subsequent paper [6].

Materials and Methods

Microorganism and Media

YM11-542 (*M. asporophorigenens*, MBIC06487^T=DSM 44955^T) was isolated from a sediment sample collected from a marine lake in the northern part of Urukthapel Island in the Republic of Palau. This strain has been reported as a mesophilic member of the novel taxon, *Mechercharimyces* gen. nov., that is distinguished from members of the related genera in *Thermoactinomyces* fam. nov. [7].

YM11-542 was harvested from marine agar (DIFCO) and used to inoculate 2216 marine broth (MB, DIFCO) to study the antibiotic production. Fermentation was carried out in different modifications of a P2 medium (10～30 g of Pharmamedia (Traders Protein), 1.0 g of yeast extract, 1.0 g of sucrose and 0.1 g of Fe·citrate-nH_2O in 75% seawater at pH 7.6). Each culture was incubated at 30°C for 5～14 days on a rotary shaker at 100 rpm. In most cases, 100-ml flasks containing 40 ml of the medium or 1000-ml baffled flasks (Shibata Scientific Technology) containing 500 ml of the medium were used.

Antibiotic Production and HPLC Analysis

The antibiotic production with the addition of nutrients and beads was studied in duplicate in 100-ml Erlenmeyer flasks with 40 ml of MB, 1.0 g/liter of sucrose containing MB or the P2 medium, and 3.0 ml of HP20 polystyrene divinylbenzene beads (Mitsubishi Chemical Co.) or four types of chitin-based beads (Chitopearl HP3020, SH3503, HP3520 or HP5020; Fujibo Holdings). Each of these flasks was inoculated with 1.0 ml of a 2-day-old culture in MB and incubated at 30°C for 10 days. The antibiotic present in the cells and beads was extracted by adding 5.0 ml of $CHCl_3$/MeOH (1/1) and sonicating for 10 minutes, this being followed by centrifugation (8,000 rpm, 15 minutes) and lyophilization.

The time-course characteristics of antibiotic production were also studied in 1000-ml baffled Erlenmeyer flasks with 500 ml of MB, 1.0 g/liter of sucrose containing MB or the P2 medium and 30 ml of Chitopearl SH3503. Each of these flasks was inoculated with 10 ml of a 2-day-old culture in MB and incubated at 30°C. Three aliquots (5.0 ml each) from each flask were collected after 5, 7 and 10 days of culture, and the antibiotic present in the cells and beads was extracted in the same manner.

An HPLC analysis was performed with a D-7000 HPLC-PDA system (Hitachi), using TSK gel ODS-80Ts (Tosoh). A 100-μl aliquot of each sample was directly injected, before centrifuging (15,000 rpm, 5 minutes), eluting with a gradient of 66～76% acetonitrile for 10 minutes at a flow rate of 1 ml/minute, and monitoring for **1** (rt=8 minutes) at UV 290 nm. The production (mg/liter) of **1** was calculated by a standard curve (not shown) from the total area of serially diluted pure **1** on HPLC charts.

Biological Assays

A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The cells were seeded in a 96-well microplate (4,000 cells/200 μl/well) and then cultured for 14 hours at 37°C in a CO_2 incubator (5% CO_2-air). Serially diluted samples were added to each well, and the cells were cultured for a further 48 hours. The number of cells was counted by the Alamar Blue method, and the IC_{50} value was determined from three independent experiments.

The human cancer cell line panel assay was carried out by the Cancer Chemotherapy Center at the Japanese Foundation for Cancer Research. The precise methods for the experiments and the data analysis have been described elsewhere [5]. The screening panel consisted of the following 39 human cancer cell lines: breast cancer (Br) HBC-4, BSY-1, HBC-5, MCF-7 and MDA-MB-231; brain cancer (CNS) U251, SF-268, SF-295, SF-539, SNB-75 and SNB-78; colon cancer (Co) HCC2998, KM-12, HT-29, HCT-15 and HCT-116; lung cancer (Lu) NCI-H23, NCI-H226, NCI-H522, NCI-H460, A549, DMS273 and DMS14; melanoma (Me) LOX-IMVI; ovarian cancer (Ov) OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8 and SK-OV-3; renal cancer (Re) RXF-631L and ACHN; stomach cancer (St) St-4, MKN1, MKN7, MKN28, MKN45 and MKN74; and prostate cancer (xPg) Du-145 and PC-3. The GI_{50} (50% growth inhibition), TGI (total growth inhibition) and LC_{50} values for these cell lines were determined by using the sulforhodamine B (SRB) colorimetric method. Computer processing of these values produced differential activity patterns against the cell lines, from which mean graphs were constructed. These mean graphs were compared with those for standard compounds, including

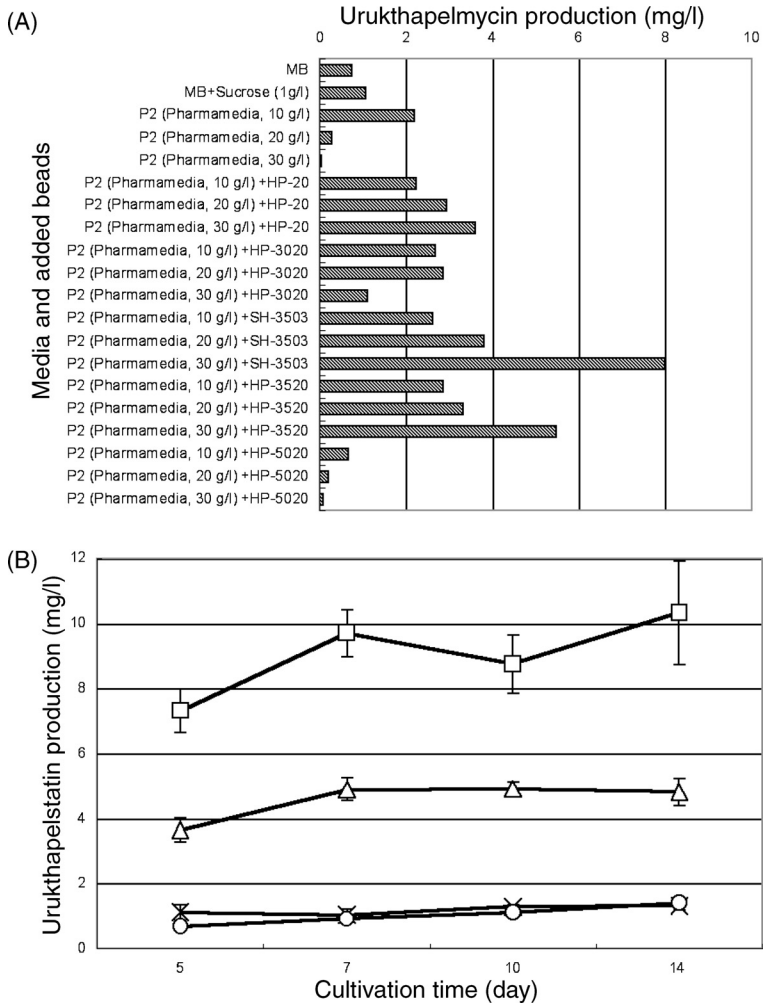

Fig. 2 (A) Antibiotic production in the P2 medium with polymer beads. (B) Time-course characteristics for antibiotic production in the P2 medium with SH3503.

× MB, ○ MB+Sucrose (1.0 g/liter), △ P2 (Pharmamedia, 30 g/liter), □ P2 (Pharmamedia, 30 g/liter)+SH-3503.

various anticancer drugs, by using a COMPARE analysis [5].

Telomerase Inhibition Assay

Telomerase activity was estimated by Telomeric Repeat Amplification Protocol (TRAP) assay which was the modified version of Kim *et al.* [8], the details having been described in the previous paper [9].

Results and Discussion

Antibiotic Production

Antibiotic production was studied in several complex media, the P2-based medium giving the best results. In order to optimize the production in the P2 medium, we studied the effect of adding Pharmamedia and polymer beads. Concentrations ranging from 1～3% of Pharmamedia and five types of beads (HP20, HP3020, SH3503, HP3520 and HP5020) were prepared with the P2 medium, and the antibiotic production after 7 days was analyzed by HPLC (Fig. 2A). The best production without beads was obtained in 1.0% Pharmamedia. A clear positive effect was observed when beads were then added to the P2 medium. With beads, the best production was obtained in 3.0% Pharmamedia containing SH3520 beads.

A fermentation time-course study was performed in 1000-ml baffled Erlenmeyer flasks as described in the Materials and Methods section, and the antibiotic present in the cells and beads of 5～14-day cultures was analyzed by HPLC (Fig. 2B). After 7 days, the production of antibiotic in MB, MB+sucrose and P2 with or without the SH3503

beads had almost reached a plateau and remained stable for 14 days of incubation. Maximum production was observed in P2 (3.0% Pharmamedia) with the Chitopearl SH3520 beads. The allowable error for **1** production by P2 (30 g/liter of Pharmamedia)+SH3520 in Fig. 2B was increased by the number of days of cultivation, resulting in the formation of a protein aggregate of cells and beads. We decided to process the 10～14-day culture as giving acceptable production for antibiotic purification.

Isolation and Purification

The procedure used for isolating the antibiotic activity is summarized in Fig. 3. After 10 days, the culture (10 liters) was centrifuged, and the mycelial cake and beads were lyophilized. The precipitate was repeatedly extracted with $CHCl_3$/methanol (1 : 1, 1.0 liter×3 times). After centrifugation, the organic layer was concentrated under reduced pressure and partitioned between $CHCl_3$ and water, the $CHCl_3$ layer being dried over anhydrous Na_2SO_4 and then evaporated to dryness to give a blackish residue (7.18 g). The extract was chromatographed in a silica gel column with $CHCl_3$/methanol as the eluent. The fractions containing urukthapelmycin A were eluted with 96 : 4～90 : 10 $CHCl_3$/methanol and further purified by reversed-phase FCC in a Cosmosil 75 C18-OPN column, with methanol/water used as the eluent. Final purification was carried out by C_{18} preparative HPLC, and the fraction containing **1** was crystallized from CH_2Cl_2/methanol to afford 35 mg of the pure compound.

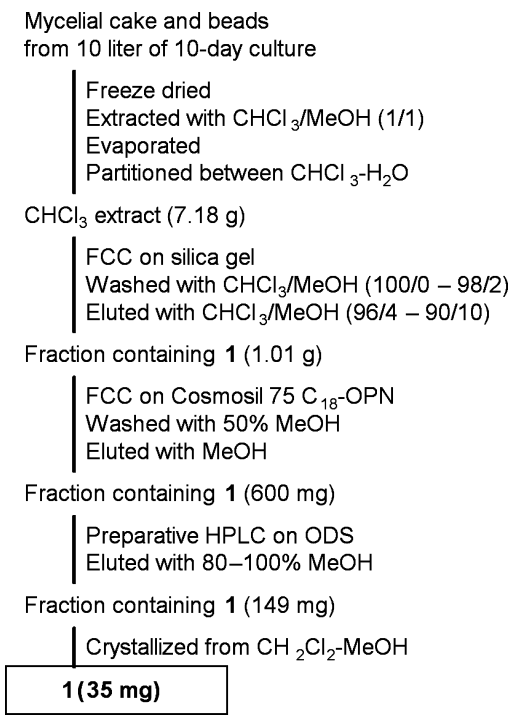

Fig. 3 Procedure for the isolation of **1**.

Biological Activities

To investigate the profile of the protein's anticancer activity *in vitro*, we used a human cancer cell line panel combined with a COMPARE analysis [5, 10].

1 showed differential growth inhibition, and seemed to be most effective against the lung and ovarian cancer cell lines, the MCF-7, HCT-116, A549, DMS114 and NCI-H460 cell lines with the log GI_{50} value between −8.28M and −8.46M. The mean log GI_{50} value for **1** was −7.81M, which indicates quite strong activity compared to the anticancer drugs in clinical use on the panel. The COMPARE analysis revealed that **1** had a low correlation coefficient with the anticancer reagent cytarabine (0.636) and enocitabine (0.594), respectively. They are all known as antimetabolite antineoplastic agents that inhibit the synthesis of DNA. It has been suggested that YM-216391 and mechercharstatin were DNA synthesis inhibitors by the results of a COMPARE analysis and cell sorting experiments, respectively [2, 3].

The effect of **1** on histone deacetylase (HDAC) [11], farnesyl transferase (FPTase) [12] and the proteasome [13] were investigated, but **1** did not show any potent activity at 10 μM.

The telomerase inhibitory activity was also tested, because of the structural similarity to telomestatin, the well-known telomerase inhibitor [14]. However, **1** did not show any telomerase inhibitory activity in TRAP assay at 2 μM, similar to mechercharstatin [2]. Since, the latest study to develop telomestatin-derivatives disclosed that plane nature of whole compound structure is important to exert telomerase inhibitory activity [15], it should be reasonable that **1** did not show inhibitory effects on telomerase.

1 do not exhibited any inhibitory activity against the tested microorganisms (*Escherichia coli* IFO3301, *Bacillus subtilis* IFO3134, *Staphylococcus aureus* IFO12732, and *Candida albicans* IFO1060) by the paper disc method (10 μg/disc).

The correlation coefficient in the COMPARE analysis was weak, which indicates that **1** may have a unique mode of action. Further pharmacological studies and an investigation of the mechanism of action are now underway.

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