UCH9, a New Antitumor Antibiotic Produced by *Streptomyces*: I. Producing Organism, Fermentation, Isolation and Biological Activities

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We developed a microbial prescreen using *Bacillus stearothermophilus* NUB3620 and bacteriophage TP-68 to detect potential antitumor compounds acting on DNA or topoisomerases. During the course of screening microbial cultures for their antibacteriophage activities, we found that *Streptomyces* sp. isolated from a soil sample collected in Iwakuni city, Yamaguchi prefecture, Japan, produced a new antitumor antibiotic, UCH9. UCH9 was isolated from culture broth by a combination of EtOAc extraction and column chromatography. UCH9 has a new structure related to the antitumor antibiotic chromomycins. It exhibited antimicrobial activity against Gram-positive organisms. UCH9 also showed cytotoxic activity against HeLa S3 cells with an IC_{50} value of 13 nM and exhibited antitumor activity *in vivo* against mouse leukemia P388.

DNA topoisomerases have been shown to be the principal targets for a number of antitumor drugs[1,2]. We screened cultures of actinomycetes and fungi for their ability to induce topoisomerase I and II mediated DNA cleavage *in vitro*, and have identified several microbial metabolites as new antitumor antibiotics[3~5].

The T4 phage DNA topoisomerase II is sensitive to many antitumor agents such as *m*-AMSA and 2-me-9-OH-ellipticinium acetate that inhibit the mammalian enzyme[6,7]. In addition, DNA intercalators including topoisomerase II inhibitors have been shown to inhibit selectively the growth of bacteriophage at the concentration that shows no apparent antibacterial activity. With this knowledge, we developed a microbial prescreen to detect potential antitumor compounds acting on DNA or DNA topoisomerases based on their ability to inhibit the growth of bacteriophage. In the course of screening microbial cultures for their antibacteriophage activity using *Bacillus stearothermophilus* NUB3620 and bacteriophage TP-68, we found that *Streptomyces* sp. isolated from a soil sample produced a new antitumor antibiotic, UCH9 (Fig. 1).

In this paper, we report the taxonomy of the producing strain, fermentation, isolation, and biological activities of UCH9. Physico-chemical properties and structure determination of this compound are reported in the following paper[8].

Materials and Methods

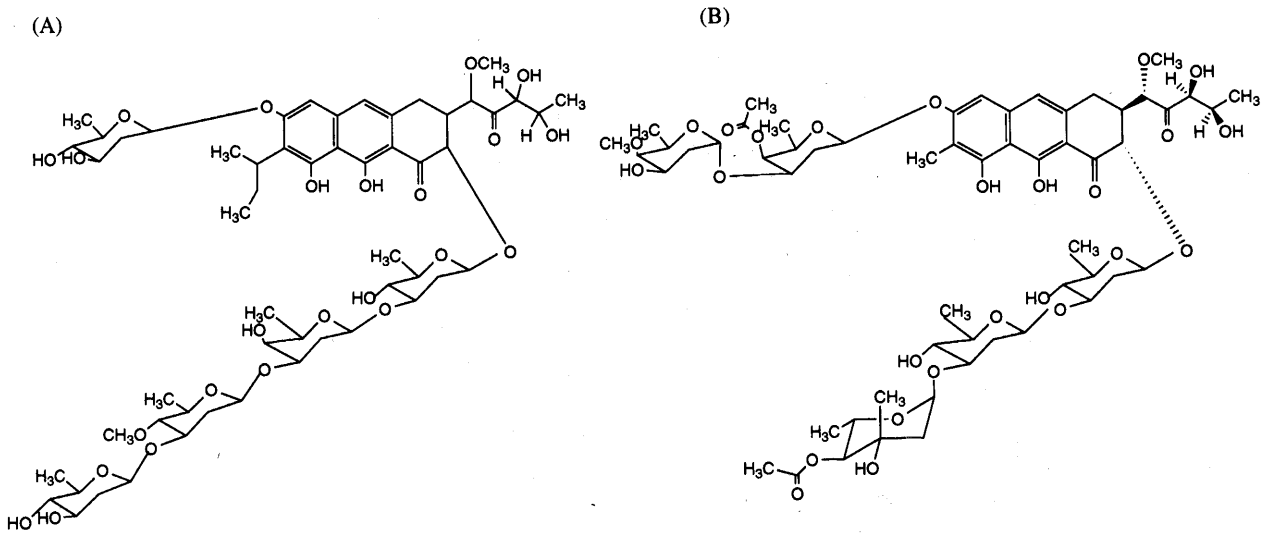
Characterization of the Producing Strain

Strain UOH9 that produced UCH9, a new antitumor antibiotic, was isolated from a soil sample collected in Iwakuni city, Yamaguchi prefecture, Japan. For cultural and physiological characterization of the producing strain, the methods of SHIRLING and GOTTLIEB[9] were employed. The cultural and physiological characterization was determined after incubation at 28°C for 2 weeks. The temperature range for growth of strain UOH9 was determined after submerged cultivation for 1 week. For analysis of the conformation of diaminopimeric acid in a whole-cell hydrolysate of strain UOH9, the method of HASEGAWA, TAKIZAWA, and TANIDA[10] was employed.

Fermentation

The media for seed and fermentation cultures are: SRIII medium; glucose 1%, soluble starch 1%, Bacto tryptone 0.5%, beef extract 0.3%, yeast extract 0.5%

Fig. 1. Structures of UCH9 (A) and chromomycin A_3 (B).



and $Mg_3(PO_4)_2 \cdot 7H_2O$ 0.05% (pH 7.2 prior to sterilization). DF2YX medium; soluble starch 5%, yeast extract 1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% (pH 7.0 prior to sterilization).

A 5-liter jar fermentation was performed as follows: A 300 ml Erlenmeyer flask containing 50 ml of SRIII medium was inoculated with a loopful of well-sporulated stock culture. The flask was incubated at 28°C on a rotary shaker for 72 hours. The 6.25 ml of seed culture was transferred to a 2-liter Erlenmeyer flask containing 125 ml of the same medium. Following 48 hours of incubation at 28°C, the second stage seed culture was used as the inoculum to initiate the fermentation in a 5-liter jar fermenter batched with 2.5 liter of a DF2YX medium. The fermentation was carried out at 28°C with 2.5 liter of air per minute and agitation at 400 rpm. Culture growth was evaluated by centrifuging fermentation broth in 10 ml test tube at 300 rpm for 10 minutes. The packed cell volume was recorded as % of total volume.

Antiphage Activity

Bacillus stearothermophilus phage TP-68[11] (virulent phage) was used in the course of screening. *Bacillus stearothermophilus* NUB3620 was cultured in LB medium supplemented with 1.05 mM nitrilotriacetate and harvested at log-phase. The bacterial culture (1.7 ml) was mixed with 300 μl of diluted phage lysate in 3.8 ml melted LB top agar. The mixture was poured onto the surface of a LB bottom agar plate. After solidification, paper discs impregnated with test samples were applied on agar plates, and the plates incubated at 60°C for 12 hours[12]. Antibacteriophage activities were detected as cell survival zones around the discs.

Antimicrobial Activity

The *in vitro* antimicrobial activity of UCH9 was determined on nutrient agar by a 2-fold serial dilution method. The lowest concentration that inhibited growth of a bacterial strain after 18 hours incubation at 37°C was recorded as the MIC.

Anticellular and Antitumor Activity

Human uterine cervix carcinoma HeLa S3 cells were obtained from American Type Culture Collection through Dainippon Pharmaceutical Co. (Osaka, Japan). Cytotoxic activity of UCH9 was determined as described previously[13]. *In vivo* antitumor activity was measured and calculated as follows[14]. P388 cells (1×10^6 cells/mouse) were transplanted i.p. into $CD2F_1$ mice on day 0, and i.p. administration of drugs was started on day 1. Antitumor efficacy was expressed as a percentage of increase in life span (ILS).

Results

Characterization of the Producing Strain

Vegetative hyphae of strain UOH9 were well developed, branched, and did not fragment into bacillary or coccoid elements. Aerial mycelia were formed on some agar media. Spore chains were born on aerial mycelia, and were flexuous or spiral with rows of 10 to 50 spores. The spores were smooth-surfaced and non-motile. The

Table 1. Cultural characteristics of strain UOH9.

Medium	Substrate mycelium	Aerial mycelium	Soluble pigment
Yeast extract - malt extract agar (ISP No. 2)	Moderate, light gold (2ic)	Scanty, oyster white (b)	None
Oatmeal agar (ISP No. 3)	Poor, bamboo (2gc)	Absent	Pale brown
Inorganic salts-starch agar (ISP No. 4)	Moderate, cream (11/2ca)	Absent	None
Glycerol - asparagine agar (ISP No. 5)	Moderate, light ivory (2ca)	Absent	None

Color names and numbers used in this table were based on Color Harmony Manual (Container Corporation of America).

Table 2. Physiological characteristics of strain UOH9.

Temperature for growth	15 ~ 47°C
Optimum temperature	ca. 30°C
Formation of Melanin on;	
Peptone-yeast extract-iron agar (ISP No. 6)	+
Tyrosine agar (ISP No. 7)	-
Utilization of:	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Fructose	+
Sucrose	+
Inositol	+
L-Rhamnose	+
Raffinose	-
D-Mannitol	+

+, Positive; -, negative.

formation of sporangia or synnemata was not observed. The conformation of diaminopimelic acid in a whole-cell hydrolysate of strain UOH9 was LL. Based on taxonomic characteristics described above, strain UOH9 is considered to belong to the genus *Streptomyces*. The cultural characteristics of the producing strain are shown in Table 1. The aerial mass color was white. Brown soluble pigment was produced on some agar media. The physiological characteristics of strain UOH9 are shown in Table 2. The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as *Streptomyces* sp. UOH9 with the accession No. FERM BP-4392.

Fermentation

As a result of a systematic study on carbon and

nitrogen sources, soluble starch and yeast extract were suitable carbon and nitrogen source for UCH9 production. Fig. 2 shows a typical time course of UCH9 production in a 5-liter jar fermentation under optimum conditions. The production of UCH9 started at the third day and gradually increased for additional 3 days.

Isolation

UCH9 was isolated from 6 liters of cultured broth by the following procedure under dark conditions because UCH9 was unstable to light. Cultured broth was mixed with 6 liter of *i*-propyl alcohol and stirred for 2 hours to extract UCH9. After filtration, the *i*-propyl alcohol extract was concentrated, and then applied to a column of Diaion HP-20 (Mitsubishi Chemical Industries Limited). The column was washed with water-MeOH (5:5) and UCH9 was eluted with water-MeOH (2:8). The eluate was concentrated and extracted with EtOAc. The extract was concentrated and then applied to a Sephadex LH-20 column. The column was eluted with

Fig. 2. Time course of UCH9 fermentation in a 5-liter jar fermenter.

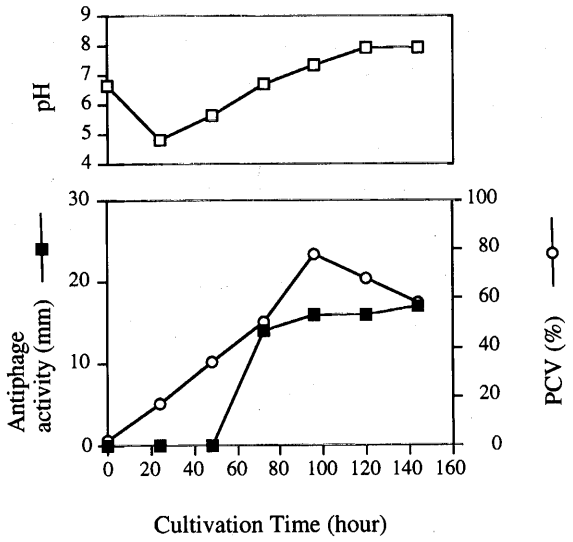


Table 3. Antibacteriophage activities of UCH9 and chromomycin A_3.

Compounds	Antibacteriophage activities (diameter: mm)		
	500 ng/disc	50 ng/disc	5 ng/disc
UCH9	22	18	14
Chromomycin A3	22	17	14

Table 4. Antimicrobial activities of UCH9 and chromomycin A_3.

Test microorganisms	MIC (μg / ml)	
	UCH 9	Chromomycin A3
Bacillus subtilis No.10707	< 0.04	< 0.04
Staphylococcus aureus subsp. *aureus* ATCC6538P	5.2	0.04
Enterococcus hirae ATCC10541	41.6	0.16
Escherichia coli ATCC26	>100	>100
Klebsiella pneumoniae subsp. *pneumoniae* ATCC10031	>100	>100
Pseudomonas aeruginosa BMH No.1	>100	>100
Proteus vulgaris ATCC6897	>100	>100
Shigella sonnei ATCC9290	>100	>100
Salmonella choleraesuis subsp. *choleraesuis* ATCC9992	>100	>100
Candida albicans ATCC10231	>100	>100

water - MeOH (3 : 7). The active fractions were combined and concentrated to yield 27.5 mg of pure UCH9 as a greenish yellow powder.

Biological Activities

Antibacteriophage activity: Antibacteriophage activity of UCH9 was compared with structurally related antibiotic, chromomycin A_3. As shown in Table 3, both compounds have the same inhibitory activity on the growth of *Bacillus stearothermophilus*-infective phage.

Antimicrobial activity: UCH9 exhibited a weak antimicrobial activity against Gram-positive bacteria but was inactive against Gram-positive bacteria and fungi (Table 4).

Cytotoxic activity: UCH9 was cytotoxic at the nM range against HeLa S3 cells *in vitro* as shown in Table 5.

Antitumor activity: Antitumor activity of UCH9 against a murine experimental tumor is shown in Table 6. UCH9 prolonged the life span (ILS 57%) of mice bearing P388 leukemia using five daily consecutive i.p. doses of 1 mg/kg, while it shortened the life span at a dose of 2 mg/kg.

Table 5. HeLa S3 cytotoxicities of UCH9 and chromomycin A_3.

Compounds	IC_{50} (nM)
UCH9	13
Chromomycin A3	1.4

Discussion

We developed a microbial prescreen using *Bacillus stearothermophilus* NUB3620 and bacteriophage TP-68 to detect potential antitumor compounds acting on DNA or topoisomerases. Using this assay, we isolated UCH9 from the cultured broth of *Streptomyces* sp. UOH9. Although a number of antibacteriophage compounds *e.g.* tomaymycin[15], RK-1441[16], and enopeptin[17] derived from *Streptomyces* sp. have been described, UCH9 is structurally distinct from these compounds. Adriamycin and *m*-AMSA, both of which act on DNA topoisomerase II, also showed antibacteriophage activity in this assay system, while UCH9 did not have topoisomerase I or II-mediated DNA cleavage activity *in vitro*. Since UCH9 showed weak, but detectable DNA intercalation activity (data not shown), it is likely that antibacteriophage activity of UCH9 is due to this DNA intercalation activity.

UCH9 is structurally related to chromomycin A_3[18] (Fig. 1). Chromomycin A_3 was isolated from *Streptomyces griseus* in 1960[19], and has been used in cancer chemotherapy. UCH9 showed cytotoxicity with an IC_{50} value of 13 nM, which was less potent than that of chromomycin A_3. UCH9 exhibited significant antitumor activity in a murine syngenic model, indicating that UCH9 could be a new antitumor agent with chromomycin-related structure. Further studies on the antitumor activity of UCH9 in human tumor xenograft model are in progress.

Table 6. Antitumor activity of UCH9 against murine P388 leukemia.

Compounds	Dose (mg / kg)	Schedule	Mean survival days ± SD	ILS (%)
Control			9.2 ± 0.4	0
UCH 9	0.13	Days 1-5	11.0 ± 0.7	20
	0.25		11.0 ± 1.2	20
	0.50		11.6 ± 1.5	26
	1.00		14.4 ± 1.7[a]	57
	2.00		5.8 ± 0.9	-39
Mitomycin C	2.00	Day 1	16.6 ± 1.1	80

P388 cells (1×10^6/mouse) were inoculated i.p. into 6-week-old male CD2F1 mice weighing 22 - 24 g on day 0. Compounds were administered i.p. on each day following indicated schedule.

a, ILS (%) ≥ 25, and $p < 0.05$ by Mann-Whitney 's U-test as compared with the control group.

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