STUDIES ON TUBERACTINOMYCIN (TUBERACTIN), A NEW ANTIBIOTIC. I

TAXONOMY OF PRODUCING STRAIN, ISOLATION AND CHARACTERIZATION

AKIO NAGATA, TAKUJI ANDO, ROKURO IZUMI, HIDEO SAKAKIBARA, TERUO TAKE, KAZUO HAYANO and JIN-NOSUKE ABE

Research Laboratory, Toyo Jozo Co., Ltd., Ohito-cho, Shizuoka-ken, Japan

(Received for publication October 14, 1968)

A new antibiotic, tuberactinomycin, has been isolated from fermentation broth of a streptomyces named *Streptomyces griseoverticillatus* var. *tuberacticus*. Tuberactinomycin is a water-soluble antibiotic belonging to the basic peptide antibiotic group, and markedly stable in acid or neutral solution. Tuberactinomycin is recovered from fermentation broth by means of a cation exchange resin process. Tuberactinomycin shows low toxicity and the activity against pathogenic tubercular bacilli both *in vitro* and *in vivo*. Taxonomical study of the producing strain, the isolation and properties of the antibiotic are reported in this paper.

A water-soluble basic antibiotic was obtained from the broth filtrate of a streptomyces strain which was isolated from a soil sample collected at Ohito-cho, Shizuoka-ken, Japan in 1966 and designated as No. B-386 in our culture collection. The antibiotic was effective against tubercular bacilli, and named tuberactinomycin. This antibiotic was previously reported as tuberactin[8,9,10], though a new generic name, tuberactinomycin, was given by the Japanese rules for non-proprietary names for drugs.

Taxonomical Studies on the Streptomyces B-386

(1) Morphological characteristics: The B-386 strain grew well on various media such as BENNETT's agar, CZAPEK's glycerine agar and starch agar. Morphological properties were observed on these media after incubation at 30°C for three weeks. The macro-colony of the strain showed a chrysanthemum pattern, somewhat delta-like and cottony white growth at initial stage, then revealed pinkish or beige color with many droplets. On microscopic observation, many primary and secondary whorls were observed, but no spiral was formed as shown in Plate 1. On electron microscopic observation, the spores were short cylindrical or ellipsoidal, and the surface was smooth as shown in Plate 2 (0.5~0.7μin ~~(1.27~1.78 cm)~~ width and 0.8~1.8μin ~~(2.03~4.57 cm)~~ length).

(2) Cultural and physiological characteristics: Cultural and physiological characteristics of the B-386 strain on various media are summarized in Table 1 and Table 2. The color names described in the table were taken from "Color Harmony Manual"*.

* Container Corporation of America, 1958.

Plate 1. Aerial mycelia of B-386 strain

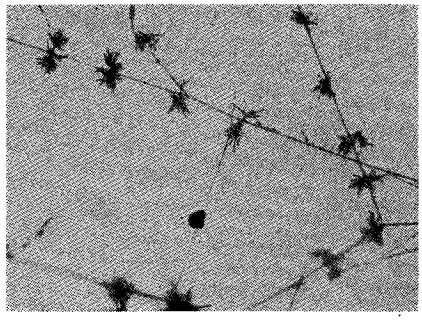


Plate 2. Spores of B-386 strain

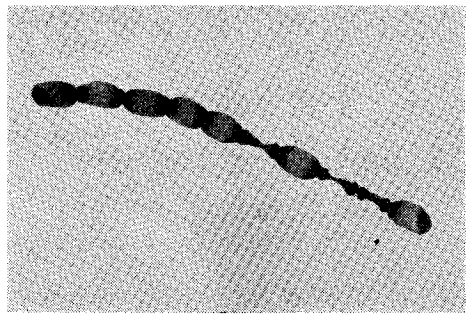


Table 1. Cultural characteristics of the strain B-386

Medium	Growth	Aerial mycelium	Substrate mycelium (Reverse)	Soluble pigment
Glycerin-Czapek agar	Good	Abundant, cottony, pearl Pink (3 ca)~Pearl (3 ba), many droplets	Light Tan (3 gc)-Cinnamon (314)	Biscuit (2 ec) ~Bamboo (2 gc)
Ammonium-Czapek agar	Poor	Short, Light Rose-Beige (4 ec)	Short mycelium penetrate into the medium, Biscuit (3ec)~Light Rose Beige (4 ec)	None
Starch agar	Moderate	Good, Fresh Pink (4 ca)~ Light Rose-Beige (4 ec)	Sometimes penetrate into the medium, Fresh Pink (4 ca)~ Light Rose Beige (4 ec)	None
Glycerin-starch-glutamate agar	Abundant	Cottony with many droplets, Biscuit (3 ec)~Light Tan (3 gc)	Good, penetrate deeply into the medium, Light Tan (3 gc)	Biscuit (2 ec)
Urea-glycerin-agar	Good	Cottony with many droplets, Light Rose-Beige (4 ec)	Light Wheat (2 ea)~Light Spice Brown (4 lg)	Putty ($1\frac{1}{2}$ ec) or Biscuit (2 ec)
Asparagine-glucose agar	Moderate	Moderate, many droplets. Shell Pink (5 ba)~Fresh Pink (5 ca)	Penetrate deeply into the medium. Pearl Pink (3 ca)~ Light Beige (3 ec)	None
Tyrosin agar	Moderate	Trace or none	Light Wheat (2 ea)~Cinnamon (3 le)	None
Ca-Malate agar	Moderate	Poor, very short, Light Beige (3 ec)	Shell Tint (3 ba)	None
Nurient agar	Poor	None	Colorless~Putty ($1\frac{1}{2}$ ec)	None
Bennett's agar	Abundant	Abundant, cottony with many droplets, Fresh Pink (4 ca)~ Light Rose Beige (4 ng)	Bamboo (2 gc)~Light Brown (4 ng)	None
Potato-glucose agar	Good	Good, many droplets, Light Rose Beige (4 ec)	Light Amber (3 ic)~Cinnamon (3 le)	None
Oatmeal agar	Abundant	Abundant, cottony with many droplets, Light Rose-Beige (4 ec)	Pearl Pink (3 ca)~Fresh Pink (4 ca)	None
Gelatin		None		None
Loeffler's blood serum	Poor	None		None
Egg	Good	Good. White-Pearl (2 ba)		None
Milk	Moderate	Moderate, White~Light Wheat (2 ea) or Biscuit (3 ec)		None
Potato plug	Moderate	White short mycelium are formed later		None
Carrot plug	Moderate	White mycelium covered the colonies slowly		None

The utilization of carbon sources by the strain was studied on PRIDHAM-GOTTLIEB's basal agar and CZAPEK's solution. D-Glucose, mannose, maltose, inositol, glycerine, dextrin and starch were utilized; fructose, rhamnose, xylose, galactose, lactose, raffinose, sucrose, sorbitol and salicin were not utilized.

Table 2. Physiological characteristics of B-386 strain

Property	Result
Nitrate reduction	Negative
H_2S production	Negative
Hydrolysis of starch	Positive
Cellulase reaction	Negative
Melanin formation	Negative

From the above characteristics, the B-386 strain was considered to belong to the genus *Streptomyces*, and to resemble the known species *St. cinnamomeus*[1], *St. hachijoensis*[2], *St. luteochromogenes*[3], *St. olivoreticuli*[4] and *St. griseoverticillatus*[5] in respect to typical whorl formation and similarities of cultural characteristics; nevertheless, B-386 strain was differentiated from those species by further studies:

(a) *St. cinnamomeus*[1] shows differences from the B-386 strain in dirty grayish white aerial mycelium and dirty grayish black vegetative mycelium on tyrosin agar, and pale violet to faint cinnamon growth with yellowish green to cream yellow vegetative mycelium on oatmeal agar. Differences are also found in some physiological properties such as rapid gelatin liquefaction and milk peptonization, utilization of xylose, fructose and galactose, and the production of sulfur-containing antibiotics, cinnamycin and duramycin.

(b) *St. hachijoensis*[2] is different from the B-386 strain in the yellow to brown growth, pinkish to orange colored soluble pigment formation, coagulation and peptonization on milk medium. Differences are also found in rapid liquefaction of gelatin and the production of an antifungal antibiotic, trichomycin.

(c) *St. luteochromogenes*[3] differs markedly from the B-386 strain in the brown soluble pigment production on calcium-malate agar, and the dark brown or black ring growth with brown soluble pigment formation on milk medium.

(d) *St. olivoreticuli*[4] shows glossy brown colonies on nutrient agar, and yellowish growth and aerial mycelium on starch agar, whereas the B-386 strain shows colorless putty colonies, and fresh pink or beige growth and aerial mycelium. Differences are also shown in brown soluble pigment formation of the former strain on many media such as nutrient agar, gelatin medium, egg medium, potato plug and carrot plug.

(e) The characteristic differences between *St. griseoverticillatus*[5] and the B-386 strain are given in Table 3. However, these two strains resemble each other in other taxonomical characteristics such as physiological properties and morphological properties on various media.

From these results, it was considered that the B-386 strain is most closely related to *St. griseoverticillatus* than other species cited above, however, it is not clearly distinguished as a different species from this streptomyces. Therefore the tuberactinomycin producing streptomyces, the B-386 strain, was designated as a variety of the species *griseoverticillatus*, and named *Streptomyces griseoverticillatus* var. *tuberacticus*.

Production and Isolation of Tuberactinomycin

Fermentation of the B-386 strain: The culture grew luxuriantly at 26~30°C in submerged culture. An inoculum was prepared by growing a vegetative suspension of the culture in a medium containing 1.0% corn starch (pH 7.0). The seed inoculum was transferred to a main fermentor which contained production medium of the following composition: 3.0% soy bean meal, 3.0% corn starch, 2.0% dextrose

and 1.5% NaCl (pH 7.0). The fermentation was carried out at 29°C for 4~6 days, and the antibiotic was assayed by a cup plate method using *Mycobacterium* ATCC 607 as a test micro-organism. In this assay, an inhibition zone of about 2.5 mm was obtained with a 250 mcg/ml solution of tuberactinomycin hydrochloride. In a typical fermentation, over 3,000 mcg/ml peak concentration of the antibiotic was achieved.

Table 3. Characteristic differences between B-386 strain and *St. griseoverticillatus*.

Characteristics	B-386 strain	*St. griseoverticillatus*
Urea-glycerol agar	SP: None or light putty or ecru.	SP: Light brown or light grayish brown
Milk peptonization	Negative	Positive
Growth on organic media (BENNETT'S, CZAPEK, potato glucose *etc.*)	AM: Cottony growth with many droplets.	AM: Sometimes cottony growth, droplets on some media.
Antibiotic elaborated	Tuberactinomycin	Takacidin

SP: Soluble pigment AM: Aerial mycelium

Isolation of tuberactinomycin: The antibiotic was recovered from the fermentation broth by means of a cation-exchange resin. The broth filtrate was passed through a column of Amberlite IRC-50 (Na^+). The resin was washed with water, then eluted with 1 N HCl. The active eluate was collected and neutralized with NaOH, then concentrated *in vacuo*. The concentrate was decolorized with active carbon (Shirasagi). The clear solution thus obtained was poured into eight volumes of cold methanol to precipitate the crude antibiotic hydrochloride. The precipitation was repeated again, and the antibiotic was dried *in vacuo*.

Further purification of the antibiotic was by ion-exchange chromatography with CM-Sephadex C-25 or Amberlite CG-50 buffered and developed with ammonium acetate buffer (0.6 M, pH 8.6). The main active fraction was collected and then passed through an Amberlite IRC-50 (Na^+) column. The antibiotic was eluted with 1 N HCl, and precipitated with methanol. The crystalline powder thus obtained was homogeneous by thin-layer chromatography.

Physico-chemical Properties of Tuberactinomycin

The physical and chemical properties of the purified tuberactinomycin are as follows:

(a) Solubility: Soluble in water. Slightly soluble in methanol, chloroform, *n*-butanol, ethyl acetate, dioxane, ethanol, pyridine. Insoluble in acetone, benzene, petroleum ether.

(b) Basicity: Basic (pKa 7.2, *ca.* 10.3 and >10).

(c) Melting point (as tuberactinomycin hydrochloride): 244~264°C (decomposed, not clearly observed).

(d) Elemental analysis (as hydrochloride): Found: C 33.95, H 5.66, N 22.69, Cl 12.40
Calculated for $C_{16}H_{31}N_9 \cdot 2HCl$: C 33.92, H 5.87, N 22.25, Cl 12.51

(e) Optical rotation (as hydrochloride): $[\alpha]_D^{25}$ −31.5° (*c* 1, H_2O)

(f) Ultraviolet absorption spectrum (as hydrochloride, shown in Fig. 1):
max: 268 mμ, $E_{1cm}^{1\%}$=330 (in H_2O)
max: 268.5 mμ, $E_{1cm}^{1\%}$=313 (in 0.1 N HCl)
max: 285.5 mμ, $E_{1cm}^{1\%}$=206.5 (in 0.1 N NaOH)

(g) Infrared absorption spectrum (as hydrochloride, in KBr tablet): Characteristic peaks at 3250, 1660, 1495, 1225, 1154 and 1045 cm^{-1} (Fig 2).

Fig. 1. Ultraviolet absorption spectrum of tuberactinomycin (in H_2O)

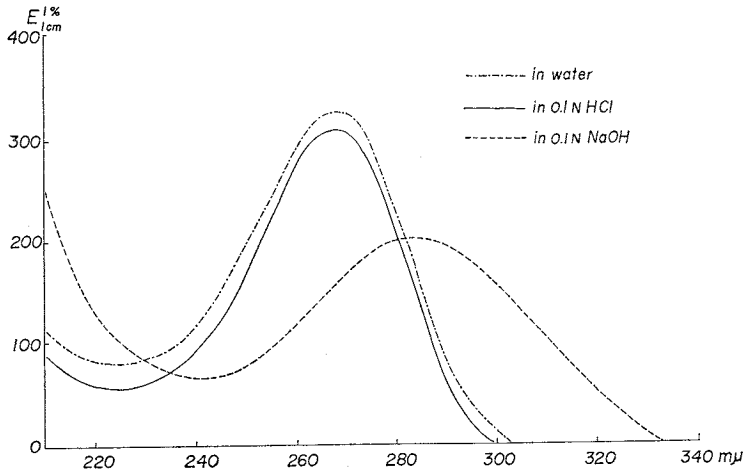
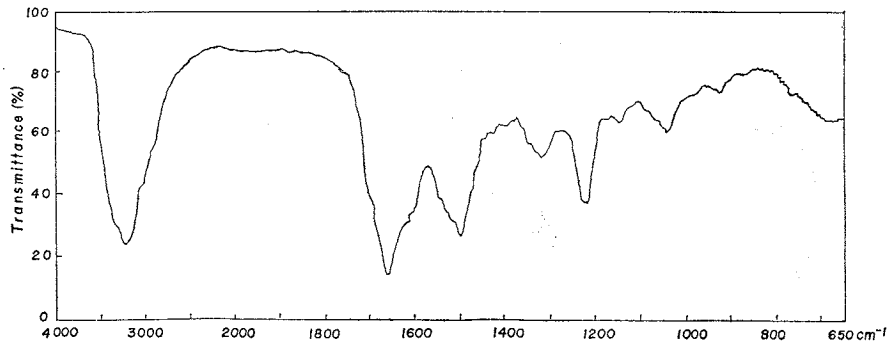


Fig. 2. Infrared absorption spectrum of tuberactinomycin (in KBr)



(h) Color reaction: Positive to ninhydrin, SAKAGUCHI, biuret reactions. Negative to isatin, PAULI, MOLISCH, ELSON-MORGAN, EHRLICH (yellow colored) reactions.

(i) Amino acid analysis: Serine, diamino-propionic acid and two unknown basic components are observed in the acid hydrolyzate (6 N HCl, 100°C, 24 hrs.) by TNBS method using an amino acid autoanalyzer[6].

From the data above, it was considered that tuberactinomycin belonged to the basic peptide antibiotic group including capreomycin[7] and viomycin[8]. However, tuberactinomycin was clearly different from those antibiotics by the following characteristics and considered to be a novel antibiotic.

Thin-layer chromatography: The Rf values of capreomycin and viomycin in thin-layer chromatography using a silica-gel plate (Kieselgel-G, Merck) developed with mixture of 10% aqueous ammonium acetate, acetone and 10% NH_4OH (9 : 10 : 0.5) were 0.15 and 0.24 respectively, whereas the Rf value of tuberactinomycin in the same system was 0.44.

Amino acid composition: It was reported that serine, diaminopropionic acid (DAPA) and β-lysine are found in both capreomycin and viomycin but alanine was found in only capreomycin; on the other hand, tuberactinomycin does not contain β-lysine and alanine in the acid hydrolyzate using Technicon's amino acid

Table 4. Minimum inhibitory concentration of tuberactinomycin

Media ♯	Test organisms	mcg/ml
A	*Pseudomonas aeruginosa*	12.5
	Escherichia coli NIHJ	25
	Escherichia coli B	100
	Salmonella paratyphi A	25
	Salmonella paratyphi B	100
	Salmonella enteritidis	100
	Shigella dysenteriae	100
	Shigella flexneri	12.5
	Shigella sonnei	>100
	Staphylococcus aureus FDA 209P	50
	Staphylococcus aureus Yoshioka	>100
	Staphylococcus albus	>100
	Staphylococcus citreus	50
	Micrococcus flavus	25
	Sarcina lutea ATCC 1001	100
	Vibrio comma (A)	>100
	Bacillus subtilis PCI 219	12.5
	Nocardia asteroides	3.2
B	*Mycobacterium* ATCC 607	12.5
	Mycobacterium phlei	3.2
	Mycobacterium avium F	6.3
C	*Mycobacterium tuberculosis* $H_{37}Rv$	4
	Mycobacterium tuberculosis SMR*	4
	Mycobacterium tuberculosis KMR*	4
	Mycobacterium tuberculosis CPMR*	32
	Mycobacterium tuberculosis VMR*	>32
D	*Aspergillus fumigatus*	200
	Aspergillus niger ATCC 6275	200
	Trichophyton rubrum	100
	Trichophyton asteroides	200
	Microsporum gypseum	50
	Cryptococcus neoformans	200
	Saccharomyces dreuisiae	200
	Candida albicans ATCC 7491	200
	Torula utilis (*Torulopsis utilis*)	200
	Trichoderma I-I ATCC 9245	200
	Piricularia oryzae NI 4192	200
	Penicillium chrysogenum Q-176	200

♯ Media and culture condition;
(A) Nutrient agar, pH 7.0, 37°C, 24-hour culture.
(B) Nutrient agar with 1 % glycerin, pH 8.0, 37°C, 48 hours.
(C) KIRCHNER medium with 10 % horse serum, 37°C, 3 weeks[10].
(D) Potato-dextrose agar, pH 7.0, 30°C, 48 hours.
* SMR, KMR, CPMR, VMR: $H_{37}Rv$ strains resistant to SM, KM, CPM, VM, respectively.

autoanalyzer. Further studies have shown that hydroxy-β-lysine homologue is present in tuberactinomycin instead of the β-lysine found in capreomycin and viomycin. (Personal communication from Dr. T. SHIBA, Osaka University.)

Biological Properties of Tuberactinomycin

The biological properties of purified tuberactinomycin may be summarized as follows[9]:

(1) Antimicrobial spectrum: The minimum inhibitory concentration of tuberactinomycin against a variety of microorganisms was examined by a serial agar streak dilution method. The results were given in Table 4. Tuberactinomycin inhibited the growth of some gram-positive and gram-negative bacteria, but it did not show activity against fungi and yeasts. It was particularly active against mycobacteria and pathogenic tubercular bacilli including strains resistant to streptomycin or kanamycin[10]. However, cross-resistance seemed to exist between tuberactinomycin and capreomycin or viomycin.

(2) Stability: Tuberactinomycin was markedly stable in acid or neutral solutions, and no reduction in the acitivity was observed after incubation with blood components.

(3) Toxicity: The acute toxicity (LD_{50}) in mice and rats by intramuscular injection was over 1,600 mg and 800 mg/kg respectively, and no adverse effects were observed after 30-day daily administrations of 200 mg/kg to rats by the same route.

Preliminary studies on the ototoxicity revealed that the effect of this antibiotic on the auditory function in guinea pigs is very weak.

(4) Chemotherapeutic effect: The effectiveness of tuberactinomycin on the therapy of experimental tuberculosis of mice and guinea pigs was tested by M. TOYOHARA[10] and Y. KOSEKI *et al.*[11] The results of their experiments were quite successful and suggested that the clinical application of this antibiotic might be promising.

Acknowledgement

The authors wish to express their sincere thanks to Dr. R. SHINOBU, Osaka University of Education, and to Dr. A. MATSUMAE, Kitasato Institute, for their kind suggestions in taxonomical studies. Thanks are due to Dr. T. WATANABE, this laboratory, for his valuable discussions, and to Mr. K. ASANO for his assistance in the fermentation work.

References

1) DVONCH, W.; O. L. SHOTWELL, R. G. BENEDICT, T. G. PRIDHAM & L. A. LINDENFELSER: Further studies on cinnamycin, a polypeptide antibiotic. Antibiot. & Chemoth. 4 : 1135~1142, 1954
2) YAMAGUCHI, T.: Studies on the antibiotic substance-producing strains. H-2075, H-2609 (*S. hachijoensis* nov. sp.) and H-3030. J. Antibiotics, Ser. A 7 : 10~14, 1954
3) MAEDA, K.; Y. OKAMI, R. UTAHARA, H. KOSAKA & H. UMEZAWA: An antibiotic, phthiomycin. J. Antibiotics, Ser. A 6 : 183, 1953
4) ARAI, T.; T. NAKADA & M. SUZUKI: Production of viomycin-like substance by a *Streptomyces*. Antibiot. & Chemoth. 7 : 435~442, 1957
5) SHINOBU, R. & Y. SHIMADA: On a new whirl-forming species of *Streptomyces*. Bot. Mag. Tokyo 75 : 170~175, 1962
6) SATAKE, K.; T. TAKE, A. MATSUO, K. TAZAKI & Y. HIRAGA: Amino acid analyzer using 2,4,6-trinitrobenzene sulfonic acid. J. Biochem. 60 : 12~16, 1966
7) HERR, E. B. Jr.: Chemical and biological properties of capreomycin and other peptide antibiotics. Antimicr. Agents & Chemoth. 1962 : 201~212, 1963
8) HASKELL, T. H.; S. A. FUSARI, R. P. FROHARDT & Q. R. BARTZ: The chemistry of viomycin. J. Am. Chem. Soc. 74 : 599~602, 1952
9) NAGATA, A; K. HAYANO & Y. HOSHINO: Biological and pharmacological studies on tuberactin, a new antibiotic. (in Japanese) Tuberculosis 43 : 249~253, 1968
10) TOYOHARA, M.: Study on the antituberculous activity of tuberactin, a new antibiotic. (in Japanese) Tuberculosis 43 : 245~248, 1968
11) KOSEKI, Y.; S. OKAMOTO, K. KANAI & T. MUROHASHI: Study on the experimental antituberculous activity of tuberactin. (Abstract) Tuberculosis 43 : 55~56, 1968