UK-2A, B, C and D, Novel Antifungal Antibiotics from *Streptomyces* sp. 517-02

I. Fermentation, Isolation, and Biological Properties

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Novel antifungal antibiotics, UK-2A, B and a mixture of C and D, were obtained from the mycelial cake of *Streptomyces* sp. 517-02. All of the UK-2 compounds were similar in structure to antimycin A. The antifungal activities of UK-2 compunds were as strong as that of antimycin A. However, the UK-2 compounds demonstrated weak cytotoxicity compared to antimycin A.

Streptomyces sp. 517-02, from which a novel cytotoxic benzoxazole UK-1 was isolated previously[1], was found to produce several antifungal antibiotics in the mycelium. We obtained the active principles, UK-2A, B and a mixture C and D (Fig. 1), from acetone extracts of the mycelium. This paper describes the fermentation, isolation and biological properties of the UK-2 compounds. Their structural elucidation will be reported in the accompanying paper[2].

Materials and Methods

Chemicals

Antimycin A was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 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 morphological, cultural and physiological characteristics, strain 517-02 seemed to be closely related to *Streptomyces morookaense*. Details of the taxonomic studies of strain 517-02 were previously reported[1].

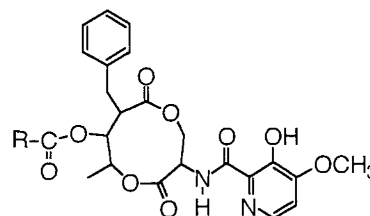
Fermentation Studies

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 48-hour incubation at 30°C under aeration at 3 liters/minute and agitation at 500 rpm, the entire cultured broth from the jar fermentor was transferred into a 600-liter tank fermentor containing 300 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). Fermentation was carried out for 48 hours at 30°C under aeration at 300 liters/minute and agitation at 250 rpm.

Fermentation Analysis

The growth of strain 517-02 was monitored by packed cell volume (PCV) measurement. The amount of UK-2 in the mycelium was determined by HPLC using a Shimadzu LC-6A pump (column, Develosil ODS-5; solvent, 60% aq CH_3CN; flow rate, 1.0 ml/minute; detection, UV at 300 nm). The sample for the HPLC assay was prepared as follows: 10 ml of the culture broth was sampled and centrifuged. The mycelium was extracted with 1 ml of acetone for 1 hour at room temperature. After centrifugation, 5 μl of the supernatant

Fig 1. Structures of UK-2A, B, C and D.



UK-2A: R = $-CH(CH_3)_2$
UK-2B: R = $-CH(CH_3)\overset{*}{=}CHCH_3$
UK-2C: R = $-CH_2CH(CH_3)_2$
UK-2D: R = $-CH(CH_3)CH_2CH_3$
* *trans*

was injected on to HPLC column.

Biological Activities

In the *in vitro* antimicrobial assay, each UK-2 compound was first dissolved in *N,N'*-dimethylformamide. The MIC's of the UK-2 compounds were measured by 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.

In the cytotoxic assay using mouse melanoma B16, mouse leukemia P388, mouse fibroblast 3T3, human colon adenocarcinoma COLO201 and human oral epidermoid carcinoma KB cells, each UK-2 compound 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 atomsphere 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-2 compounds for 72 hours, the cytotoxic effects were determined by the MTT colorimetric method[3]. The concentration that inhibits 50% of control growth (ED_{50}) was calculated to assess the potency of the inhibitory effect of the drug.

Growth Studies on *Saccharomyces cerevisiae*

A 10-hour culture of *S. cerevisiae* IFO 0203 was diluted with semisynthetic medium[4] (containing, per liter, 3 g Bacto-yeast extract, 1 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.5 g NaCl and 0.6 g $MgCl_2 \cdot 6H_2O$) supplemented with 2% lactate and 0.05% glucose (the pH of the medium was adjusted to 5.5 with NaOH) to give approximately 10^5 cell/ml. A 10-ml portion of this cell suspension was dispensed into each L-tube. After 2 hours of shaking cultivation, known concentrations of UK-2A and antimycin A were added to these tubes, which were then shaken again. Portions of the culture were withdrawn at intervals to measure the colony forming units (CFU). CFUs were counted by plating dilutions of the culture in saline on malt agar plates and incubating at 25°C for 24 hours.

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 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 cell/ml and incubated with shaking at 25°C. After 10 minutes, each UK-2A and antimycin A was added and, at 5-minute intervals, portions of the incubation mixture were withdrawn. The respiratory activity of yeast cells in the mixture was measured polarographically at 25°C with a Yanagimoto PO-100A oxygen electrode[5,6].

Results

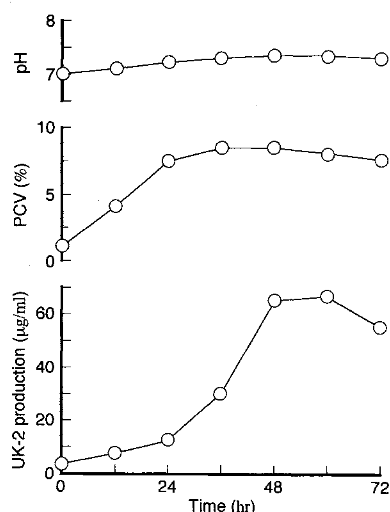
Fermentation

A typical time course for the production of the UK-2 complex is shown in Fig. 2. The growth of strain 517-02 gradually increased in the first 36 hours, and the UK-2 complex production started at approximately 24 hours, reaching its maximum at 48 hours after incubation.

Isolation

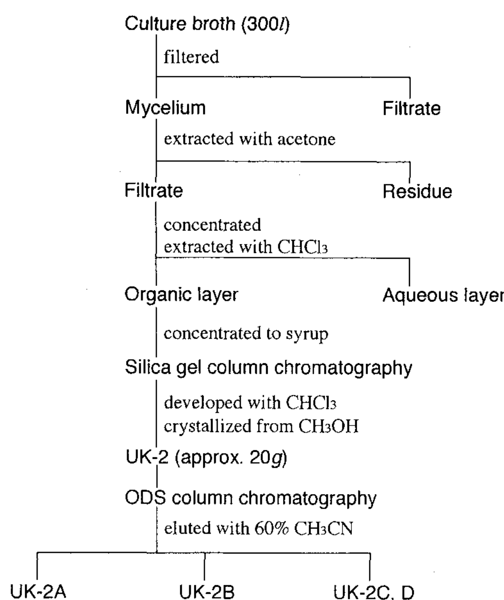
A flow diagram of the isolation procedure of UK-2 compounds is shown in Fig. 3. The culture broth (300 liters) thus obtained was filtered with the aid of diatomaceous earth. The mycelial cake was extracted with acetone (110 liters) and filtered. The filtrate was concentrated *in vacuo* to give an aqueous 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 silica gel column (Wakogel C-200). Absorbed material was developed with chloroform. After the elution of UK-1[1], fractions containing antifungal activity against *S. cerevisiae* were collected and concentrated *in vacuo*. The crude UK-2 complex was dissolved in methanol and allowed to stand for 1～2 days at 4°C to yield colorless needles (approximately 20 g of the UK-2 complex). Then 55 mg of the complex was dissolved in a small amount of 60% aqueous CH_3CN, applied on a preparative HPLC (Develosil ODS HG-5) and developed with 60% aqueous CH_3CN. The HPLC profile of UK-2A, B, C and D is shown in Fig. 4. The

Fig. 2. Time course of UK-2 complex production.



portions corresponding to A, B and a mixture of C and D were collected and lyophilized to obtain 34.9 mg of A, 2.1 mg of B, and 13.6 mg of C and D. The details of their structural elucidation will be reported in the accompanying paper[2].

Fig. 3. Isolation procedure of UK-2A, B, C and D.

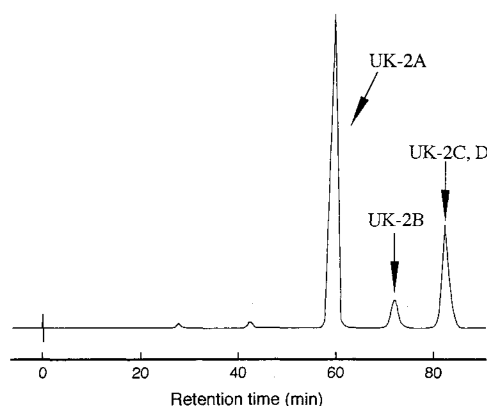


Antimicrobial Activity

UK-2A, B, a mixture of C and D, and antimycin A (a structural relative to UK-2 compounds) did not show any growth inhibitory activity against Gram-negative and Gram-positive bacteria up to 100 μg/ml.

However, these compounds strongly inhibited the growth of various kinds of yeasts and filamentous fungi. The activities evaluated after incubation for 24 hours are shown in Table 1. The MIC of each test compound was generally the same as that of another (MIC ranges were approximately 0.78 ~ 0.0125 μg/ml). Against *Torulaspora*

Fig. 4. HPLC separation of UK-2 complex.



Column, develosil ODS HG-5 i.d.20 × 250 mm; mobile phase, 60% acetonitrile; flow rate, 5 ml/minute; detection, 300 nm.

Table 1. Antimicrobial activities of UK-2A, B, a mixture of C and D, and antimycin A.

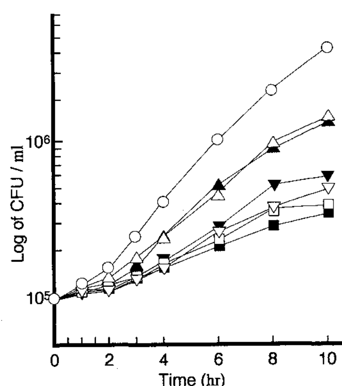
Tested Organism	MIC (μg/ml)			
	UK-2A	UK-2B	UK-2CD[a]	Antimycin A
Escherichia coli IFO 3545	>100	>100	>100	>100
Proteus vulgaris IFO 3851	>100	>100	>100	>100
Pseudomonas aeruginosa IFO 3080	>100	>100	>100	>100
Bacillus subtilis IFO 3007	>100	>100	>100	>100
Micrococcus luteus IFO 3333	>100	>100	>100	>100
Staphylococcus aureus NCTC 8530	>100	>100	>100	>100
Saccharomyces cerevisiae IFO 0203	0.05	0.1	0.05	0.025
Candida albicans IFO 1061	0.39	0.78	0.39	0.1
Rhodotorula rubra IFO 0001	0.78	0.78	1.56	1.56
Schizosaccharomyces pombe IFO 0342	0.1	0.1	0.025	0.025
Hansenula anomala IFO 0136	3.13	0.78	0.78	1.56
Torulaspora delbrueckii DSM 70504	1.56	3.13	1.56	0.05
Aspergillus fumigatus IFO 5840	0.0125	0.39	0.2	25
Aspergillus niger ATCC 6275	0.39	1.56	0.1	0.39
Fusarium oxysporum IFO 7152	>100	>100	>100	>100
Mucor javanicus IFO 4569	>100	>100	>100	>100
Mucor mucedo IFO 7684	0.025	0.0125	0.0125	0.00625
Neurospora sitophila DSM 1130	0.1	0.1	0.1	0.2
Penicillium chrysogenum IFO 4626	0.39	0.39	0.39	0.39
Phycomyces nitens IFO 5694	0.025	0.1	0.1	0.1
Rhizopus formosaensis IFO 4732	0.0125	0.0125	0.0125	>100
Sclerotinia sclerotiorum IFO 5292	0.05	0.1	0.025	0.05
Thamnidium elegans IFO 6152	0.00156	0.00156	0.00156	0.00313
Trichophyton mentagrophytes IFO 6124	0.1	0.1	0.1	0.1

[a] A mixture of UK-2C and D.

Table 2. Cytotoxic activities of UK-2A, B and CD.

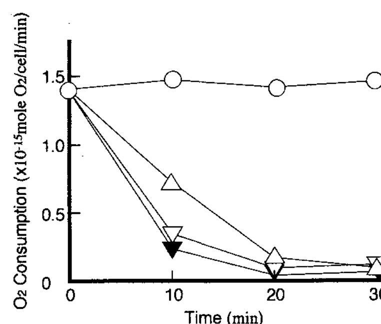
	ED_{50} (μg/ml)				
	P-388	B-16	KB	COLO201	3T3
UK-2A	100	100	17	35	100
UK-2B	28	30	20	70	95
UK-2CD	35	60	10	30	95
Antimycin A	0.015	0.02	0.063	0.018	15

Fig. 5. Effects of UK-2A and antimycin A on growth of *S. cerevisiae* IFO 0203 measured in terms of cell viability.



UK-2A and antimycin A were added at 0-hour at the following concentrations (μg/ml) UK-2A: ○, 0; △, 0.01; ▽, 0.1; □, 1.0. Antimycin A: ▲, 0.01; ▼, 0.1; ■, 1.0.

Fig. 6. Effects of UK-2A and antimycin A on O_2 consumption by *S. cerevisiae* IFO 0203 cells.



UK-2A was added at the following concentration (μg/ml): ○, 0; △, 0.01; ▽, 0.1. Antimycin A was added at 0.1 μg/ml, ▼.

delbrueckii DSM 70504, UK-2 compounds were less active than antimycin A. In contrast, although UK-2 compounds inhibited the growth of *Rhizopus formosaensis* IFO 4732 at 0.0125 μg/ml, antimycin A showed no effect on it up to 100 μg/ml. Interestingly, such antifungal activities of these compounds decreased gradually with prolonged incubation.

Cytotoxic Activity

UK-2A, B, a mixture of C and D, and antimycin A were tested for cytotoxicity against P388, B16, KB, COLO201 and 3T3 cells. The results are shown in Table 2. Though antimycin A markedly inhibited the growth of these cells (ED_{50} ranges were 0.015 ~ 0.063 μg/ml) with the exception of 3T3 cells, UK-2 compounds showed slight inhibition (ED_{50} ranges were 10 ~ 100 μg/ml). Because of their poor solubility in water, the culture media became turbid after the addition of UK-2 compounds at final concentrations of more than 25 μg/ml.

Effects on Growth of *S. cerevisiae*

The growth inhibitory effects of UK-2A and antimycin A on *S. cerevisiae* are shown in Fig. 5. When exponentially growing cells were exposed to UK-2A and antimycin A at 0.01 μg/ml, the growth rate measured in terms of turbidity was reduced. However, even at higher concentrations of UK-2A and antimycin A, drastic reduction of the growth rate was not observed. The actions of UK-2A and antimycin A seemed to be fungistatic but not fungicidal.

Effects on Cellular Respiration

As shown in Fig. 6, when cell suspensions of *S. cerevisiae* were incubated with UK-2A or antimycin A, the respiratory activity quickly decreased; incubation periods resulting in 50% inhibition by UK-2A and antimycin A at 0.1 μg/ml were 4.0 and 3.5 minutes, respectively.

Discussion

Many antimycin group antibiotics produced by *Streptomyces* sp. have been reported, and they possess saturated alkane side chains. UK-2 compounds are the first antimycin type antibiotics whose side chain contains a benzyl group. Moreover, they are the first reported antimycins possessing a pyridyl group.

UK-2 compounds and antimycin A markedly inhibited the growth of various yeasts and filamentous fungi at similar low concentrations. However, their effects were not durable and seemed to be fungistatic. UK-2A and antimycin A quickly inhibited yeast cell respiration. The main site of the antifungal action of UK-2 compounds appears to be the mitochondrial electron transport system, the same as that of antimycin A.

Antimycin A strongly inhibited the growth of P388, B16, KB and COLO201 cells, while UK-2 compounds weakly inhibited the growth of these cells. It is still uncertain why the difference in cytotoxic activities exists between UK-2 compounds and antimycin A.

The recent studies[7,8] on antimycin A demonstrated

that the natural 9-membered ring is not essential for the activity, but this moiety may regulate the tight fitting of the salicylic acid moiety into the cavity in the cytochrome bc_1 complex by fixing the entire molecular configuration and enhancing the hydrophobicity of the molecule. As for antimycin A, the presence of a 3-formylamino group in its proper conformation is essential for tight binding. However, the pyridine ring in UK-2 compounds is substituted for the salicylic acid moiety in antimycin A, and the 3-methoxy group for the 3-formylamino group. These substitutions might bring about the decrease in cytotoxicity.

The origin of the pyridyl group is unknown. Studies on the biosynthetic pathway of UK-2 are now in progress.

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