UK-3A, a Novel Antifungal Antibiotic from *Streptomyces* sp. 517-02: Fermentation, Isolation, Structural Elucidation and Biological Properties

MASASHI UEKI, ATSUSHI KUSUMOTO, MUHAMMAD HANAFI, KOZO SHIBATA, TOSHIO TANAKA and MAKOTO TANIGUCHI*

Faculty of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558, Japan

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A novel antifungal antibiotic, UK-3A, was obtained from the mycelial cake of *Streptomyces* sp. 517-02. UK-3A was very similar in structure to UK-2A, a structural relative of antimycin A. The antifungal spectrum of UK-3A was relatively broad (MICs for yeasts and filamentous fungi: 1.56~6.25 and 0.39~1.56 μg/ml, respectively). The cytotoxic activity of UK-3A was weak (IC_{50}: 18~100 μg/ml).

Streptomyces sp. 517-02, from which a novel cytotoxic benzoxazole UK-1 and antifungal nine-membered dilactones UK-2A, B, C and D were previously isolated[1~4], was found to produce several other antifungal antibiotics in the mycelium. We obtained one of the active principles, UK-3A (Fig. 1), from the acetone extracts of the mycelium. This paper describes the fermentation, isolation and structural elucidation of UK-3A. The antifungal and cytotoxic activities of UK-3A will be also discussed as compared with those of UK-2A and antimycin A.

Materials and Methods

Chemicals

Antimycin A was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemical reagents were of commercial grade.

Producing Organism

The organism strain 517-02 was isolated from a soil sample collected at the Sugimoto campus of Osaka City University. Based on its morphological, cultural and physiological characteristics, strain 517-02 seemed to be closely related to *Streptomyces morookaense*. Details of the taxonomic studies were previously reported[1].

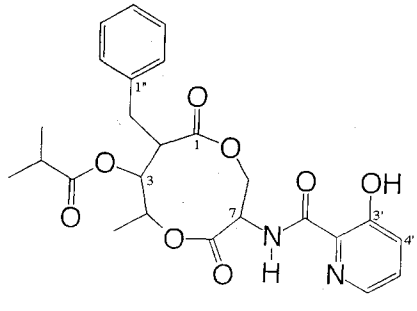
Fermentation Studies

The stock culture of the producing organism was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium composed of 1% glucose, 1% soluble starch, 0.6% wheat germ, 0.5% peptone, 0.3% yeast extract, 0.2% soybean meal and 0.2% $CaCO_3$ (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 broth was transferred into a 5-liter jar fermentor containing 3 liters of the seed medium. Following a 48-hour incubation at 30°C under aeration at 3 liters/minute and agitation at 500 rpm, the entire cultured broth of the jar fermentor was transferred to a 50-liter tank fermentor containing 30 liters of the production medium composed of 3.0% glucose, 0.5% malt extract, 0.5% yeast extract and 0.2% $CaCO_3$ (pH 7.0 before sterilization). The above production medium was supplemented with 2 mM 3-hydroxypicolinic acid (3HP) after sterilization. Fermentation was carried out for 48 hours at 30°C under aeration at 30 liters/minute and agitation at 250 rpm.

Isolation

The culture broth (33 liters) thus obtained was filtered with the aid of diatomaceous earth. The mycelial cake

Fig. 1. Structure of UK-3A.



was extracted with acetone (10 liters) and filtered. The filtrate was concentrated *in vacuo* to give an aqueous solution (1 liter), which was extracted with chloroform (2 liters). The organic layer was concentrated *in vacuo* to yield an oily material, which was dissolved in a small volume of dichloromethane and applied on a silica gel column (Wakogel C-200). Absorbed material was developed with dichloromethane. The first fractions containing antifungal and cytotoxic activities were collected, concentrated *in vacuo* and dissolved in a small amount of methanol. After removal of the principle (UK-1[1]) responsible for the cytotoxic activity from this solution by recrystallization, the mother liquor was chromatographed on a column of Toyo Pearl HW-40 developed with methanol so that UK-1 was completely removed. Fractions containing another principle (UK-3 complex) responsible for the antifungal activity were collected and concentrated *in vacuo*. The complex was recrystallized from methanol as colorless needles (9.6 mg). The crystals were then dissolved in a small amount of 60% aqueous CH_3CN, applied on preparative HPLC (Shiseido, Capcell Pac C18 AG120, i.d. 10×250 mm) and eluted with 60% aqueous CH_3CN. As shown in Fig. 2, the HPLC profile indicated that the UK-3 complex was composed of five components, A, B, C, D and E. Each component showed antifungal activity. The main component, UK-3A, was characterized by NMR and MS. Details of the physico-chemical properties and the total synthesis of UK-3A will be described elsewhere.

Biological Activities

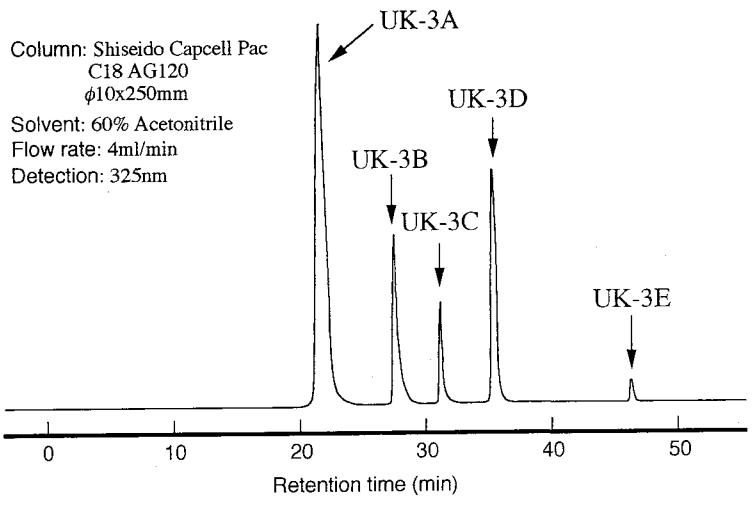
For the *in vitro* antimicrobial assay, UK-3A was first dissolved in *N,N'*-dimethylformamide. The MIC's of the UK-3A were measured using the serial 2-fold agar dilution method in 3% nutrient agar at 30°C for bacteria and in Sabouraud dextrose agar at 25°C for yeasts and fungi.

For the cytotoxic assay using mouse melanoma B16, mouse leukemia P388, mouse fibroblast 3T3, human colon adenocarcinoma COLO201 and human oral epidermoid carcinoma KB cells, UK-3A was first dissolved in acetone. B16, 3T3, COLO201 and KB 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. After washing with PBS (containing, per liter, 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g EDTA·2Na), the cells were trypsinized and seeded at 2×10^4 cells in each well of a 96-well multiplate. After the challenge with serially diluted UK-3A for 72 hours, the cytotoxic effects were determined using the MTT colorimetric method[5]. The concentration that inhibits 50% of control growth (IC_{50}) was calculated to assess the potency of the inhibitory effect of the drug.

Respiratory Activity of *S. cerevisiae*

S. cerevisiae IFO 0203 was cultured in Sabouraud dextrose with shaking at 25°C for 24 hours. The cells were harvested and washed with 0.9% NaCl by centrifugation. The cells were then suspended in 100 mM citrate-$NaHPO_4$ buffer (pH 6.0) containing 28 mM glucose, 10 mM KCl and 0.1 mM EDTA at a final cell concentration of 10^6 cells/ml and incubated with shaking at 25°C. After 10 minutes, UK-3A was added and, at 5-minute

Fig. 2. HPLC separation of UK-3 complex.



intervals, portions of the incubation mixture were withdrawn. The respiratory activity of the yeast cells in the mixture was polarographically measured at 25°C with a Yanagimoto PO-100A oxygen electrode[6,7].

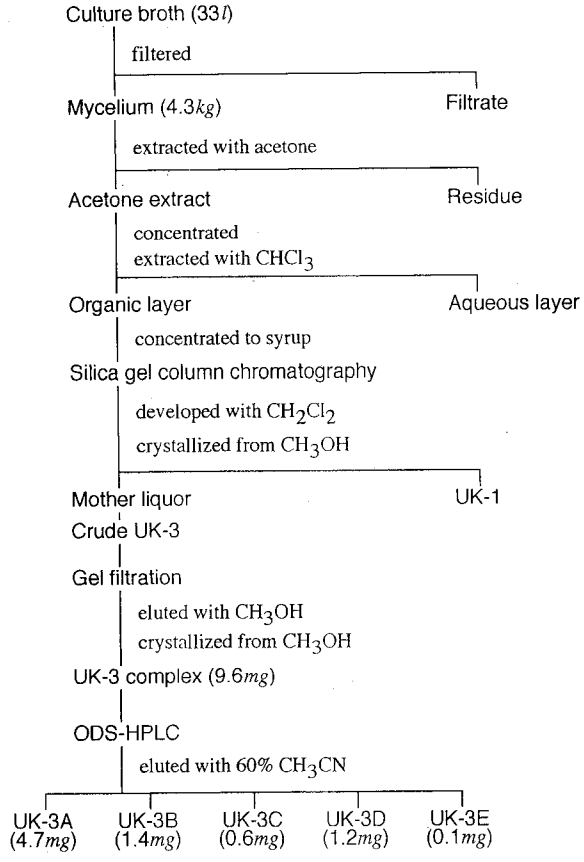
Results and Discussion

Fermentation and Isolation

To increase the production of the UK-3 complex, the addition effects of various kinds of compounds in the producing medium were examined. Among them, 3HP was the most effective and doubled the quantities of both UK-2 and UK-3 complexes in the mycelium at a final concentration of 2 mM (data not shown).

A flow diagram of the isolation procedure of UK-3A, B, C, D and E is shown in Fig. 3. From the mycelium of strain 517-02 cultured in 33 liters of the producing medium, 4.7 mg of UK-3A, 1.4 mg of UK-3B, 0.6 mg of UK-3C, 1.2 mg of UK-3D and 0.1 mg of UK-3E were obtained.

Fig. 3. Isolation procedure of UK-3 complex from *Streptomyces* sp. 517-02.



Structure Elucidation

UK-3A was isolated as colorless needles and showed the molecular ion at *m/z* 484. EI-MS indicated the absence of a methoxyl group in UK-2A (M^+ *m/z* 514). As shown in Table 1, the NMR spectrum of UK-3A resembled that of UK-2A except for the disappearance of the signal based on the methoxyl group on the picolynyl residue linked to the nine-membered dilactone moiety in UK-2A. Furthermore, the chemical shifts in the ^{13}C NMR spectrum and the *J* values in the ^1H NMR spectrum assigned to the picolynyl group of UK-3A were in accord with those of the *N*-(3-hydroxypicolynyl)serine methyl ester prepared by dehydration with DCC from 3-hydroxypicolynic acid and the serine methyl ester. On the basis of the above results, the structure of UK-3A was elucidated as the demethoxyl UK-2A.

Though structures of UK-3B, C, D and E have not been elucidated yet because of their small quantity, their structures may be different in the short chain fatty acid ester group attached to the C-3 hydroxy group similar to the same structural difference in the UK-2 compounds.

Antimicrobial Activity

UK-3A did not show any growth inhibitory activity against Gram-negative and Gram-positive bacteria up to 100 μg/ml.

However, UK-3A inhibited the growth of various kinds of yeasts and filamentous fungi. The activity evaluated after incubation for 24 hours is shown in Table 2. The MICs for yeasts and fungi were 1.56~6.25 and 0.39~1.56 μg/ml, respectively. Although these MIC values were larger than those of UK-2A and antimycin A, the antimicrobial spectra of these compounds were similar. *Fusarium oxysporum* IFO 7152 was insensitive to these compounds. Furthermore, such antifungal activities of these compounds gradually decreased with prolonged incubation (data not shown).

Cytotoxic Activity

UK-3A, UK-2A and antimycin A were tested for cytotoxicity against P388, B16, KB, COLO201 and 3T3 cells. The results are shown in Table 3. Though antimycin A markedly inhibited the growth of these cells (IC_{50} ranges were 0.015~0.063 μg/ml) with the exception of the 3T3 cells, UK-3A and UK-2A showed no significant cytotoxic activity (IC_{50} ranges were 18~100 and 17~100 μg/ml, respectively).

Table 1. ^{1}H and ^{13}C NMR spectral data for UK-3A, UK-2A and *N*-(3-hydroxypicolynyl)serine methyl ester (in C_6D_6 at 40°C).

	UK-3A		UK-2A		*N*-(3-Hydroxypicolynyl)serine methyl ester	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		171.74		171.76		
2	2.91 (td, 9.9, 3.3)	52.47	2.90 (td, 9.9, 2.9)	52.45		
3	5.35 (t, 9.9)	75.47	5.35 (t, 9.9)	75.53		
4	4.97 (dq, 9.9, 6.2)	74.86	4.97 (dq, 9.9, 6.2)	74.83		
6		169.92		169.98		170.34
7	5.03 (br t)	50.53	5.03 (br)	50.60	4.82 (dt, 8.2, 3.7)	52.82
8	5.13 (br)		5.14 (br)	65.31	4.13 (dd, 11.3, 3.7)	62.93
			3.12 (br)		4.03 8dd, 11.3, 3.7)	
3-OCO-		175.03		175.06		
4-CH_3	1.10 (d, 6.2)	17.75	1.10 (d, 6.2)	17.76		
$(CH_3)_2$-*CH*-	2.25 (septet, 7.0)	34.18	2.25 (septet, 7.0)	34.20		
$(CH_3)_2$-CH-	0.98 (d, 7.0)	18.89	0.97 (d, 7.3)	18.93		
	0.96 (d, 7.0)	18.81				
-CONH-	8.55 (br d, 7.3)	168.72	8.72 (d, 7.7)		8.77 (br d, 7.6)	168.91
2′		131.28		129.56		130.97
3′-OH	12.07 (s)	158.52	12.34 (s)	140.23	11.72 (s)	157.79
4′	6.90 (dd, 8.4, 1.5)	126.94		149.29	7.29 (dd, 8.6, 1.5)	126.11
4′-OCH_3			3.14 (s)	55.36		
5′	6.56 (dd, 8.4, 4.4)	129.48	6.08 (d, 5.2)	156.18	7.34 (dd, 8.6, 4.3)	128.90
6′	6.67 (4.4, 1.5)	139.63	7.67 (d, 5.2)	109.74	8.08 (dd, 4.3, 1.5)	139.82
1″		138.48		138.52		
2″/6″	7.09 (5H, bs)	128.82	6.99 (d, 7.3)	128.82		
3″/5″		128.54	7.01 (t, 7.3)	128.56		
4″		126.00	7.02 (t, 7.3)	126.92		
Ph-CH_2-	3.12 (dd, 13.3, 11.7)	35.15	3.15 (dd, 13.4, 9.9)	34.20		
	2.74 (dd, 13.3, 3.3)		2.73 (dd, 2.9, 13.4)			

Table 2. Antimicrobial activities of UK-3A, UK-2A and antimycin A.

Tested Organism	MIC (μg/ml)		
	UK-3A	UK-2A	Antimycin A
Escherichia coli IFO 3545	>100	>100	>100
Proteus vulgaris IFO 3851	>100	>100	>100
Pseudomonas aeruginosa IFO 3080	>100	>100	>100
Bacillus subtilis IFO 3007	>100	>100	>100
Micrococcus luteus IFO 3333	>100	>100	>100
Staphylococcus aureus NCTC 8530	>100	>100	>100
Saccharomyces cerevisiae IFO 0203	1.56	0.05	0.025
Candida albicans IFO 1061	3.13	0.39	0.1
Rhodotorula rubra IFO 0001	3.13	0.78	1.56
Schizosaccharomyces pombe IFO 0342	1.56	0.1	0.025
Hansenula anomala IFO 0136	6.25	3.13	1.56
Aspergillus niger ATCC 6275	0.78	0.39	0.39
Fusarium oxysporum IFO 7152	>100	>100	>100
Mucor mucedo IFO 7684	0.78	0.025	0.00625
Neurospora sitophila DSM 1130	0.39	0.1	0.2
Penicillium chrysogenum IFO 4626	1.56	0.39	0.39
Phycomyces nitens IFO 5694	0.39	0.025	0.1
Rhizopus formosaensis IFO 4732	0.39	0.0125	>100

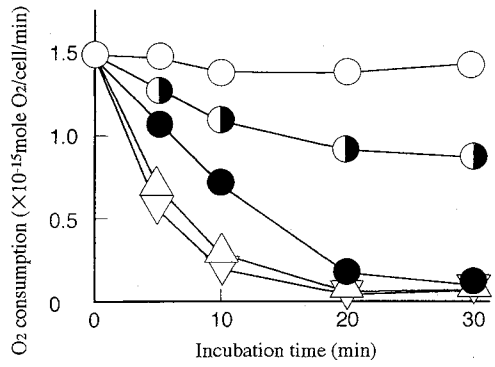
Table 3. Cytotoxic activities of UK-3A, UK-2A and antimycin A.

	IC_{50} (μg/ml)				
	P-388	B-16	KB	COLO201	3T3
UK-3A	38	18	20	45	100
UK-2A	100	100	17	35	100
Antimycin A	0.015	0.02	0.063	0.018	15

Effects on Cellular Respiration

As shown in Fig. 4, when cell suspensions of *S. cerevisiae* were incubated with UK-3A, UK-2A and antimycin A, the respiratory activity was inhibited; the incubation period resulting in a 50% inhibition by UK-3A at 2.0 μg/ml, UK-2A at 0.1 μg/ml and antimycin A at 0.1 μg/ml were 10, 4.0 and 3.5 minutes, respectively. Details of the action mode of UK-2A and UK-3A will be described in an accompanying paper.

Fig. 4. Effects of UK-3A, UK-2A, and antimycin A on O_2 consumption by *S. cerevisiae* IFO 0203 cells.



○: control, ◑: UK-3A 0.5 μg/ml, ●: UK-3A 2.0 μg/ml, △: UK-2A 0.1 μg/ml, ▽: antimycin A 0.1 μg/ml.

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