# THE KAPURIMYCINS, NEW ANTITUMOR ANTIBIOTICS PRODUCED BY *STREPTOMYCES*

## PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

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As a result of screening for antitumor agents from actinomycetes, the kepurimycins were isolated from *Streptomyces* sp. DO-115. The antibiotics were produced in a fermentation medium supplemented with high porous polymer resin which adsorbs antibiotics and results in an increase of titer. The active complex was isolated from the polymer resin by a solvent extraction procedure and was separated by column chromatography, into two minor and one major component, named A1, A2 and A3. The kepurimycins were active against bacteria, particularly Gram-positive organisms, and were cytotoxic to HeLa $S_3$ human cerivical cancer cells and T24 human bladder carcinoma cells *in vitro*. Among the individual components of the kapurimycins, kapurimycin A3 exhibited the strongest antibacterial and cytotoxic activities. It showed a potent antitumor activity against murine leukemia P388 *in vivo*.

In the course of our search for novel antitumor antibiotics produced by microorganisms, a culture designated DO-115 was isolated from a soil sample and was found to produce new antitumor compounds, kapurimycins. These compounds exhibited significant antimicrobial activity against Gram-positive bacteria and showed antitumor activity. The antibiotics were isolated and separated into three related components named A1, A2 and A3. As described in an accompanying paper[1], the kapurimycins constitute a new class of polycyclic microbial metabolite possessing the tetrahydroanthrapyrone skeleton, and the individual components of the kapurimycins differ from one another in the side chains at the pyrone ring of the molecule.

This paper describes the taxonomy of the producing strain, fermentation, isolation and biological activities of the kapurimycins. Physico-chemical properties and structure determination of these compounds are reported in the following paper[1].

## Materials and Methods

### Microorganism

Strain DO-115 was isolated from a soil collected at Kanazawa-city, Ishikawa Prefecture, Japan.

### Taxonomic Studies

Growth characteristics and carbohydrate utilization were determined by the methods of the International Streptomyces Project (ISP)[2]. Color codes were assigned to the substrate and aerial mass pigments according to the Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America,

Chicago). The spores and mycelia of the strain were observed with a scanning electron microscope (model S-570, Hitachi Co., Ltd.).

Diaminopimelic acid of the cell wall was analyzed on the hydrolysate of cultures grown in a medium (glucose 10 g, starch 10 g, beef extract 3 g, yeast extract 5 g, $CaCO_3$ 2 g per liter of tap water, pH 7.0) for 48 hours at 28°C.

Fermentation

The media for seed and fermentation cultures are: DS1 medium; dextrin 20 g, glucose 10 g, peptone 10 g, corn steep liquor 5 g, yeast extract 1 g, $KH_2PO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 1 g per liter (pH 7.2 prior to sterilization), DF2 medium; soluble starch 50 g, dry yeast 15 g, $KH_2PO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 5 g and antifoam agents LG109 (Asahi Denka Kogyo) 0.3 ml and KM70 (Shinetsu Kagaku) 0.3 ml per liter (pH 7.0 prior to sterilization). A dry mixture of trace element was prepared as described by HUNTER[3].

Culture growth was evaluated by centrifuging untreated fermentation broth in 10 ml conical tubes at $1,200 \times g$ for 10 minutes. The peaked cell volume was reported as % of total broth volume.

Antibiotic production was monitored by the paper-disc method on nutrient agar using *Bacillus subtilis* No. 10707 as the test organism.

Antimicrobial Activity

The *in vitro* antimicrobial activity of kapurimycins 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.

Antitumor Activity

HeLa $S_3$ human cerivical cancer cells were cultured in modified EAGLE's minimum essential medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal bovine serum (FBS). T24 human bladder carcinoma cells were cultured in F10 medium (Gibco Laboratories) containing 10% FBS, 100 units/ml benzylpenicillin and 100 $\mu$g/ml streptomycin. Cytotoxic activity of kapurimycins was determined as described previously[4]. *In vivo* antitumor activity was measured and calculated as follows. P388 cells ($10^6$) were transplanted ip into $CD2F_1$ mice and ip administration of drugs was started the day after tumor transplantation. Antitumor efficacy was expressed as a percentage of the mean survival time (MST) of the control group. Sarcoma 180 ($5 \times 10^6$ cells/mouse) was inoculated sc at the axillary region in *dd*Y mice. Drugs were administrated iv starting the day after tumor inoculation and the tumor volume was measured on day 10. The antitumor activity was evaluated by the T/C (%), where T and C represents the mean size of tumor of the treated animal and that of control animal, respectively[5].

## Results and Discussion

### Taxonomy of the Producing Strain

The appearance of strain DO-115 on nine solid media is presented in Table 1. The vegetative mycelia grew on both synthetic and complex media. The aerial mycelium was gray or white colored and well developed, branched but not fragmented. It bore chains of 10 to 30 or more spores, which were flexous or loop-like, belonging to type *Rectiflexibiles* or *Retinaculiaperti*. Scanning electron micrographs indicated that the spore was ovalin shape, $0.5 \times 0.7$ $\mu$m in size, and spiny-surfaced (Fig. 1). The substrate mycelium was branched but not fragmented. Melanoid pigments were formed in oatmeal agar and peptone-yeast extract-iron agar. Soluble pigments of pale yellow were formed in servaral agar media as shown in Table 1. Analysis of the whole cell hydrolysate by TLC revealed the presence of the L,L-isomer of diaminopimelic acid, indicating that the cell wall belongs to type I.

In Table 2, the physiological properties of strain DO-115 are presented. The strain grew at temperature ranging from 16 to 37°C with the optimum range from 28 to 32°C, peptonized milk and decomposed

Table 1. Cultural characteristics of strain DO-115.

| | |
|---|---|
| Sucrose-nitrate agar | G: Poor |
| | AM: Scant, covert gray (2fe) |
| | SM: White |
| | P: None |
| Glucose-asparagine agar | G: Moderate |
| | AM: Abundant, dark covert gray (2ih) |
| | SM: Golden brown (3pi) to light brown (3lg) |
| | P: Pale yellow |
| Glycerol-asparagine agar | G: Moderate |
| | AM: Scant, white |
| | SM: Bisque (3ec) |
| | P: None |
| Starch agar | G: Moderate |
| | AM: Scant, white |
| | SM: Clove brown (3ni) |
| | P: Pale yellow |
| Tyrosine agar | G: Poor |
| | AM: None |
| | SM: Camel (3ie) |
| | P: Pale yellow |
| Nutrient agar | G: Moderate |
| | AM: Fair, griege (1fe) |
| | SM: Light brown (3lg) |
| | P: Pale yellow |
| Yeast extract-malt extract agar | G: Poor |
| | AM: None |
| | SM: Light mustard tan (2ie) |
| | P: Pale yellow |
| Oatmeal agar | G: Good |
| | AM: Scant, white |
| | SM: Clove brown (3pl) |
| | P: Brown |
| Peptone-yeast extract-iron agar | G: Poor |
| | AM: None |
| | SM: White |
| | P: Brown |

Abbreviations: G, degree of growth; AM, formation of aerial mycelium and its color; SM, color of substrate mycelium; P, formation of soluble pigment and its color.

Fig. 1. Scanning electron micrographs of strain DO-115.

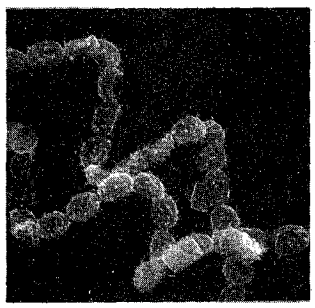


Table 2. Physiological properties of strain DO-115.

| | |
|---|---|
| Liquefaction of gelatin | Negative |
| Coagulation of milk | Negative |
| Peptonization of milk | Positive |
| Decomposition of cellulose | Positive |
| Hydrolysis of starch | Positive |
| Formation of melanoid pigment | Positive |
| Optimum growth temperature | 28~32°C |
| Optimum growth pH | 6.8~7.5 |

Table 3. Carbohydrate utilization by strain DO-115.

| | |
|---|---|
| L-Arabinose | − |
| D-Xylose | − |
| D-Glucose | + |
| D-Fructose | − |
| D-Mannitol | − |
| Sucrose | − |
| *m*-Inositol | − |
| Raffinose | − |
| L-Rhamnose | − |

+: Utilized, −: not utilized.

cellulose. The strain grew well on glucose, glycerol and starch. Growth was poor on the other carbon sources tested (Tables 2 and 3).

From the morphological, cultural and physiological characteristics observed, strain DO-115 is considered to belong to the genus *Streptomyces* Waksman and Henrici 1943[6~10]. The strain was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the name of *Streptomyces* sp. DO-115 under the accession No. FERM BP-2408.

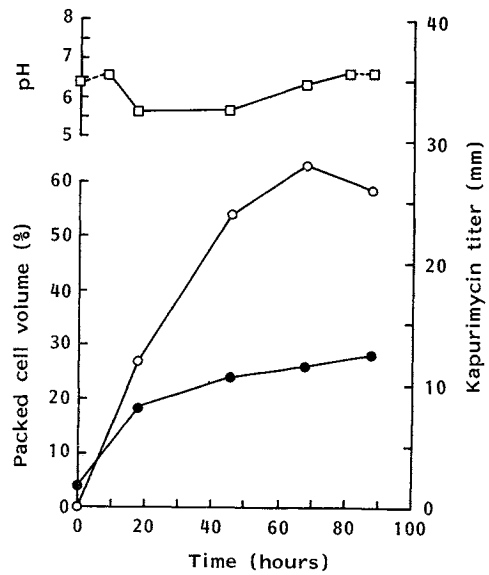
## Fermentation

In the early experiments for optimum production of kapurimycins, DF2 medium (see Materials and

Methods) was selected as a basal medium to increase titer in liquid agitated fermentation. As the additives in the culture medium for the higher production of kapurimycins, the mixture of trace metals[3] and yeast extract were found to be effective. Based on these results, DF2TY medium was found to support higher titers. DF2TY medium; soluble starch 50 g, dry yeast 15 g, yeast extract 5 g, dry mixture of trace element[3] 0.5 g, $KH_2PO_4$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 5 g and antifoam agents LG109 0.3 ml and KM70 0.3 ml per liter (pH 7.0, prior to sterilization). The examination of growth in shake flasks indicates that the addition of high porous polymer resin Diaion HP-20 in the production medium, which adsorbs kapurimycins, results in a significant increase in the total accumulation of the kapurimycins. Therefore, Diaion HP-20 resin was added at 24 hours of fermentation. Fig. 2 shows a typical time course for the production of kapurimycin in a 30-liter jar fermentation under optimum conditions. The kapurimycins were found to be unstable in culture broth above pH 7; pH of the medium was controlled under 6.5 with 4 N $H_2SO_4$ aqueous solution. The antibiotic production increased steadily for 3 days concomitantly with a gradual increase of mycelium volume.

Fig. 2. Time course of kapurimycins fermentation in a 30-liter jar fermenter.

pH (□), packed cell volume (●) and activity (○) were included. pH of the medium was controlled under 6.5 with 4 N $H_2SO_4$ aqueous solution (---).




The inoculum for antibiotic production was prepared in DS1 medium. A 300-ml Erlenmeyer flask containing 50 ml of the above medium was inoculated with a loopful spores of strain grown on agar slants and was incubated at 28°C on a rotary shaker for 48 hours. Ten-ml of the seed culture was transferred to a 2-liter Erlenmeyer flask containing the same medium. Following 24 hours of incubation at 28°C, the second stage seed culture (0.9 liter) was used as the inoculum to initiate the fermentation in 30-liter jar fermenters bathed with 15 liters of DF2TY medium. The fermentation was carried out at 28°C with 1.8 liters of air per minute and agitation at 300 rpm. Diaion HP-20 resin (10%) was added at 24 hours after inoculation.

## Isolation

The kapurimycins were isolated from 60 liters of culture broth by the following procedure. The filtered mycelial cake containing Diaion HP-20 was suspended in acetone (0.1% AcOH) and stirred for 1 hour to elute kapurimycins. Then mycelium and Diaion HP-20 were removed by filtration and the filtrate containing kapurimycins was concentrated. This concentrate was diluted with an equal volume of MeOH and was applied to a column of Diaion HP-20. The column was washed with 50% aqueous MeOH (0.1% AcOH), 20% aqueous acetone (0.1% AcOH) and kapurimycins were eluted with 80% aqueous acetone (0.1% AcOH). The fractions containing kapurimycins were concentrated, diluted with an equal volume of MeOH and were applied to a column of Diaion HP-20 SS. The column was washed with 45% aqueous acetone (0.1% AcOH) and kapurimycins were eluted with 50% aqueous acetone (0.1% AcOH). The active eluate was concentrated, extracted with EtOAc at pH 3 and concentrated to dryness. Further purification was effected by silica gel chromatography using toluene - acetone (2 : 1) as eluents. Three main fractions containing kapurimycin A1, A2 or A3 were obtained. Each of the active fractions were concentrated to dryness, redissolved in MeOH and further

Table 4. Antimicrobial activity of the kapurimycins.

| Organisms | MIC (μg/ml) | | |
|---|---|---|---|
| | A1 | A2 | A3 |
| *Staphylococcus aureus* ATCC 6538P | 10 | 2.6 | 0.3 |
| *Enterococcus faecium* ATCC 10541 | 21 | 5.2 | 0.3 |
| *Bacillus subtilis* No. 10707 | 21 | 21 | 1.3 |
| *Klebsiella pneumoniae* ATCC 10031 | >100 | 21 | 5.2 |
| *Escherichia coli* ATCC 26 | >100 | >100 | 21 |
| *Pseudomonas aeruginosa* Bin H No. 1 | >100 | >100 | 83 |
| *Salmonella typhi* ATCC 9992 | >100 | >100 | >100 |
| *Proteus vulgaris* ATCC 6897 | >100 | >100 | 83 |
| *Shigella sonnei* ATCC 9290 | >100 | >100 | 42 |
| *Candida albicans* ATCC 10231 | >100 | >100 | >100 |

Table 5. Cytotoxic activity of the kapurimycins.

| Compound | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|
| | HeLa $S_3$ | | T-24 | |
| | 1 hour | 72 hours | 1 hour | 72 hours |
| Kapurimycin A1 | 4.66 | 0.43 | 4.23 | 0.30 |
| Kapurimycin A2 | 3.85 | 0.77 | 6.90 | 0.94 |
| Kapurimycin A3 | 0.61 | 0.28 | 1.34 | 0.85 |
| Doxorubicin | 1.90 | 0.05 | 0.93 | 0.12 |

purified by Sephadex LH-20 column chromatography in MeOH to afford pure compounds. Kapurimycins A1 (20 mg), A2 (20 mg) and A3 (700 mg) were obtained as yellow powders.

## Biological Activities

### Antimicrobial Activity

The antimicrobial activity of the kapurimycins is shown in Table 4. The kapurimycins exhibited antimicrobial activity against Gram-positive bacteria. The antibacterial activity of kapurimycin A3 was above one order greater than that of kapurimycins

Table 6. Antitumor activity of kapurimycin A3 against P388 leukemia.

| Compounds | Dosage (mg/kg) | MST[a] | ILS (%) |
|---|---|---|---|
| Kapurimycin A3 | 4 | 5.2±0.4 | −48 |
| | 2 | 10.8±2.2 | 8 |
| | 1 | 13.2±1.6 | 32 |
| | 0.5 | 13.0±1.2 | 30 |
| | 0.25 | 11.0±1.4 | 10 |
| | 0.13 | 11.4±1.1 | 14 |
| | 0.063 | 10.6±1.3 | 6 |
| Mitomycin C | 4 | 18.0±3.0 | 80 |
| | 2 | 16.4±1.8 | 64 |
| Control | | 10.0±0.9 | 0 |

[a] MST of deceased mice. Mean±SD.

Table 7. Antitumor activity of kapurimycin A3 on murine sarcoma 180.

| Compounds | Schedule | Dosage (mg/kg) | T/C | WBC[a] | BW (g) |
|---|---|---|---|---|---|
| Kapurimycin A3 | Day 1 | 0.63 | 0.93 | 58.2 | +5.8 |
| | | 1.25 | 0.76 | 62.5 | +5.4 |
| | | 2.5 | 0.68 | 55.0 | +5.8 |
| | | 5.0 | 0.58 | 58.8 | +5.2 |
| | | 10 | Toxic | | |
| Mitomycin C | Day 1 | 6 | 0.32 | 12.8 | +1.2 |
| Control[b] | | | 1.00 | 79.0 | +5.8 |
| Kapurimycin A3 | Day 1~5 | 0.63 | 0.92 | 50.4 | +7.0 |
| | | 1.25 | 0.81 | 60.4 | +3.5 |
| | | 2.5 | 0.61 | 40.0 | +3.7 |
| | | 5 | 0.28 (1/5)[c] | 27.3 | −1.5 |
| | | 10 | Toxic | | |
| Mitomycin C | Day 1 | 6 | 0.28 | 17.0 | +0.5 |
| Control[d] | | | 1.00 | 83.8 | +7.2 |

Sarcoma 180 cells ($5\times10^6$/mouse) were inoculated sc on day 0. Drugs were administrated iv on each day.

[a] Number of white blood cells (WBC) in peripheral blood ($\times10^2/mm^3$).

[b] Mean±SD of tumor volume was 1,831.7±214.1 $mm^3$.

[c] Number of toxic mouse death.

[d] Mean±SD of tumor volume was 1,787.9±137.9 $mm^3$.

A1 and A2. Kapurimycin A3 was also active against the Gram-negative bacterium, *Klebsiella pneumoniae* ATCC 10031 with an MIC value in the $\mu$g/ml range.

### Cytotoxic Activity

Kapurimycins were cytotoxic at the $\mu$M range against HeLa $S_3$ and T24 cells *in vitro* (Table 5). When cells were incubated with antibiotics for 1 hour, kapurimycin A3 inhibited cell growth better than kapurimycins A1 and A2. When cells were incubated with antibiotics for 72 hours, however, the $IC_{50}$ of each antibiotic was approximately the same.

### Antitumor Activity

Kapurimycin A3, which is the major component, showed the strongest cytotoxicity *in vitro* and was evaluated in murine tumor models *in vivo*. $LD_{50}$ of kapurimycin A3 was 1.8 mg/kg by single intraperitoneal administration. As shown in Tables 6 and 7, kapurimycin A3 showed antitumor activity against P388; ILS 32% at a single dose of 1 mg/kg (ip). Kapurimycin A3 at a single dose of 5 mg/kg induced the marginal regression of sarcoma 180 (T/C 0.58). Further studies on the antitumor activity of kapurimycin A3 are in progress.

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