Louisianins A, B, C and D: Non-steroidal Growth Inhibitors of Testosterone-responsive SC 115 Cells

I. Taxonomy, Fermentation, Isolation and Biological Characteristics

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In the course of screening for non-steroidal growth inhibitors of testosterone-responsive Shionogi carcinoma 115 cells, louisianins A, B, C and D, were isolated from fermentation broth of *Streptomyces* sp. WK-4028. Louisianin A remarkably inhibited the growth of SC 115 cells in the presence of 10^{-7} M testosterone at an IC_{50} value of 0.6 μg/ml, whereas, no inhibition was observed on the other cell lines. Furthermore, inhibitory activity of testosterone 5α-reductase and antimicrobial activity have not been observed at a concentration of 50 μg/ml and 1,000 μg/ml, respectively.

Androgens play a key role in the differentiation and maintenance of the mammalian prostate. Several steroidal antiandrogens have been used as hormonal therapy for benign prostatic hyperplasia and prostatic cancer, but new types of drugs for prostatic cancer must be developed to minimize side effects and improve efficacy.

In a continuing search for non-steroidal antiandrogens of microbial origin, louisianins A (**1**), B (**2**), C (**3**) and D (**4**) were isolated from the cultured broth of *Streptomyces* sp. WK-4028, which had been isolated from a soil sample collected in the state of Louisiana, U.S.A. In this paper, we describe the taxonomy of the producing strain, fermentation, isolation and biological properties of louisianins.

Materials and Methods

General Experimental Procedures

Kieselgel 60 (70~230 mesh, 230~400 mesh, Merck) were used for column chromatography and DC-Alufolien Kieselgel 60 F_{254} (Merck) was used for TLC analysis. Analytical HPLC was carried out with CH_3CN-H_2O (40:60) using a Senshu Pak Pegasil ODS (5 μm, i.d. 4.6×250 mm) column employing a UV monitoring system (210 nm) at a flow rate of 0.8 ml/minute. Preparative HPLC was performed using a Senshu Pak Pegasil ODS (5 μm, i.d. 20×250 mm) column with a solvent system of CH_3CN-H_2O (40:60) at 7 ml/minute.

Taxonomic Studies

The International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[1] and media of WAKSMAN[2] were used to investigate the cultural and physiological characteristics. Cultures were routinely observed after 2-week incubation at 27°C. Color names and hue numbers were determined according to the Color Harmony Manual[3]. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% carbon at 27°C[4]. Morphological properties were observed with a scanning electron microscope (Hitachi, model S-430). The type of diaminopimelic acid (DAP) isomers was determined by the method of BECKER *et al.*[5].

Inhibitory Effect on Mammalian Cell Growth

Testosterone-responsive Shionogi carcinoma 115 (SC 115) cells were kindly supplied by Shionogi Pharmaceutical Co. Ltd., and maintained in monolayer cultures in GIT medium supplemented with 5% fetal calf serum and 10^{-7} M testosterone. HeLa human carcinoma cells, B16 mouse melanoma cells and L929 mouse fibroblast cells were maintained in RPMI 1640 medium containing 10% fetal calf serum.

For the experiment, stock culture of SC 115 cells was suspended in GIT medium containing 2% dextran-coated charcoal treated-5% fetal calf serum (DCC-serum) and 10^{-7} M testosterone at 2×10^4 cells/ml. The cell suspension (200 μl) was plated in a 96-well culture plates and incubated at 37°C in 5% CO_2-95% air atmosphere. After one-day of incubation, medium in each well was replaced with fresh medium containing 10^{-7} M testosterone and a specific concentration of the

test materials. Then, after 6-days of incubation, cells were counted by a hemocytometer.

The other three cell lines, B16 melanoma, HeLa carcinoma and L929 fibroblast cells, were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum at $2\sim4\times10^4$/ml. Cell suspension (200 μl) was plated in a 96-well microplate and incubated at 37°C in 5% CO_2-95% air atmosphere. After one day of incubation, compound **1**, an active component, was added to the culture, it was reincubated for 3 days and the cells were counted by a hemocytometer.

Testosterone 5α-Reductase Assay

The assay mixture consisting of 30 μl of 0.25 M sucrose-0.1 M HEPES buffer (pH 7.4), 10 μl of 50 mM NADPH, 10 μl of 0.75 nmol [^{14}C]-testosterone, 30 μl of the test sample, and 20 μl of the enzyme solution prepared from the prostate of Donryu male rats[6] was incubated for 2 hours at 37°C. *n*-Hexane (400 μl) was added, and the mixture was shaken. The *n*-hexane layer (20 μl) was applied to 0.25 mm-precoated Silica gel plates (Merck), and the plates were developed with a solvent system of cyclohexane-ethyl acetate (1 : 1). The radioactive areas of [^{14}C]-testosterone and [^{14}C]-5α-dihydrotestosterone were determined using an AMBIS image analyzer.

Antimicrobial Activity Tests

The antimicrobial spectra of the test materials were determined using 6 mm paper disks (Toyo Seisakusho Co., Ltd.). Test organisms were as follows, bacteria; *Staphylococcus aureus* KB 210 (ATCC 6538p), *Micrococcus luteus* KB40 (ATCC 9341), *Bacillus subtilis* KB27 (PCI 219), *Mycobacterium smegmatis* KB 42 (ATCC 607), *Escherichia coli* KB 8 (NIHJ), *Escherichia coli* KB176 (NIHJ JC-2), *Pseudomonas aeruginosa* KB 105 (PCI 602), *Xanthomonas oryzae* KB 88, *Bacteroides fragilis* KB169 (ATCC 23745), *Acholeplasma laidlawii* PG 8, fungi; *Aspergillus niger* KF 103 (ATCC 6275), *Piricularia oryzae* KF 180, *Mucor racemosus* KF 223, yeast; *Candida albicans* KF 1 and *Saccharomyces sake* KF 26. Bacteria were grown on Mueller-Hinton agar medium (Difco) and fungi or yeast were grown on potato-broth agar medium. Antimicrobial activity was observed after 24 hours of incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Results and Discussion

Taxonomy of the Producing Strain WK-4028

The vegetative mycelia grew abundantly on yeast extract-malt extract agar, inorganic salts-starch agar and other agar media, and did not show fragmentation into coccoid forms or bacillary elements. The aerial mycelia grew abundantly on yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar, glycerol-asparagine agar and glucose-asparagine agar. The spore chains were *Spirales* type and each had more than 20 spores per chain. The spores were cylindrical in shape, 0.9×0.7 μm in size and had a smooth surface (Fig. 1). Whirls, sclerotic granules, sporangia and flagellate spores were not observed. The isomer of DAP in whole-cell hydrolysates of strain WK-4028 was determined to be the LL-form.

The cultural characteristics, the physiological properties and the utilization of carbon sources are shown in Tables 1, 2 and 3, respectively. The color of vegetative mycelia was yellow to brown. The aerial mass color was gray on various media. Melanoid pigment and other soluble pigment were not produced. The strain utilized D-glucose, D-xylose, D-mannitol and *i*-inositol well, but did not utilize raffinose, melibiose and sucrose.

Based on the taxonomic properties described above, strain WK-4028 is considered to belong to the genus *Streptomyces*[7]. The strain was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. WK-4028 and the accession No. is FERM P-14742.

Fermentation and Isolation of the Active Components

A stock culture of the producing organism was inoculated into a test tube (i.d. 2×20 cm) containing 10 ml of seed medium consisting of 2% glucose, 0.5% peptone, 0.3% dry yeast, 0.5% meat extract, 0.5% NaCl and 0.3% $CaCO_3$ (pH 7.0 before sterilization). The tube was incubated at 27°C for 96 hours on a reciprocal shaker. Then, 2 ml portions of the growth were transferred to 500-ml Erlenmeyer flasks containing 100 ml of the seed medium. The flasks were incubated at

Fig. 1. Scanning electron micrograph of spore chains of *Streptomyces* sp. WK-4028 grown on glycerol-asparagine agar for 14 days.

Bar represents 1.0 μm.

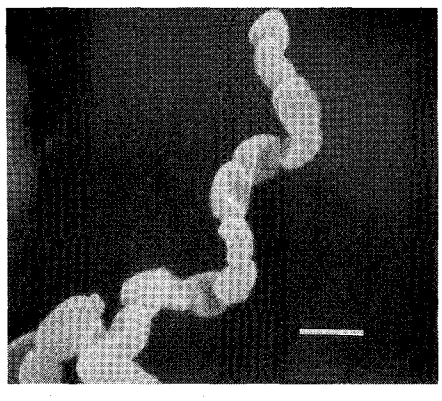


Table 1. Cultural characteristics of strain WK-4028.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract-malt extract agar [a]	G: Good, adobe brown (3lg) R: Clove brown (3pl) AM: Abundant, natural (2dc) SP: None	Tyrosine agar [a]	G: Moderate, pearl pink (3ca) R: Beige (3ge) AM: Moderate, ashes (5fe) SP: None
Oatmeal agar [a]	G: Good, bamboo (2gc) R: Adobe brown (3lg) AM: Abundant, natural (3dc) SP: None	Sucrose-nitrate agar [b]	G: Moderate, pearl (2ba) R: Natural (2dc) AM: Moderate, natural (3dc) SP: None
Inorganic salts-starch agar [a]	G: Good, clove brown (3ni) R: Clove brown (3ni) AM: Abundant, silver gray (3fe) SP: None	Glucose-nitrate agar [b]	G: Poor, bamboo (2fb) R: Light wheat (2ea) AM: Poor, light ivory (2ca) SP: None
Glycerol-asparagine agar [a]	G: Good, clove brown (3ni) R: Cinnamon (3le) AM: Abundant, natural (3dc) SP: None	Glycerol-calcium malate agar [b]	G: Good, honey gold (2ic) R: Adobe brown (3lg) AM: Moderate, ashes (5fe) SP: None
Glucose-asparagine agar	G: Good, bamboo (2gc) R: Camel (3ie) AM: Abundant, covert gray (2fe) SP: None	Glucose-peptone agar [b]	G: Moderate, no name (7po) R: Chocolate (5nl) AM: Moderate, ashes (5fe) SP: None
Peptone-yeast extract-iron agar [a]	G: Moderate, bamboo (2fb) R: Honey gold (2ic) AM: Moderate, alabaster tint (13ba) SP: None	Nutrient agar [b]	G: Moderate, bamboo (2fb) R: Light amber (3ic) AM: Moderate, ashes (5fe) SP: None

[a] Medium recommended by ISP.
[b] Medium recommended by S. A. Waksman.
Abbreviations: G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

Table 2. Physiological properties of strain WK-4028.

Melanin formation	
Tyrosine agar	—
Peptone-yeast extract-iron agar	—
Tryptone-yeast extract broth	—
Reduction of nitrate	—
Liquefaction of gelatin (21~23°C)	+
Hydrolysis of starch	+
Coagulation of milk (37°C)	+
Peptonization of milk (37°C)	+
Decomposition of cellulose	—
Temperature range for growth	13~38°C

+: Positive, — : Negative.

Table 3. Utilization of carbon sources by strain WK-4028.

Utilized:	D-Glucose, D-Xylose, D-Mannitol, *i*-Inositol
Weakly utilized:	L-Arabinose, D-Fructose, L-Rhamnose
Not utilized:	Raffinose, Melibiose, Sucrose

27°C for 48 hours on a rotary shaker (210 rpm), and 400 ml of the resulting cultured broth was transferred to a 30-liter fermenter containing 20 liters of a producing medium consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract, 0.4% $CaCO_3$, 0.5% Sekado (100 mesh) and 5 ml/liter of trace metals. The fermentation was carried out at 27°C for 72 hours using an agitation rate of 160 rpm and an aeration rate of 60 liters per minute.

The fermentation broth of *Streptomyces* sp. WK-4028 (20 liters) was extracted with EtOAc (20 liters), and the EtOAc layer was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield a brown oily material (6.1 g). The material was subjected to silica gel column chromatography (70~230 mesh, i.d. 5×30 cm) using $CHCl_3$ and $CHCl_3$-$(CH_3)_2CO$ (9:1) as the developing solvents. Fractions exhibiting an inhibitory effect on the growth of SC 115 cells were collected. Two active fractions (I: 103 mg, II: 34 mg) were eluted with $CHCl_3$-$(CH_3)_2CO$ (9:1). Further separation of active fraction I by silica gel column chromatography (230~400 mesh, i.d. 1.5×20 cm) eluted with $CHCl_3$-$(CH_3)_2CO$ (9:1) gave pure **1** (25 mg) and a rich fraction of **2** (23 mg), respectively. Final purification of **2** was performed using

preparative HPLC. Active fraction II was also separated by preparative HPLC. Pure **1** (colorless needles), **2** (colorless plates), **3** (dark brown oil), and **4** (colorless plates) were obtained in yields of 25 mg, 8.0 mg, 12 mg and 10 mg, respectively.

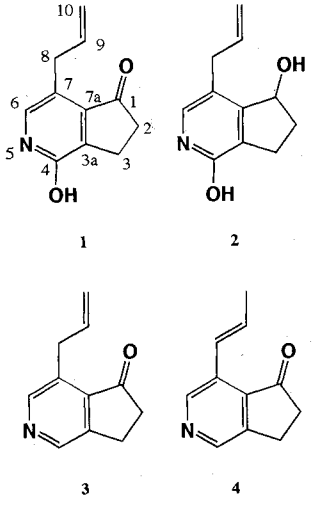
Structure of Louisianins

Structures of louisianins are shown in Fig. 2. Studies on the structural determination of these metabolites will be reported in a separate paper[8].

Biological Activity of Louisianins

Louisianin A significantly inhibited the growth of SC 115 cells at a concentration of 0.6 μg/ml (IC_{50}), whereas, the growth of HeLa cells (IC_{50} 3.0 mg/ml), B16 melanoma cells (IC_{50} >6.3 μg/ml) and L929 (IC_{50} >6.3 μg/ml) cells were not affected at this concentration. According to a brief experiment, among these analogs (**1**~**4**), compound **1** is the most active against the growth of SC 115 cells. These compounds (**1**~**4**) did not inhibit the activity of testosterone 5α-reductase derived from rat prostate (data not shown). Because growth of SC 115 cells was thought to be stimulated only by androgens *in vivo*[9] and in cell culture[10], the study of specific activity of **1** on this cell line is of interest. Louisianins did not show any antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi and yeasts at a concentration of 1,000 μg/ml.

Fig. 2. Structure of Louisianins **1**~**4**.



1 2 3 4

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