REVEROMYCINS, NEW INHIBITORS OF EUKARYOTIC CELL GROWTH

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES

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New antibiotics named reveromycins A, B, C and D were isolated as inhibitors of mitogenic activity induced by epidermal growth factor (EGF) in a mouse epidermal keratinocyte. Reveromycins were produced by a soil actinomycete (strain SN-593) which belongs to the genus *Streptomyces*.

The action of most of antitumor agents depends on the difference of cell proliferation rate between normal cells and tumor cells. These drugs directly act on function and synthesis of DNA or RNA. Intracellular signaling pathways induced by growth factors and modulation of the functions of oncogenes are considered to be targets for the development of a new type of antitumor drugs. For instance, an inhibitor of mitogenic activity of transforming growth factor α (TGF-α) is a possible candidate for the target of antitumor drugs. Because epidermal growth factor (EGF)[1] shares the same receptor with TGF-α[2], we aimed our screening effort at inhibitiors of EGF.

In previous papers, we have already reported epiderstatin[3] and actiketal[4] as inhibitors of EGF. Here we report isolation and characterization of new antibiotics, reveromycins A, B, C and D. This paper deals with taxonomy and fermentation of the producing organism. A main component, reveromycin A was reported preliminarily[5]. Biological activities and the structure elucidation of reveromycins will be reported in the succeeding papers[6,7].

Materials and Methods

Taxonomy

Physiological characterization of strain SN-593 was determined by the methods and media recommended by International Streptomyces Project (ISP)[8]. Color names of mycelial and soluble pigments were assigned according to the Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Chicago, Illinois).

Whole-cell hydrolysates were analyzed by the methods of BECKER *et al.*[9] and LECHEVALIER and LECHEVALIER[10].

Fermentation

Strain SN-593 was cultured in the seed medium consisting of glucose 2%, soluble starch 1%, meat

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extract 0.1%, dried yeast 0.4%, soybean meal 2.5%, NaCl 0.2% and K_2HPO_4 0.005% (adjusted at pH 7.0 before sterilization). The seed culture was carried out on a rotary shaker at 250 rpm for 72 hours in 500-ml Erlenmeyer flasks containing 70 ml of the seed medium. Then, 1.7 liters of the culture was inoculated in a 200-liter fermentation tank containing 120 liters of the same medium with 0.01% of DF 40P antifoam. The fermentation was carried out at 27°C under constant agitation at 300 rpm and aerated 120 liters per minutes.

Production of inhibitors in fermentation broth were monitored by both the inhibitory activity of incorporation of [^{3}H]thymidine into quiescent Balb/MK cells[11] induced by EGF and HPLC analysis of solvent extract from fermentation broth. The bioassay was described in a previous paper[3]. HPLC analysis was performed with a Capcell Pak NH_2 column (Shiseido, 4.6 × 250 mm), eluted with MeOH - H_2O - 1% NH_4OH (18 : 81 : 1), at the flow rate of 1 ml/minute and monitored with a UV detector set at 240 nm.

Isolation

Whole broth was filtered and the filtrate (100 liters) was adsorbed on Diaion HP-20 (Mitsubishi Chemical Industries Ltd.) column, which was eluted with 30% and 100% MeOH. The activity appeared in the 100% MeOH fraction. The active fraction was evaporated *in vacuo* to dryness and the residue was suspended in acidic water adjusted to pH 3 with 1 N HCl and then, extracted with EtOAc. The organic layer was concentrated *in vacuo* and applied onto a silica gel (Silica gel 60, Merck) column. After washing with $CHCl_3$ - MeOH (2 : 1) to remove impurities, the active principle was eluted with 100% MeOH. The concentrated residue was further purified by Sephadex LH-20 (Pharmacia Fine Chemicals) column chromatography with 20% MeOH to yield crude active mixture.

The crude mixture was subjected to the preparative HPLC using a Capcell Pak C_{18} column (Shiseido, 100 × 500 mm, monitored by UV at 240 nm), eluted with MeOH - H_2O - 1% NH_4OH (18 : 81 : 1) to separate main product, reveromycin A, from minor components, reveromycins B, C and D. The combined minor compounds were further purified by the preparative HPLC performed with a Capcell Pak C_{18} column (Shiseido, 30 × 250 mm, monitored by UV at 240 nm) using the same solvent system. The active fractions were collected and concentrated *in vacuo* to remove MeOH. The resulting aqueous solutions were extracted with EtOAc after adjusting to pH 3 with 1 N HCl. The organic layers were concentrated *in vacuo* and the concentrated materials were lyophilized.

Instrumental Analyses

The mp was measured with a Büchi 535 melting point apparatus. Optical rotation was determined on a Jasco DIP-181 polarimeter. UV and IR spectra were taken with a Hitachi U-3210 spectrophotometer and Shimadzu IR27G recording IR spectrophotometer, respectively. High resolution fast atom bombardment mass spectrum (HRFAB-MS) was obtained on a Jeol JMS DSX-300 or HX-110 (HS) mass spectrometer.

Results and Discussion

Taxonomy

Strain SN-593 was isolated from a soil sample collected in Gunma Prefecture, Japan. Cultural characteristics of the strain on various ISP media are summarized in Table 1. No soluble pigments were produced on these media. The strain utilized D-glucose, L-rhamnose and L-arabinose, but did not utilize D-xylose, *i*-inositol, D-mannitol, D-fructose, sucrose and raffinose. The strain had rectiflexible spore chains with more than 50 spores per chain. The ornamentation of the spore surface was smooth (Fig. 1). The whole-cell hydrolysate contained the L,L isomer of DAP which corresponds to cell-wall type I[10]. Based on these morphological and chemotaxonomic characteristics, it was concluded that the strain belongs to the genus *Streptomyces*.

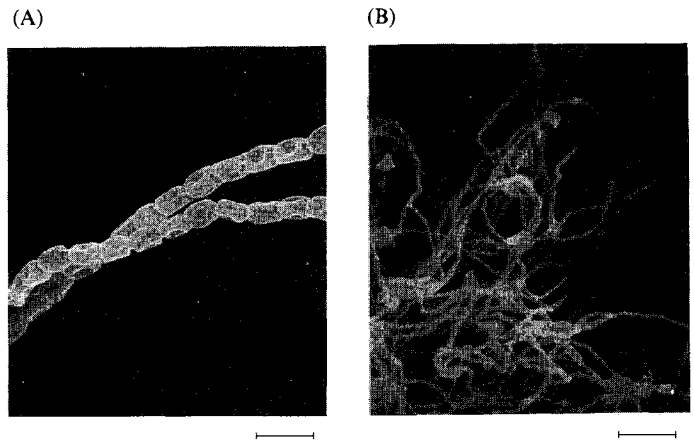
According to the cultural characteristics, *Streptomyces* sp. SN-593 belongs to the gray color series. Among the described species, the ones most closely resembling this strain are *Streptomyces aburaviensis*,

Table 1. Cultural characteristics of strain SN-593.

Media	Substrate mycelium	Aerial mycelium
Yeast extract-malt extract agar (ISP No. 2)	Abundant, 5 po (Chocolate brown)	Abundant, 3 ih (Beige gray)
Oatmeal agar (ISP No. 3)	Abundant, 2 ne (Mustard gold)	Abundant, 2 ml
Inorganic salts-starch agar (ISP No. 4)	Abundant, 4 ni (Chestnut brown)	Abundant, 3 ih (Beige gray)
Glycerol-asparagine agar (ISP No. 5)	Moderate, 3 ng (Yellow maple)	Moderate, 2 dc (Natural)
Yeast extract-starch agar	Abundant, 3 li (Beaver)	Abundant, 2 ih (DK covert gray)
Glucose-asparagine agar	Good, 2 ne (Mustard gold)	Good, 2 fe (Covert gray)
Sucrose-nitrate agar (WAKSMAN's No. 1)	Moderate, colorless	Good, 2 ge (Covert tan)
V8 juice agar	Poor, 2 po (Ebony)	Moderate, 3 ih (Beige gray)
Potato-carrot agar	Good, colorless	Good, 5 fe (Ashes)

Fig. 1. Scanning electron micrograph of strain SN-593.

Bars in pictures indicate 1.2 μm (A) and 6 μm (B), respectively.



Streptomyces omiyaensis and *Streptomyces gelaticus*[1,2].

Fermentation and Isolation

During the fermentation, pH of the broth gradually decreased to around 5.8 and the production titer of inhibitors reached almost 100 μg/ml after 117 hours (Fig. 2).

After Sephadex LH-20 column chromatography, about 8 g of a mixture of reveromycins was obtained. The first preparative HPLC profile exhibited one main peak corresponding to reveromycin A and three minor peaks corresponding to reveromycins B, C and D. Reveromycin A was separated from minor components by the first preparative HPLC. Reveromycins B, C and D were separated each other by the second preparative HPLC.

Approximate yields of reveromycins A, B, C and D were 3 g, 12, 80 and 14 mg, respectively.

Physico-chemical Properties

The physico-chemical properties of reveromycins are summarized in Table 2.

Reveromycins were soluble in MeOH, EtOAc and alkaline water, but insoluble in acidic water. They

Table 2. Physico-chemical properties of reveromycins.

	A	B	C	D
Appearance	White powder	White Powder	White powder	White powder
MP	95°C	78~79°C	78~79°C	78~80°C
$[\alpha]_D^{20}$ (*c* 0.1, MeOH)	−115°	−66°	−90°	−112°
Molecular formula	$C_{36}H_{52}O_{11}$	$C_{36}H_{52}O_{11}$	$C_{37}H_{54}O_{11}$	$C_{37}H_{54}O_{11}$
HRFAB-MS Obsd $(M+Na)^+$:	683.3496	683.3433	697.3574	697.3575
Calcd:	683.3407	683.3407	697.3564	697.3564
UV λ_{max}^{MeOH} nm (ε)	238 (25,300)	239 (28,800)	239 (30,800)	238 (30,400)

showed positive color reactions for iodine, anisaldehyde-H_2SO_4 and bromocresol green, but negative to ninhydrin.

The molecular formula of reveromycin A was established as $C_{36}H_{52}O_{11}$ based on HRFAB-MS and elemental analysis[5]. The molecular formulas of reveromycins B, C and D were determined as $C_{36}H_{52}O_{11}$, $C_{37}H_{54}O_{11}$ and $C_{37}H_{54}O_{11}$, respectively, based on HRFAB-MS.

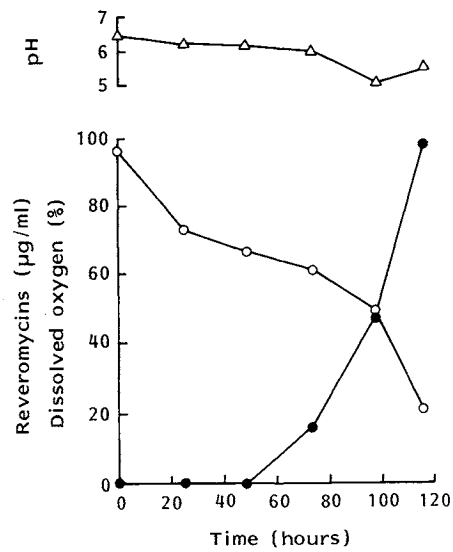
Reveromycins A, B, C and D have the same UV spectrum having absorption maximum at 238~239 nm. The IR spectra of reveromycins indicated the presence of hydroxyl (3430 cm^{-1}) and carbonyl (1690 cm^{-1}) groups. The retention times of reveromycins A, B, C and D on HPLC analysis under the described conditions were 9.5, 13.5, 10.3 and 12.1 minutes, respectively.

No microbial products which have similar physico-chemical properties have ever been reported. Therefore, we concluded that reveromycins are novel compounds.

Fig. 2. Time course of reveromycins production.

● Production titer of reveromycins, ○ dissolved oxygen, △ pH.

Production titer of reveromycins was calculated as that of reveromycin A which exists as overwhelming main product in the fermentation broth.



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