STUDIES ON TUBERACTINOMYCIN. II

ISOLATION AND PROPERTIES OF TUBERACTINOMYCIN-N, A NEW TUBERACTINOMYCIN GROUP ANTIBIOTIC

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A new basic peptide antibiotic of the tuberactinomycin group has been isolated from the culture broth of an artificial mutant derived from the streptomyces producing tuberactinomycin[1]. The antibiotic, named tuberactinomycin-N, showed somewhat stronger antituberculous activity and lower ototoxicity than the tuberactinomycin obtained from the parent strain. The isolation, characterization and properties of tuberactinomycin-N are described in this paper.

In the previous paper[1], we reported a new peptide antibiotic, tuberactinomycin (tuberactin), from *Streptomyces griseoverticillatus* var. *tuberacticus*. In the course of mutation study on this strain, a mutant obtained by nitrosoguanidine (N-methyl-N'-nitro-N-nitrosoguanidine) treatment, was found to produce a new peptide antibiotic different from tuberactinomycin in the culture. The antibiotic was isolated from the broth filtrate by means of ion-exchange resin adsorption, characterized as a new tuberactinomycin group antibiotic and named tuberactinomycin-N. At the same time, the tuberactinomycin[1] was renamed tuberactinomycin-A.

Method, Material and Result

1 Isolation of the Mutant Strain and its Taxonomy

The spore suspension of *Streptomyces griseoverticillatus* var. *tuberacticus* grown on BENNETT's agar slant was treated with nitrosoguanidine under the following conditions:

Spore count: 4.5×10^4/ml
Reagent concentration: 100 mcg/ml (in 0.1 M acetate buffer, pH 6.0)
Temperature: 37°C (in dark place)
Contacting period: 120 minutes

After the treatment, the spore suspension was diluted with water to give a mono spore culture on BENNETT's agar plate at 30°C. The survival rate was about 1.0 %. The isolated colonies were transferred to a shaking culture and tested for antibiotic production. From these mono-spore cultures, a few mutant strains were obtained which produce a different antibiotic than the original tuberactinomycin.

One of the strains, designated N 6-130, showed stable productivity of the new antibiotic and was compared for fermentation and taxonomical characteristics to the parent streptomyces.

Plate 1

In Plate 1 is shown a photograph of aerial mycelia of N 6-130 strain, and in Tables 1 and 2 are summarized the cultural and physiological characteristics. These characteristics are similar to those of the parent strain, *S. griseoverticillatus* var. *tuberacticus*, however, some minor differences were found between these strains as shown in Table 3. Thus the N6-130 strain was finally given the name of *S. griseoverticillatus* var. *tuberacticus* N 6-130.

Table 1. Cultural characteristics of N6-130 strain

Medium	Growth	Aerial mycelium	Substrate mycelium (Reverse)	Soluble pigment
CZAPEK agar	good	good, pearl pink~light rose beige	light maize~bamboo	none
Asparagine glucose agar	moderate	moderate, shell pink~fresh pink	pearl pink~biscuit	none
Tyrosin agar	moderate	very poor	light wheat~cinnamon	none
Urea glycerin agar	abundant	many droplets, good, light rose beige	light wheat~light spice brown	none~very slight putty ecru
Ca-malate agar	moderate	moderate, biscuit	pearl~shell tint	none
BENNETT's agar	abundant	fresh pink~light rose beige many droplets	bamboo~light brown	none
Nutrient agar	poor	none	putty	none
Peptone-glucose agar	good	light rose beige~rose beige	butterscotch~golden brown	none
Oatmeal agar	good	light rose beige, many droplets	pearl pink	none
Potato glucose agar	good	light rose beige, many droplets	light amber~cinnamon	none
Glucose bouillon	good	none	colorless mycelia at bottom of tube	none
Starch agar	moderate	good, fresh pink~light rose beige		none
Potato plug	moderate	white short mycelium are formed later		none
Carrot plug	none			
Gelatin	trace	none	ligh brown~cocoa brown slow liquefaction	none
LOEFFLER's blood serum	moderate		light maize~bamboo with opalescence, no liquefaction of coagulated serum	none
Egg	good	white~pearl		none
Milk	moderate	ring forming at surface, weakly coagulation of milk		none
Cellulose	none			

2 Production and Isolation of Tuberactinomycin-N

Fermentation of N6-130 strain.

An inoculum of N6-130 strain was prepared from a freeze-dried stock by growing in a shaking culture using a seed medium of the following composition; starch 1.0 %, peptone 1.0 %, meat-extract 1.0 %, molasses 1.0 % (pH 7.0 adjusted by NaOH).

The inoculum was tarnsferred into a 250-liter fermentation vessel containing a production medium: soy bean meal 4.0%, starch 3.0 %, dextrose 2.0 %, NaCl 1.5 % (pH 6.2). A typical time course of the fermentation is shown in Fig. 1.

Table 2, Physiological characteristics of N6-130 strain

Property	Result
Liquefaction of gelatin	Positive
Hydrolysis of starch	Positive
Nitrate reduction	Negative
Cellulase action	Negative
H_2S production	Negative
Melanin formation	Negative
Carbon utilization	Positive ; glucose, maltose, mannose, innositol, glycerin, dextrin, starch
	Negative ; xylose, fructose, rhamnose, raffinose, arabinose, salicin
	Uncertain ; lactose, saccharose, mannitol

Table 3. Comparison of N6-130 strain with *S. griseoverticillatus* var. *tuberacticus*

Items	N6-130 strain	*S. griseoverticillatus* var. *tuberacticus*
Growth on carrot plug	None	Moderate, white mycelium covered the colonies slowly
Milk coagulation	Weakly positive	Negative
Gelatin liquefaction	Positive	Negative
Soluble pigment production	On urea-glycerin agar	On glycerin-Czapek, glycerin-starch-glutamate, urea-glycerin agar
Antibiotic elaborated	Tuberactinomycin-N	Tuberactinomycin-A

The antibiotic was assayed by a cup plate method using *Mycobacterium* ATCC 607 as test microorganism. In a typical fermentation, the maximum production was achieved in 84 hours and reached 3,780 mcg/ml.

Fig. 1. Time course of 250-liter fermentation

Temperature 29°C
Aeration 50 % v/v. Agitation 300 r.p.m.

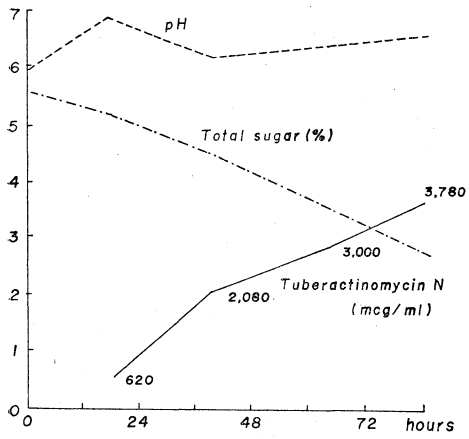
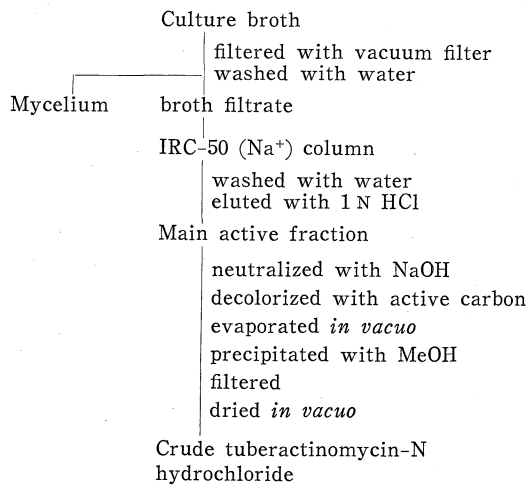


Fig. 2. Scheme of isolation



Isolation of tuberactinomycin-N.

The antibiotic was recovered from the culture filtrate by means of a cation-exchange resin, Amberlite IRC-50, as usually employed for an isolation of the water-soluble basic antibiotic. The isolation scheme is shown in Fig. 2.

The crude antibiotic bulk thus obtained was further purified by an ion-exchange chromatography using Amberlite CG-50 resin column buffered and developed with 0.6 M ammonium acetate (pH 9.0). The main active eluate was collected and charged again on Amberlite IRC-50 column. Then the antibiotic was eluted with a dilute mineral acid and precipitated twice with methanol. Tuberactinomycin-N hydrochloride or sulfate thus prepared was an easily water-soluble white powder, homogeneous on thin-layer chromatogram.

3 Physico-chemical Properties of Tuberactinomycin-N

Physico-chemical proporties of the tuberactinomycin-N hydrochloride are summarized in Table 4.

Table 4. Physico-chemical properties of tuberactinomycin-N hydrochloride

Solubility	very soluble in water, slightly soluble in methanol and ethanol, insoluble in common organic solvents
Basicity	$PKa_1=7.25$, $PKa_2=10.05$, $PKa_3>11$
Melting point	m. p. $\geqq$245°C (decomp.)
Optical rotation	$[\alpha]_D^{21}$ −19.1 (c 1.0, H_2O)
Elemental analysis	Found : C 37.70, H 6.12, N 22.50, Cl 13.32 Calcd. for $C_{25}H_{43}N_{13}O_{10}\cdot 3HCl$: C 37.76, H 5.83, N 22.90, Cl 13.38
Molecular weight	778 (titration)
UV spectrum	268 mμ ($E_{1cm}^{1\%}$ 342) in H_2O and N/10 HCl 288 mμ ($E_{1cm}^{1\%}$ 215) in N/10 NaOH (Fig. 3)
IR spectrum	(Fig. 4)
NMR spectrum	(Fig. 5)
Rf value on T. L. C.	0.30 (Merck, kiesel gel-G, phenol : H_2O : NH_4OH=150 : 50 : 3)
Color reaction	positive : ninhydrin, biuret negative : SAKAGUCHI, isatin
Amino acid composition	serine - α-β-diamino propionic acid - γ-hydroxy-β-lysine - capreomycidine - 3-ureido-dehydroalanine (2 : 1 : 1 : 1 : 1) (Fig. 6)

Differentiation from other related antibiotics.

(1) Difference from tuberactinomycin-A : Tuberactinomycin-N is closely related to tuberactinomycin-A which was produced by the parent strain. The differences in physico-chemical properties of these two compounds are summarized in Table 5.

(2) Difference from viomycin and capreomycins : Tuberactinomycin-N was differentiated from viomycin[2] and capreomycins[3] by thin-layer chromatography and amino acid analysis. Tuberactinomycin-N has capreomycidine as a guanidine moiety in the molecule but tuberactinomycin-A and viomycin contain tuberactidine[4]. It was also different from capreomycins by the presence of γ-hydroxy-β-lysine instead of the β-lysine found in capreomycin. Recently, the chemical structure

Fig. 3. Ultraviolet absorption spectra of tuberactinomycin-N hydrochloride (20 mcg/ml)

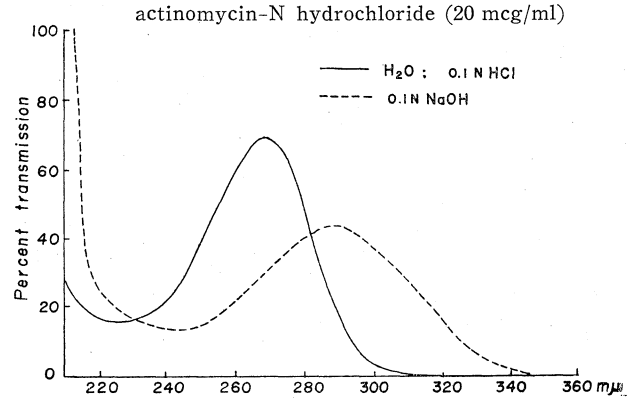


Table 5. Comparison of tuberactinomycin-N and tuberactinomycin-A

Items	Tuberactinomycin-N	Tuberactinomycin-A
Molecular formula	$C_{25}H_{43}N_{13}O_{10}\cdot 3HCl$	$C_{25}H_{43}N_{13}O_{11}\cdot 3HCl$
SAKAGUCHI reaction	negative	positive
Rf value in T. L. C.*	0.30	0.13
Guanidine moiety	capreomycidine	tuberactidine

* Solvent system : phenol - H_2O - NH_4OH (150 : 50 : 3)

Fig. 4. Infrared absorption spectrum of tuberactinomycin-N hydrochloride (KBr)

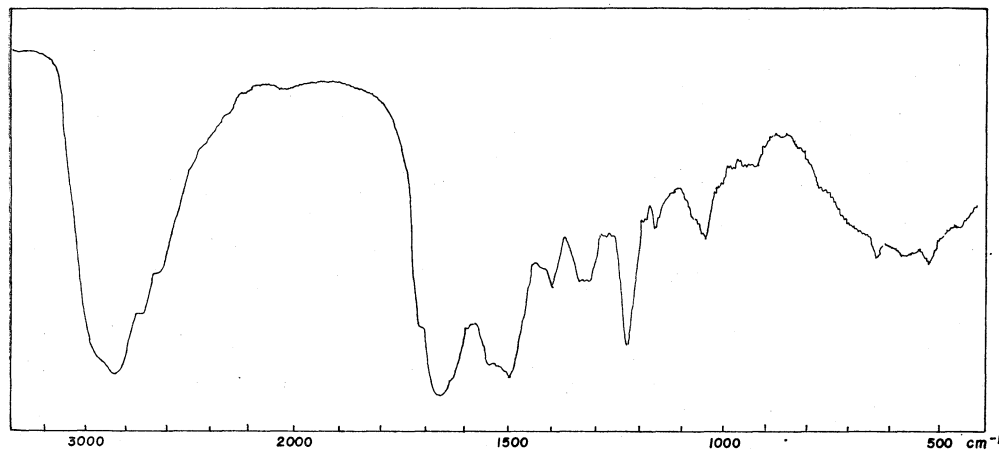


Fig. 5. NMR spectrum of tuberactinomycin-N (60 MHz, in D_2O)

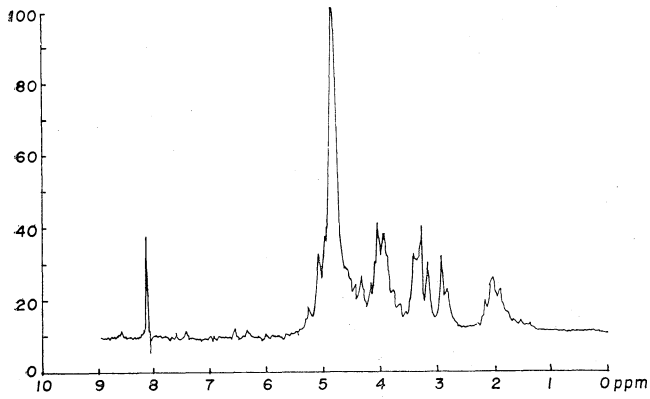
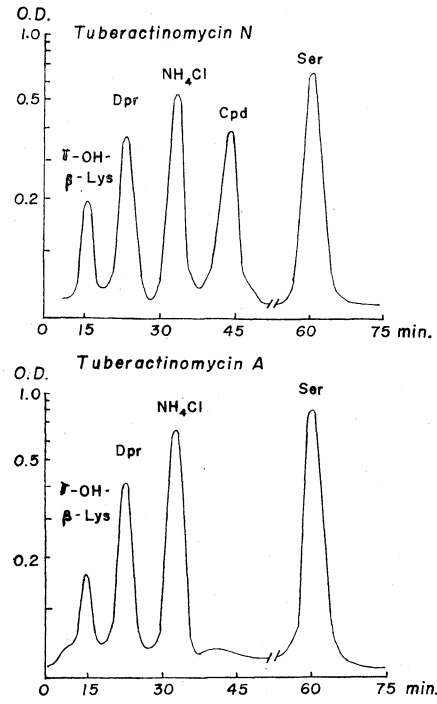


Fig. 6. Amino acid analysis of tuberactinomycin-A and -N (JEOL-JLC-5AH)



of tuberactinomycin group antibiotics was elucidated by cooperative studies with Dr. SHIBA *et al.* of Osaka University and confirmed through X-ray crystallographic study by the group of Dr. NAKATSU *et al.* of Kwansei-Gakuin University. The chemical structure is reported in another paper[6].

4 Biological Properties of Tuberactinomycin-N

(1) Antimicrobial spectrum: The minimum inhibitory concentration of tuberactinomycin-N against various microorganisms was determined by a serial agar dilution method and is shown in Table 6. Tuberactinomycin-N is active against some gram-positive bacteria and mycobacteria. Its antimycobacterial activity was somewhat stronger than that of tuberactinomycin-A, the original antibiotic.

(2) Antituberculous Activity: The effect of tuberactinomycin-N on the experimental tuberculosis in mice and guinea-pigs was studied by Dr. TOYOHARA and other groups. Tuberactinomycin-N was as effective as tuberactinomycin-A and viomycin.

(3) Toxicity: The acute toxicity (LD_{50}) in male mice was 385 mg/kg and over 2,000 mg/kg by intravenous and intramuscular administration respectively. In the 3-month toxicity study in rats at a daily dose of 400 mg/kg by intramuscular route, no adverse effects were observed on the body-weight gain, feed consumption, haematology, histology, *etc.*

(4) Ototoxicity: Dr. M. AKIYOSHI *et al.* compared the ototoxicity in guinea-pigs of tuberactinomycin-N, tuberactinomycin-A, viomycin and capreomycin. Based on the pinna reflex test and histopathological examination of the organ of Corti, it was concluded that tuberactinomycin-N is the least ototoxic of these antibiotics. The detailed data will be published in another report.

Table 6. Minimum inhibitory concentration of tuberactinomycin-N

Test organisms	Media	M. I. C. (mcg/ml)
Staphylococcus aureus FDA 209 P		>100
Staphylococcus citreus		50
Staphylococcus albus		>100
Bacillus subtilis PCI 219		12.5
Micrococcus flavus		>100
Sarcina lutea ATCC 1001		100
Vibrio comma (A)		>100
Nocardia asteroides	A	3.2
Escherichia coli NIHJ		50
Escherichia coli B		100
Salmonella paratyphi A		25
Salmonella paratyphi B		100
Pseudomonas aeruginosa		>100
Shigella sonnei		>100
Shigella flexineri		100
Mycobacterium ATCC 607		6.3
Mycobacterium avium F	B	1.6
Mycobacterium smegmatis		3.2
Mycobacterium tuberculosis $H_{37}Rv$	C	3.2

Media and culture condition;
A: Nutrient agar, pH 7.0, 37℃, 24 hours.
B: Nutrient agar with 1% glycerin, pH 8.0, 37℃, 24 hours.
C: KIRCHNER medium with 10% horse serum, 37℃, 3 weeks.

Discussion

A mutant strain of tuberactinomycin-producing streptomyces obtained with nitrosoguanidine treatment, has been shown to produce a new tuberactinomycin group antibiotic named tuberactinomycin-N.

This antibiotic shows similar properties to tuberactinomycin-A, though it contains capreomycidine as a guanidine moiety instead of tuberactidine. The isolated mutant has shown quite stable and high productivity of tuberactinomycin-N under the same culture conditions as used for the parent strain.

Tuberactinomycin-N shows somewhat stronger anti-tuberculous activity and less toxicity than tuberactinomycin-A. The ototoxic effect of tuberactinomycin-N in guinea-pigs is less than the related antituberculous peptide antibiotic, viomycin, capreomycin and tuberactinomycin-A.

The preliminary therapeutic evaluation of this antibiotic is now progressing and giving promissing results.

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