A NEW ANTIBIOTIC, ASUKAMYCIN, PRODUCED BY *STREPTOMYCES*

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Asukamycin, a new antibiotic, has been isolated from the culture broth of a streptomycete designated as *Streptomyces nodosus* subsp. *asukaensis*. The antibiotic inhibits the growth of Gram-positive bacteria including *Nocardia asteroides*. The empirical formula of antibiotic asukamycin has been proposed as $C_{29}H_{22}N_2O_9$ (M.W. 542). An acute toxicity of the antibiotic in mice is LD_{50} 48.5 mg/kg by intraperitoneal injection and it has no effect on mice when it was administered by 450 mg/kg per os.

During the course of searching for new antibiotics, we have found a new compound designated as asukamycin, which inhibits the growth of some Gram-positive bacteria, in culture broths of a streptomycete isolated from the soil sample collected in Nara Prefecture, Japan. In the present paper, the taxonomy of the producing strain, fermentation, isolation, and physico-chemical and biological properties of antibiotic asukamycin are described.

Taxonomic Studies

The antibiotic-producing strain AM-1042, which was cultured on several agar media for 2~3 weeks at 27°C, was used for taxonomic studies.

Morphological characteristics of the strain are shown in Fig. 1. A well-branched substrate mycelium was formed abundantly on most agar media, except for several natural media. Spore chain morphology belongs to section *Retinaculi-aperti* or *Spirales* having short chains of conidiospores. Spore surfaces were smooth, but they often appeared with wrinkles or folds. Cultural characteristics, physiological properties, and utilization of carbon sources of strain AM-1042 on various media are summarized in Tables 1, 2 and 3, respectively. From these data, it was concluded that strain AM-1042 belongs to the genus *Streptomyces*. Among other

Fig. 1. Morphological observations of strain AM-1042 grown on oatmeal agar for 14 days by optical and electron microscopes

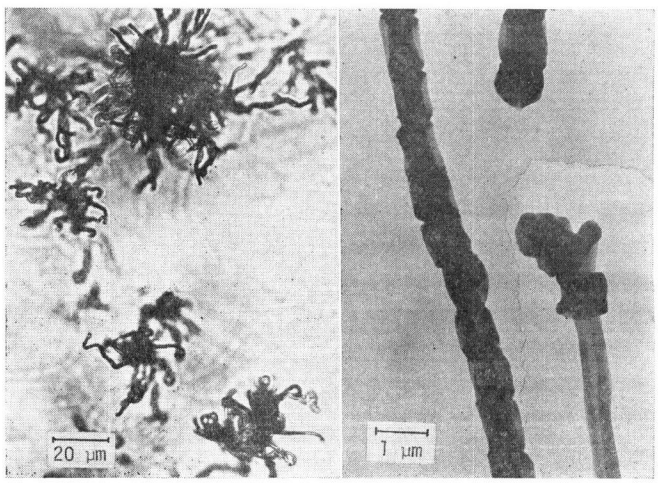


Table 1. Cultural characteristics of strain AM-1042

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	colorless	brownish gray	brownish gray	colorless to pale yellow
Glucose-nitrate agar	colorless	pale yellowish brown	brownish gray powder	yellowish brown
Glycerol-calcium malate agar	colorless	pale yellow to grayish yellow brown	light brownish gray powder	pale yellowish brown
Glucose-asparagine agar (ISP)*	colorless	grayish yellow brown	brownish gray	yellowish brown
Glycerol-asparagine agar (ISP)	colorless	pale yellowish brown to yellowish brown	brownish gray powder	yellowish brown
Inorganic salts-starch agar (ISP)	yellowish brown or dark yellow brown	yellowish brown or pale reddish brown	brownish gray	pale yellowish brown or grayish red brown
Tyrosine agar (ISP)	light reddish yellow	pale yellowish brown to light reddish yellow	grayish yellow brown	dull yellow
Peptone-yeast extract-iron agar (ISP)	colorless	yellowish brown	—	—
Peptone-beef extract agar	light brownish gray	light brownish gray	—	—
Glucose-peptone agar	colorless	dull yellowish orange	light brownish gray	yellowish brown to light brown
Yeast extract-malt extract agar (ISP)	colorless	grayish yellow brown	brownish gray	yellowish brown
Oatmeal agar (ISP)	colorless to pale yellow	light reddish yellow to light brownish gray	light brownish gray to brownish gray	pale yellowish brown to dull yellow
Tryptone-yeast extract broth		colorless	white and brownish gray	

* ISP: International Streptomyces Project

strains listed in the manuals of *Streptomycetes*[1~3], *Streptomyces nodosus* TREJO[3,4] was identified as most closely similar to strain AM-1042. A culture of *Streptomyces nodosus* TREJO ISP 5109 (KA-1293) was then compared with that of strain AM-1042. Morphological observations, cultural characteristics, and physiological properties of both strains considerably resembled each other. In particular, they showed almost identical cultural characteristics on starch-inorganic salts agar medium. However, they exhibited somewhat different behavior on utilization of carbon sources. Namely, *Streptomyces nodosus* TREJO ISP 5109 utilized mannitol and inositol, while the other did neither. On the basis of these results, it was concluded that strain AM-1042 can most reasonably be classified as one of the subspecies of *Streptomyces nodosus* TREJO ISP 5109. Strain AM-1042 was therefore named *Streptomyces nodosus* subsp. *asukaensis*. The strain has been deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Tokyo, and assigned as *Streptomyces nodosus* subsp. AM-1042 with an accession number of FERM-P 3429.

Fermentation

Spores of *Streptomyces nodosus* subsp. *asukaensis* grown on an agar medium containing 1% glucose, 0.5% peptone, 0.5% meat extract and 1.2% agar were inoculated in a 500-ml SAKAGUCHI's flask containing 100 ml of a seed medium composed of 1.0% glucose, 2.4% starch, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.4% calcium carbonate (pH adjusted to 7.0 prior to sterilization), and cultured

Table 2. Physiological properties of strain AM-1042

Melanin formation	−
Tyrosinase reaction	−
Nitrate reduction	+
H_2S production	−
Liquefaction of gelatin	+
Hydrolysis of starch	+
Coagulation of milk	±
Peptonization of milk	+
Cellulolytic activity	±

+: positive. ±: ambiguous. −: negative.

Table 3. Utilization of carbon sources

Response	Carbon source
Positive	D-Glucose, D-xylose, L-rhamnose
Ambiguous	D-Fructose, D-raffinose, L-arabinose
Negative	Mannitol, sucrose, *i*-inositol

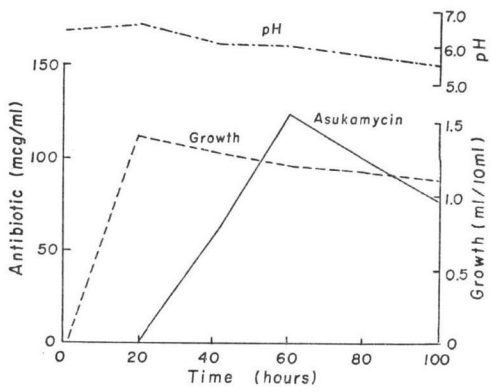
for 2 days at 27°C. The seed culture (400 ml) was transferred into a 30-liter jar fermentor containing 20 liters of the fermentation medium composed of 0.2% glucose, 2.0% dextrin, 1.5% soybean meal, 0.3% yeast extract and 0.3% calcium carbonate (pH adjusted to 7.0 prior to sterilization). It was kept for about 72 hours at 27°C under the conditions of 250 r.p.m. agitation, 10 liters/min. aeration, and 0.5 kg/cm² internal pressure.

A large scale fermentation was carried out using a 100-liter tank fermentor containing 50 liters of the fermentation medium described above. The typical time course of asukamycin production in the 100-liter tank fermentor is shown in Fig. 2. The antibiotic activity was assayed by paper disc method using *Bacillus subtilis* PCI 219 as the test organism.

Isolation and Characterization

The pH of the cultured broth (17 liters) was adjusted to 3.0 with 2 N HCl. It was filtered through diatomaceous earth to remove mycelium. The filtrate was extracted twice with ethyl acetate (5 liters × 2) and the solvent layer evaporated to dryness *in vacuo* to provide a crude powder (4.3 g). The powder was then treated with ethyl ether and the residue was dissolved in chloro form. The solvent layer was evaporated under reduced pressure. The remnant brownish syrup (1.6 g) was loaded on a silica gel (Merck, Kieselgel G) column for chromatography and eluted with chloroform - methanol (60: 1) mixture. The active fractions were collected and evaporated to dryness *in vacuo* to yield yellowish powder (350 mg). The powder was recrystallized from a mixture of chloroform and methanol to afford pale yellow needles of asukamycin (300 mg); mp 188°C (darkened, decomp.); $[\alpha]_D^{26}+181°$ (*c* 0.5, $CHCl_3$).

Fig. 2. Time course of asukamycin production by *Streptomyces nodosus* subsp. *asukaensis*



The molecular weight and the molecular formula of asukamycin were proposed on the basis of mass spectrometry of its acetyl derivative. No evidence for the presence of acetyl groups in asukamycin itself was obtained by IR and NMR spectrometry. On the other hand, the acetyl derivative of asukamycin prepared by treating the antibiotic with acetic anhydride in pyridine showed the molecular ion peak at *m/e* 626.156 (calcd. for $C_{33}H_{26}N_2O_{11}$, *m/e* 626.154) and the fragment peak (M^+-84) at *m/e* 542.136

(calcd. for $C_{29}H_{22}N_2O_9$, *m/e* 542.133) indicating that two acetyl groups were eliminated from the acetate. According to these results, the molecular formula of asukamycin was suggested to be $C_{29}H_{22}N_2O_9$ (M.W. 542).

The ultraviolet absorptions showed up at 315 nm (ε 56910) and 265 nm (sh. ε 37400) in 90% methanol solution, 305 nm (ε 56910) and 260 nm (sh. ε 40110) in 0.1 N NaOH-90% methanol solution, and 320 nm (ε 5590) in 0.1 N HCl-90% methanol solution (Fig. 3). The infrared spectrum in KBr tablet is shown in Fig. 4, which exhibited bands characteristic of amine or hydroxyl group around 3340~3330 cm^{-1}, carbonyl and double bond groups at 1660 and 1600 cm^{-1} respectively, and alkane at 2925, 2850 and 1365 cm^{-1}. The nuclear magnetic resonance spectrum of asukamycin (Fig. 5) suggested the presence of amine or hydroxyl group at δ 13.6, two aromatic

Fig. 3. Ultraviolet spectra of asukamycin

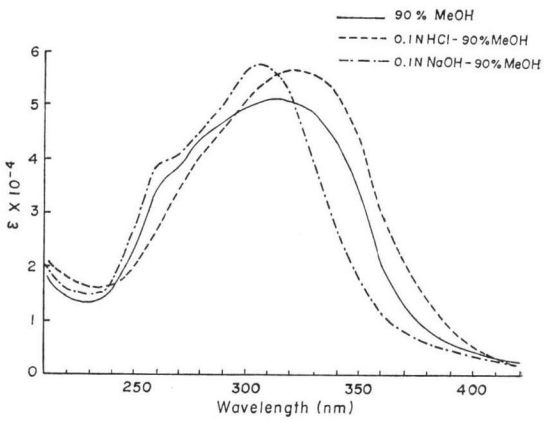


Fig. 4. IR spectrum of asukamycin (KBr)

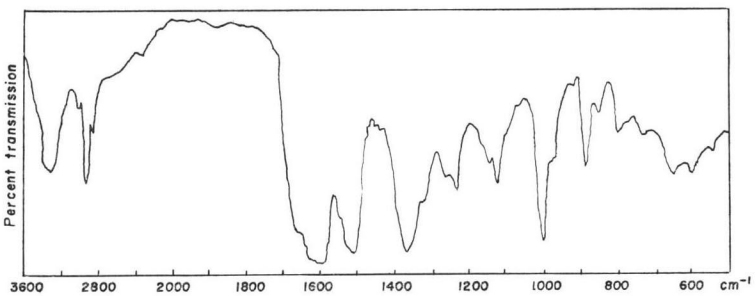
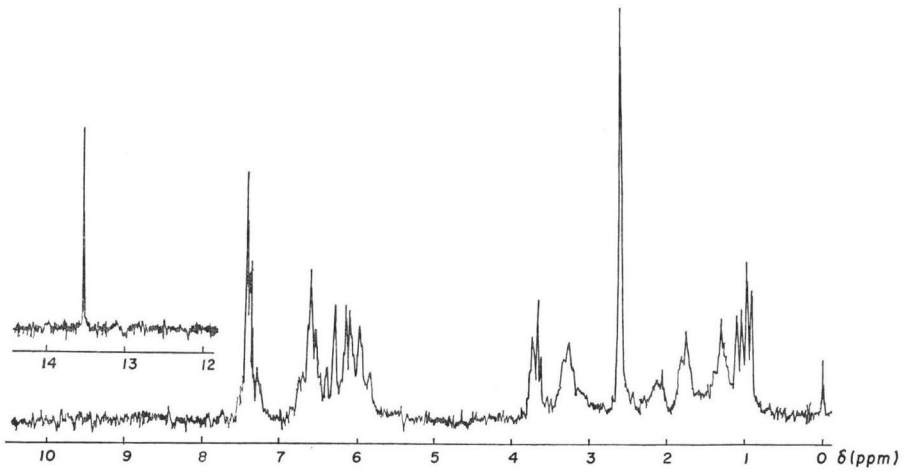


Fig. 5. NMR spectrum of asukamycin (100 MHz, $CDCl_3$)



protons at δ 7.41, nine olefinic protons at δ 7.0~5.8, a methyl group linked to a nitrogen atom or double bonds at δ 2.6, and two methyl groups at δ 1.0~0.9.

The antibiotic is readily soluble in dimethyl sulfoxide and dimethylformamide, soluble in lower alcohols, acetone, chloroform and ethyl acetate, but insoluble in petroleum ether, *n*-hexane and water. It gave a positive test with EHRLICH's, DRAGENDORFF's and iodine reagents, but it was inert to MOLISCH's, BEILSTEIN's and RYDON-SMITH's reactions, and ninhydrin reagent. The Rf values of the antibiotic on silica gel (Merck, Kieselgel G) thin-layer chromatography were as follows: Benzene - acetone (13: 7), 0.40; chloroform - methanol (10: 1), 0.56; ethyl acetate - methanol (87: 13), 0.66.

Biological Activities

Antimicrobial activity of asukamycin was determined by conventional agar dilution method using nutrient agar for bacteria (37°C, 18 hours), and glucose-potato agar for fungi (27°C, 72 hours). As shown in Table 4, the antibiotic inhibited the growth of Gram-positive bacteria within a concentration range of 0.78~12.5 mcg/ml, except for *Aerobacter aerogenes* IAM 1183 and *Mycobacterium smegmatis* ATCC 607. It had no significant effects on Gram-negative bacteria at below 100 mcg/ml. A weak antifungal activity against *Trichophyton mentagrophytes* at a concentration of 25 mcg/ml was observed; however, the antibiotic had no effects on other fungal strains at below 100 mcg/ml.

The acute toxicity (LD_{50}) of asukamycin in mice calculated by BEHRENS-KÄRBER's method was 48.5 mg/kg by intraperitoneal injection and the antibiotic had no effect on mice when it was administered by 450 mg/kg per os.

Asukamycin was found to possess anticoccidial activity in chickens. The antibiotic, when fed at the concentration of 100 ppm in

Table 4. Antimicrobial spectrum of asukamycin (MIC, mcg/ml)

Test organism	MIC	Medium*
Staphylococcus aureus FDA 209P JC-1	0.78	N
Staphylococcus aureus FDA 209P	3.12	N
Staphylococcus aureus FS-1277 (R-PC)**	6.25	N
Staphylococcus aureus KB-64 (R-TC, EM)***	6.25	N
Staphylococcus albus	12.5	N
Micrococcus flavus 16	1.56	N
Sarcina lutea PCI 1001	6.25	N
Bacillus subtilis PCI 219	3.12	N
Bacillus subtilis ATCC 6633	6.25	N
Bacillus cereus T	3.12	N
Bacillus megaterium APF	6.25	N
Bacillus anthracis	12.5	N
Bacillus agri	12.5	N
Corynebacterium paurometabolum	1.56	N
Nocardia asteroides	1.56	N
Aerobacter aerogenes IAM 1183	100	N
Mycobacterium smegmatis ATCC 607	>100	N
Escherichia coli NIHJ	100	N
Klebsiella pneumoniae PCI 602	>100	N
Proteus vulgaris IFO 3163	>100	N
Salmonella typhimurium	>100	N
Shigella sonnei E-33	>100	N
Pseudomonas aeruginosa P-3	>100	N
Candida albicans	>100	P
Saccharomyces cerevisiae	>100	P
Aspergillus niger	>100	P
Cryptococcus neoformans	>100	P
Trichophyton mentagrophytes	25	P
Piricularia oryzae	>100	P
Alternaria kikuchiana	>100	P
Fusarium oxysporum	>100	P
Xanthomonas oryzae	100	P
Sclerotinia cinerea	100	P
Botrytis cinerea	>100	P

*N: Peptone 0.5%, meat extract 0.5%, agar 1.2%, pH 7.0
P: potato extract containing glucose 1.0% and agar 1.2%, pH 6.8
**: resistant to penicillin
***: resistant to tetracycline and erythromycin

the diet to 4-day-old chicks, was effective in reducing mortality of chicks infected with *Eimeria tenella*. The details of anticoccidial activity will be described elsewhere.

Discussion

A streptomycete isolated from Nara Prefecture in Japan was found to produce a novel antibiotic, asukamycin, which showed antimicrobial activity against Gram-positive bacteria and *E. tenella*. Strain AM-1042 was named *Streptomyces nodosus* subsp. *asukaensis*. The antibiotic was obtained from the culture broth of the strain as pale yellow needles. Antibiotic asukamycin has characteristic ultraviolet absorptions at 315 nm and 365 nm (sh.). It was therefore compared with other known antibiotics that have ultraviolet absorptions around 315 nm. Several antibiotics such as viridenomycin[5] (310 nm), denamycin[6] (311 nm), azomycin[7] (313 nm), variotin[8] (320 nm) and amicetin B[9] (321 nm) were examined. However none of their physico-chemical properties were identical with those of antibiotic asukamycin. Consequently, it was reasonable to conclude that asukamycin is a new antibiotic.

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