A NEW ANTIBIOTIC ECHINOSPORIN (XK-213) — PRODUCING ORGANISM, ISOLATION AND CHARACTERIZATION

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Streptomyces echinosporus MK-213 produces a novel antibiotic echinosporin (XK-213). Isolation of echinosporin was performed by absorption on activated carbon under acidic conditions and then eluted by aqueous acetone. The compound crystallized from methanol is a water soluble white solid composed of $C_{10}H_9NO_5$.

Echinosporin exhibits weak antibacterial activities against Gram-positive and -negative microorganisms and it shows antitumor activity.

During the course of our screening program, an actinomycetes, designated as *Streptomyces echinosporus* MK-213 which was isolated from Mexican soil, produced an antibacterial substance in the fermentation beer. The active substance was easily isolated and purified to a crystalline form.

This paper describes the taxonomy of the producing microorganism, fermentation and isolation process followed by characterization of the antibiotic. The structural determination and antitumor activities of the compound will be described in separate papers.

Taxonomy of Producing Microorganism

The echinosporin producing microorganism strain MK-213 was isolated from a soil sample collected from Mexico and shows the following properties.

Morphological Properties

The aerial mass color was grayish green to grayish blue and the reverse color was tan to brown. Aerial mycelium was monopodially branched with spore chains of *spiral* type on most of the medium used. Spores were in chains of more than ten, spherical to oval in shape, $0.7 \sim 1.2 \times 0.8 \sim 1.2$ μm in size and had spiny surfaces. The spores had no flagella. No sporangia or sclerotia had formed.

Cultural and Physiological Characteristics

Cultural characteristics of MK-213 were based on observations made after 14 days at 30°C on media listed in Table 1. Color designations and color chip numbers were taken from the Color Harmony Manual (Container Corporation of America). The physiological properties of the strain are summarized in Table 2. Carbohydrate utilization was determined in the basal medium of PRIDHAM and GOTTLIEB[1]. All other properties were determined according to the procedure used in the International Streptomyces Project (ISP). The hydrolysates of purified cell walls were shown to contain LL-2,6-diaminopimelic acid and glycine.

Based on these characteristics, the strain MK-213 was assigned to the genus *Streptomyces*. Strain MK-213 showed some similarities to the eleven *Streptomyces* species reported in literatures[2~7] as shown in Table 3.

In view of the clustering with these reference strains, MK-213 is considered to be a new species designated *Streptomyces echinosporus* MK-213.

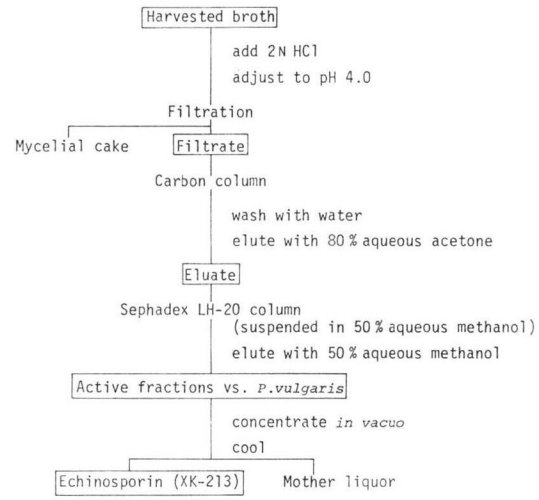
Table 1. Cultural characteristics on various media.

Agar media	Growth	Aerial mass	Aerial mass color	Reverse color	Soluble pigment
Sucrose - nitrate	good	abundant	pearl-sand (3ba-3cb)	mustard brown-deep brown (2pl-3pl)	dark olive green (24 1/2nl)
Glucose - asparagine	moderate	sparse	white-sand-dusty jade green (a-3cb-2lec)	butter scotch-yellow maple (3ne-3ng)	pale peach (5ca)
Glycerol - asparagine (ISP No. 5)	moderate	abundant	white-sand-light mistletoe green (a-3cb-24 1/2fe)	deep brown-dark spice brown (3pl-4pl)	dusty olive (11g)
Starch - inorganic salt (ISP No. 4)	abundant	abundant	dusty jade green-mist green (21ec-22ec)	oak brown-deep brown (4pi-4pl)	brick red (5ng)
Oat meal (ISP No. 3)	abundant	fair	white-sand-aqua gray (a-3cb-22dc)	oak brown-deep brown (4pi-4pl)	brick red (5ng)
Yeast extract - malt extract (ISP No. 2)	abundant	abundant	white-natural gray-aqua gray (a-2dc-22dc)	golden brown-oak brown (3pi-4pi)	dark brown (4pl-5pl)
Nutrient	poor	scant	white-sand (a-3dc)	light ivory-pearl pink (2ca-3ca)	none
Tyrosine (ISP No. 7)	poor to moderate	sparse	natural gray (2dc-3dc)	cocoa brown-chocolate (5ni-5nl)	light rose beige-rose beige (4ec-4gc)
Peptone - yeast - iron (ISP No. 6)	poor	none	—	light ivory-pearl pink (2ca-3ca)	cinnamon (3le)
BENNET'S	abundant	abundant	covert gray-celadon gray (1fe-24fe)	golden brown-oak brown (3pi-4pi)	golden brown-yellow maple (3pg-3ng)
EMERSON'S	abundant	sparse	natural gray (2dc-3dc)	light brown-oak brown (4ng-4pi)	copper brown-deep brown (5pi-5pl)

Table 2. Physiological properties.

Carbohydrate utilization	
Utilized	D-glucose, D-raffinose, D-mannitol, D-fructose, *i*-inositol, D-rhamnose, sucrose
Not utilized	D-arabinose
Weakly utilized	D-xylose
Gelatin liquefaction	weakly positive
Starch hydrolysis	positive
Milk peptonization	weakly positive
Milk coagulation	negative
Melanoid pigment	positive on tyrosine agar
Optimum temperature for growth	27~30°C

Fig. 1. Isolation of echinosporin (XK-213).



Fermentation

The medium used for the seed culture was composed of 1% dextrin, 1% glucose, 0.5% yeast extract, 0.5% polypeptone and 0.1% $CaCO_3$, pH 7.0 before sterilization. The spores of producing organism were inoculated into a 300-ml Erlenmeyer flask containing 50 ml of the seed medium and incubated at

Table 3. Comparison of strains resembling strains MK-213.

Microorganisms	Reverse side of colony (Medium in ISP number)	Soluble pigments (Medium in ISP number)	Melanoids		Carbon utilization*				
			(ISP No. 6)	(ISP No. 7)	Suc	Xyl	Rha	Raf	Glu
MK-213	no distinctive pigments (2, 3, 4, 5); not pH indicator	olive brown (5), reddish brown (3, 4), deep brown (2); not pH indicator	weakly positive	positive	±	±	+	+	+
S. chartreusis		none (2, 3, 4, 5)	positive	negative	+	+	+	+	+
S. coerulescens			positive	unknown	+	+	+	+	+
S. curacoi			positive	positive	+	+	±	±	+
S. bicolor		yellow pigment (2, 3, 4, 5); not pH indicator	positive	positive	+	+	+	+	+
S. lanatus		yellow or brown (2, 3, 4, 5)	positive	positive	+	+	+	+	+
S. cyaneus	dark grayish blue to dark grayish purple (2, 3, 4); pH indicator	blue or violet (2, 3, 4); pH indicator	positive	positive	+	+	+	+	+
S. indigocolor	very dark green blue or gray blue (2), gray blue to grayish purple (3, 4), grayish purple to reddish black (5); pH indicators	blue or violet (2, 3, 4, 5); pH indicator	positive	negative	+	+	+	+	+
S. bellus	modified by red (orange) pigment (2, 5), red or blue pigment (3, 4); not pH indicator	none (2, 3, 4), trace of red pigment (5); not pH indicator	positive	positive	+	+	+	+	+
S. coeruleorubidus	grayish yellow to yellowish brown (2), grayed yellow to yellowish green (3,4), grayed yellowish green and grayed red or orange (5); not pH indicator	red pigment (2, 4, 5); not pH indicator	positive	positive	+	+	+	+	+
S. coeruleofuscus	yellow brown modified by red (2), grayed yellow or grayed greenish yellow modified by green, red, brown (3, 4, 5)	none (2, 3, 4, 5)	positive	unknown	+	+	+	+	+
S. viridochromogenes	greenish pigment (3); pH indicator	olive brown to dark olive (2), grayish green to grayish olive (3), grayish yellow, olive brown, greenish yellow (4, 5); pH indicator	negative	variable	±	+	+	+	+

* Suc: Sucrose, Xyl: Xylose, Rha: Rhamnose, Raf: Raffinose, Glu: Glucose. +: Utilized, ±: Weakly utilized.

30°C for 2 days on a rotary shaker. Two ml of the resulting seed culture was transferred into a 300-ml Erlenmeyer flask containing 50 ml of the production medium composed of 2% soluble vegetable protein, 1% dry yeast, 2% glycerine, 0.5% $MgCl_2$, 0.3% KH_2PO_4 and 0.2% $Na_2HPO_4 \cdot 12H_2O$, pH 6.5 before autoclaving. The fermentation was conducted at 30°C for 96 hours on a rotary shaker.

Isolation

The isolation procedure of echinosporin is outlined in Fig. 1. Echinosporin was absorbed onto charcoal under acidic condition and then eluted by aqueous acetone. The eluate was monitored by antibacterial activity against *Proteus vulgaris*. The active fractions were collected and concentrated *in vacuo*. Further purification can be achieved by gel filtration with Sephadex LH-20 from which echinosporin was eluted later than most of the inactive pigments. The active fractions from LH-20 were concentrated and kept cool until the white crystals were obtained. After the first crop of echinosporin was separated by filtration, the mother liquor was concentrated and re-chromatographed through the Sephadex LH-20 column to obtain a second crop of crystals.

Characterization

The physico-chemical properties of echinosporin are summarized in Table 4. The crystals are soluble in water exhibiting the ultraviolet absorption spectrum as given in Fig. 2.

The absorption maximum is at 236 nm under neutral and acidic aqueous solutions and it shifts to 246 nm under alkaline conditions. The infrared absorption spectrum in KBr tablet is shown in Fig. 3.

According to the above physico-chemical characteristics, echinosporin was compared with the known antibiotics reported in Index of Antibiotics from Actinomycetes, vol. I, II and supplements cited in The Journal of Antibiotics.

Melanosporin, hygroscopin A and some antibiotics have reported to show the similar ultraviolet absorption maximum to echinosporin, but all of them are differed from it with the other properties.

No compounds which has the same molecular formula to echinosporin, $C_{10}H_9NO_5$, were found in the literatures and neither in our own reference library.

Chromatographic properties on paper chromatography as shown in Table 5, have also revealed the echinosporin did not resemble to the known antibiotics.

Echinosporin exhibited weak antibacterial activities towards Gram-negative and little activity against Gram-positive bacteria as shown in Table 6.

It exhibited intra peritoneal acute toxicity in mice at LD_{50} 54 mg/kg.

Table 4. Physico-chemical properties of echinosporin.

Appearance	White amorphous powder
Formula	$C_{10}H_9NO_5$
Melting point	260°C (decomposed)
$[\alpha]_D^{25}$	−400° (*c* 0.1, MeOH)
Color reaction	Ninhydrin; weakly positive
Solubility	Soluble in water, methanol Insoluble in benzene, chloroform and ethyl acetate

Fig. 2. Ultraviolet absorption spectrum of echinosporin.

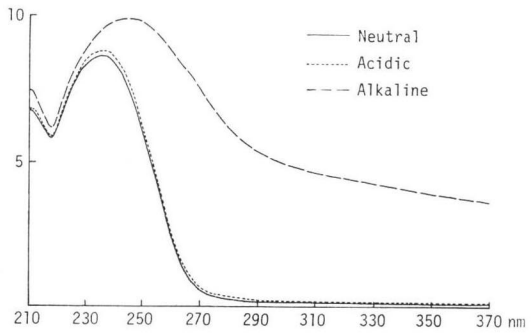


Fig. 3. Infrared absorption spectrum of echinosporin.

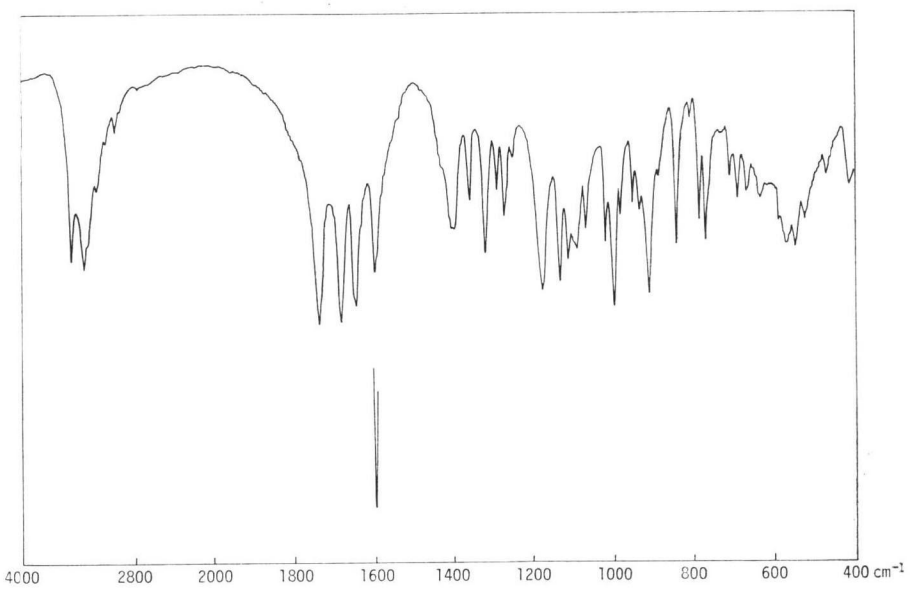


Table 5. Paper chromatographic properties of echinosporin.

Solvents	Rf
1. *n*-Butanol saturated with water	0.36
2. *n*-Butanol - acetic acid - water, 3:1:1 (v/v)	0.48
3. Ethyl acetate saturated with water	0.00

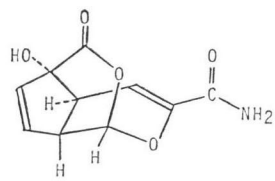
Paper : Toyo #51 (2×40 cm)
Developed ascendingly at 28°C for 15 hours (solvent 1,2) and 4 hours (solvent 3).
Assay : Bioautography *vs. Proteus vulgaris*

Table 6. Antibacterial activities of echinosporin.

Test organisms	MIC (μg/ml)
Proteus vulgaris ATCC 6897	100
Salmonella typhosa ATCC 9992	100
Shigella sonnei ATCC 9290	100
Escherichia coli ATCC 26	200
Bacillus subtilis No. 10707	400
Staphylococcus aureus ATCC 6538 p	>500

Agar dilution assay at pH 7.0.

Fig. 4. Structure of echinosporin.



The structure of echinosporin was recently reported[8] as shown in Fig. 4, and the details of the structural studies and the antitumor activities[9] of echinosporin will be reported in succeeding papers.

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