UK-1, A NOVEL CYTOTOXIC METABOLITE FROM *Streptomyces* sp. 517-02

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

MASASHI UEKI, KEIICHI UENO, SHINJI MIYADOH†, KEIICHI ABE††,
KOZO SHIBATA, MAKOTO TANIGUCHI* and SUSUMU OI

Faculty of Science, Osaka City University,
3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558, Japan
†Pharmaceutical Technology Laboratories, Meiji Seika Kaisha, Ltd.,
788 Kayama, Odawara-shi, Kanagawa 250, Japan
††Institute for Biomedical Research, Suntory Ltd.,
120-1 Takahama, Shimamoto-cho, Mishima-gun, Osaka 618, Japan

(Received for publication January 21, 1993)

A new benzoxazole, UK-1, was isolated from the mycelial cake of an actinomycete strain 517-02. Based on morphological, cultural and physiological characteristics, strain 517-02 was seemed to be a close relative of *Streptomyces morookaense*. UK-1 showed potent cytotoxic activity against B16, HeLa and P388 cells and did not show any antimicrobial activity.

In the course of our screening program for new antibiotics, an actinomycete strain 517-02 was found to produce several antifungal antibiotics in the mycelium. During the process of isolation of the active principles, an other novel benzoxazole, UK-1 (Fig. 1), was obtained. UK-1 was not responsible for antifungal activity, but did show potent cytotoxic activity against B16, HeLa and P388 cells. This paper describes the taxonomy of the producing organism, fermentation, isolation and physico-chemical and biological properties of UK-1. The structural elucidation of UK-1 will be reported in the accompanying paper[1].

Materials and Methods

Taxonomic Studies

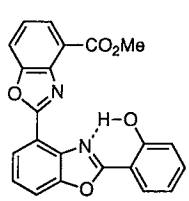
The producing organism, strain 517-02, was isolated from a soil sample collected at Sugimoto campus of Osaka City University.

The taxonomic studies were carried out as described by the International Streptomyces Project (ISP)[2]. For the evaluation of cultural characteristics, the strain was incubated for 14~28 days at 27°C. The color recorded for mature cultures was described according to the "Color Harmony Manual"[3]. Utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[4]. The type of diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[5].

Fermentation Studies

The stock culture of the producing organism was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium composed of glucose 1%, soluble starch 1%, wheat germ 0.6%, peptone 0.5%, yeast extract 0.3%, soybean meal 0.2% and $CaCO_3$ 0.2% (pH 7.0 before sterilization). After incubation at 30°C for 48 hours on a rotary shaker at 220 rpm, a 30-ml aliquot of the culture

Fig. 1. Structure of UK-1.



broth was transferred into a 5-liter jar fermentor containing 3 liters of the seed medium. Following 48 hours incubation at 30°C under aeration of 3 liters/minute and agitation of 500 rpm, whole cultured broth of the jar fermentor was transferred into a 600-liter tank fermentor containing 300 liters of the production medium composed of glucose 3.0%, malt extract 0.5%, yeast extract 0.5% and $CaCO_3$ 0.2% (pH 7.0 before sterilization). Fermentation was carried out for 48 hours at 30°C under aeration of 300 liters/minute and agitation of 250 rpm.

Fermentation Analysis

The growth was monitored by packed cell volume (PCV) measurement. The amount of UK-1 in the mycelium was quantified by HPLC using a Shimadzu LC-6A pump (column; Develosil ODS-5, solvent; 70% aq CH_3CN, flow rate; 1.0 ml/minute, detection; UV at 325 nm). The sample for the HPLC assay was prepared as follows; 10 ml of the culture broth was sampled and centrifuged. The precipitated mycelium was extracted with 1 ml of acetone for 1 hour at room temperature. After centrifugation, 5 μl of the supernatant was injected.

In Vitro Activities

In the *in vitro* antimicrobial assay, UK-1 was first dissolved in *N,N'*-dimethylformamide (DMF). The MIC's of UK-1 were determined by the serial 2-fold agar dilution method in 3% nutrient agar for bacteria and in Sabouraud dextrose agar for yeasts and fungi.

In the *in vitro* cytotoxic assay using mouse melanoma B16, human epitheloid carcinoma HeLa and mouse leukemia P388 cells, UK-1 was first dissolved in acetone. B16 and HeLa cells were cultivated in EAGLE's minimum essential medium (Nissui Seiyaku) supplemented with 10% fetal bovine serum (JRH Bioscience) and P388 cells in RPMI1640 medium (Nissui Seiyaku) with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. The cells were trypsinized and seeded at 2×10^3 cells to each well of 96-well multi plate. After the challenge with serially diluted UK-1 for 72 hours, the cytotoxic effects were determined by MTT colorimetric method[6]. The concentration that inhibits 50% of control growth (ED_{50}) was calculated to assess the potency of the inhibitory effect of the drug.

Results and Discussion

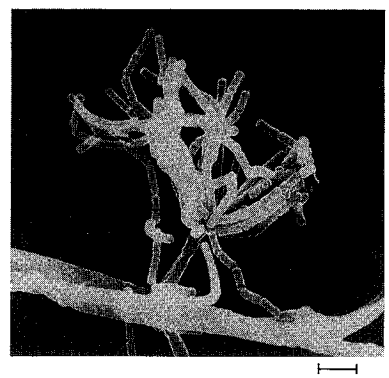
Taxonomy

Morphological and Chemical Properties

The substrate mycelia of strain 517-02 were well-branched and not fragmented. The aerial mycelia consisted of long, straight filaments bearing at regular intervals branches arranged in verticils. Each branch of the verticil produced at its apex an umbel that consisted of straight 10~20 chains of cylindrical spores. The spores were 0.5~0.6 × 1.5~2.0 μm in size. Spore surfaces were smooth. Sclerotia, sporangia and zoospores were not observed (Fig. 2).

Fig. 2. Scanning electron micrograph of aerial mycelia of strain 517-02.

Bar = 2.0 μm.



Analysis of whole cell hydrolysates showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain is classfied as type I.

Cultural Characteristics

The cultural characteristics observed on various media are summarized in Table 1. Mature aerial mycelia corresponded to both the yellow and the green color series. The reverse side of the colony was

Table 1. Cultural characteristics of strain 517-02.

Medium	Cultural characteristics	Medium	Cultural characteristics
Sucrose-nitrate agar	G: Poor	Yeast extract-malt extract agar (ISP No. 2)	G: Good
	A: None		A: Abundant, cottony, light green (1½ge)
	R: Colorless		R: Dark brown
	S: None		S: Dark brown
Glucose-asparagine agar	G: Good	Tyrosine agar (ISP No. 7)	G: Poor
	A: None		A: None
	R: Colorless		R: Colorless
	S: None		S: None
Glycerol-asparagine agar (ISP No. 5)	G: Good	Nutrient agar	G: Moderate
	A: Abundant, cottony, ivory (1½ec)		A: None
	R: Dark brown		R: Colorless
	S: Light brown		S: None
Inorganic salts-starch agar (ISP No. 4)	G: Good	Ca-malate agar	G: Poor
	A: Abundant, cottony, ivory (1½ec)		A: None
	R: Light brown		R: Colorless
	S: None		S: None
Oatmeal agar (ISP No. 3)	G: Moderate	BENNET's agar	G: Good
	A: Poor, light gray (1dc)		A: Abundant, cottony, light green (1½ge)
	R: Light brown		R: Light brown
	S: None		S: Light brown

Abbreviation: G, growth; A, aerial mycelium; R, reverse side color; S, soluble pigment.

Table 2. Physiological characteristics of strain 517-02.

Temperature:		Utilization of:	
Growth range	15~41°C	Glycerol	+
Optimum growth	30°C	L-Arabinose	+
Melanoid pigment production	Negative	D-Xylose	+
Starch hydrolysis	Positive	L-Rhamnose	−
Gelatine liquefaction	Positive	D-Glucose	+
Nitrate reduction	Positive	D-Fructose	+
Milk peptonization	Positive	Sucrose	+
Milk coagulation	Negative	Raffinose	−
NaCl tolerance	1.5%	*myo*-Inositol	+
		D-Mannitol	+

light to dark brown. Brownish soluble pigments were formed.

Physiological Properties

Gelatine liquefaction, starch hydrolysis, nitrate reduction and milk peptonization were positive, whereas milk coagulation and melanoid pigment production were negative. Temperature range for growth was 15~41°C with the optimum at about 30°C. NaCl tolerance was less than 1.5% on yeast-malt agar.

Growth of the strain was supported by the following carbohydrates as a sole carbon source; D-glucose, D-fructose, glycerol, D-xylose, D-mannitol, *myo*-inositol, sucrose and L-arabinose. L-Rhamnose and raffinose were not utilized.

Taxonomic Position

The morphological, cultural and physiological characteristics of strain 517-02 indicate that the strain

belongs to the genus *Streptomyces*. The characteristics of this strain were compared with the published description of various *Streptomyces* species. It was considered strain 517-02 to be closely related to *Streptomyces morookaense*, formerly *Streptoverticillium morookaense*[7]. Since there are some differences, therefore strain 517-02 was named *Streptomyces* sp. 517-02.

Fermentation

A typical time course for the production of UK-1 is shown in Fig. 3. The growth of strain 517-02 gradually increased in the first 40 hours, and UK-1 production started at approximately 20~24 hours, reaching its maximum at 48 hours after incubation.

Isolation

A flow diagram of the isolation procedure of UK-1 is shown in Fig. 4. The culture broth (300 liters) thus obtained was filtered with the aid of diatomaceous earth. The mycelial cake was extracted with acetone (110 liters) and filtered. The filtrate was concentrated *in vacuo* to give aqueous solution (16 liters), which was extracted with chloroform (25 liters). The organic layer was concentrated *in vacuo* to yield an oily material, which was dissolved in a small volume of chloroform and applied to a silica gel column (Wakogel C-200). Absorbed material was eluted with chloroform. Fractions containing UK-1 were collected and concentrated *in vacuo*. The crude UK-1 was dissolved in methanol and allowed to stand for 1~2 days at 4°C to yield colorless needles (550 mg of UK-1).

Physico-chemical Properties

The physico-chemical properties of UK-1 are summarized in Table 3. UK-1 is soluble in chloroform, dichloromethane, benzene and pyridine, but practically insoluble in water. The UV and IR spectra of

Fig. 3. Time course of UK-1 production.

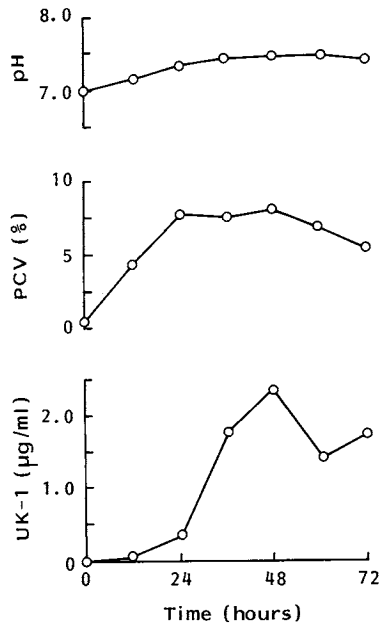


Fig. 4. Isolation procedure of UK-1.

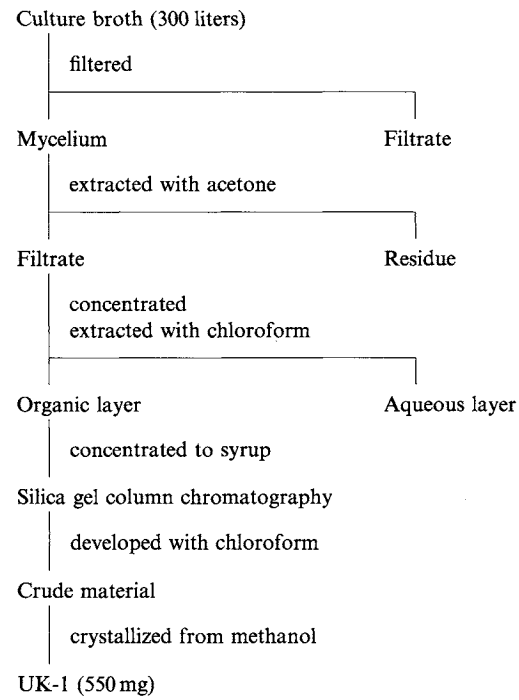


Table 3. Physico-chemical properties of UK-1.

Appearance	Colorless needles
Molecular formula	$C_{22}H_{14}N_2O_5$
HR-MS (m/z)	386.0913 (Calcd; 386.0903)
MP (°C)	217~219
Elemental analysis (%)	
Calcd:	C 68.39, H 3.65, N 7.25
Found:	C 68.36, H 3.63, N 7.23
IR ν_{max} (Nujol)	1725 cm^{-1}
UV λ_{max}^{MeOH} nm (ε)	249 (20,990), 263 (20,150), 271 (19,760), 314 (29,000), 325 (32,540), 348 (27,820), 363 (18,680), 420 (1,480)
Rf (TLC, SiO_2)	$CHCl_3$-MeOH (97:3) 0.43
	$CHCl_3$-MeOH (87:13) 0.77
Solubility	Soluble in $CHCl_3$, CH_2Cl_2, benzene, pyridine
	Slightly soluble in MeOH, DMF, EtOAc, n-C_6H_{14}
	Insoluble in water

Fig. 5. The UV spectrum of UK-1 in methanol.

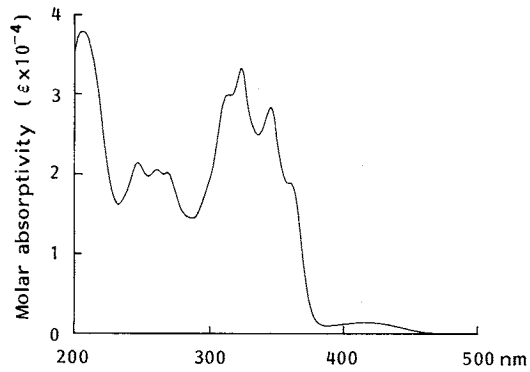
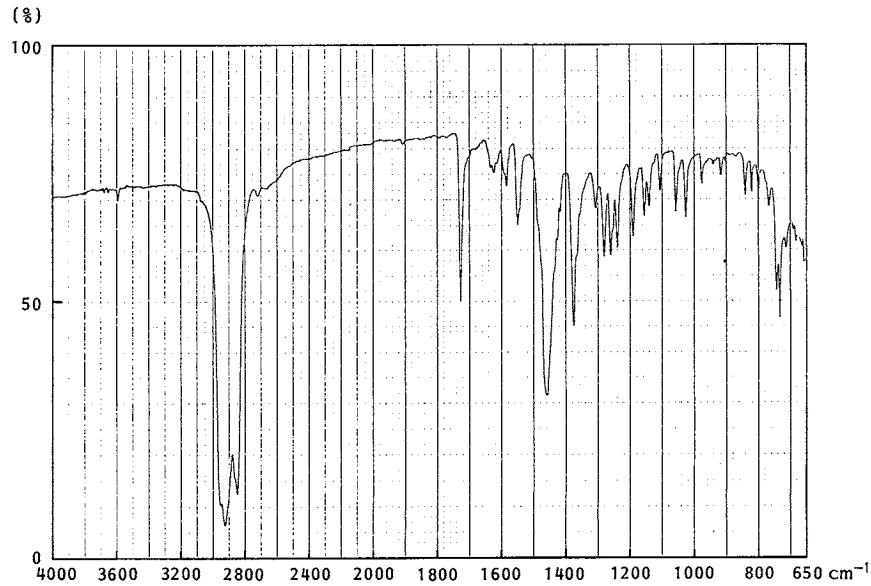


Fig. 6. The IR spectrum of UK-1 (Nujol).



UK-1 are shown in Figs. 5 and 6, respectively. The molecular formula of UK-1 was elucidated by high resolution mass spectrometry; observed: m/z 386.0913; calcd for $C_{22}H_{14}N_2O_5$: m/z 386.0903. The details of the structure elucidation of UK-1 will be reported in the accompanying paper[1].

Biological Properties

UK-1 did not show any growth inhibitory activity against Gram-negative and Gram-positive bacteria, yeasts and fungi up to 100 μg/ml.

However, UK-1 strongly inhibited growth of B16, HeLa and P388 cells: The ED_{50} values were 1.17, 1.22 and 0.11 μg/ml, respectively. Detailed studies on the mode of action of UK-1 and various biological

activities of its derivatives are now in progress.

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