STUDIES ON TUBERACTINOMYCIN. III

ISOLATION AND CHARACTERIZATION OF TWO MINOR COMPONENTS, TUBERACTINOMYCIN B AND TUBERACTINOMYCIN O

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Two minor components of tuberactinomycin have been isolated from cultures of *Streptomyces griseoverticillatus* var. *tuberacticus*[1] and named tuberactinomycin B and tuberactinomycin O. Tuberactinomycin O is a new member of the tuberactinomycin family with biological activity similar to that of tuberactinomycin N. Tuberactinomycin B, on the other hand, has properties similar to viomycin and is considered to be identical with it. The characterization and properties of these two antibiotics are reported in this paper.

In our previous report on tuberactinomycin N[2], we indicated that a minor active component could be separated from the crude bulk by means of ion-exchange resin column chromatography. Using a similar procedure, another minor component has been isolated from the crude bulk of tuberactinomycin A.

These two minor components, both active against tubercule bacilli, have been designated tuberactinomycin O and tuberactinomycin B respectively.

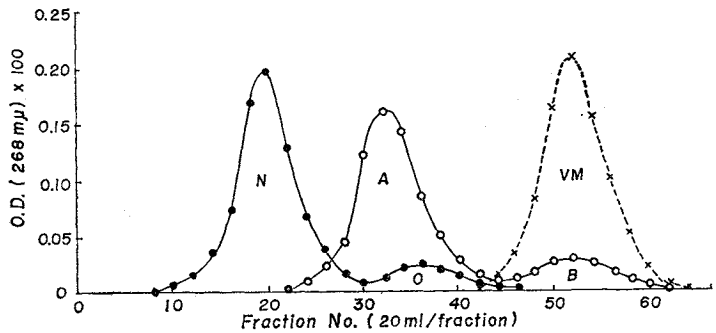
Materials, Methods and Results

1. Fractionation of the Crude Bulk Preparations

(1) Isolation of tuberactinomycin B from tuberactinomycin A bulk

Crude tuberactinomycin A[1] was processed as outlined in Fig. 2. The crude was dissolved in a small portion of 0.4 M ammonium acetate buffer (pH 9.0) and charged

Fig. 1. Elution pattern of tuberactinomycins and viomycin on CG-50 column chromatography
Buffer ; 0.4 M ammonium acetate pH 9.0 Column ; Amberlite CG-50 100~200 mesh (NH_4 form) 1×50 cm Sample ; 100 mg Flow rate ; 40 ml/hr.
A : tuberactinomycin A N : tuberactinomycin N
B : tuberactinomycin B O : tuberactinomycin O
VM : viomycin



on the Amberlite CG-50 (NH_4 form) column previously treated with the same buffer solution. The column was then developed with the buffer and the eluate was fractionated into 20-ml portions.

All fractions were assayed for biological activity against *Bacillus subtilis* PCI 219 and also by measuring the ultraviolet absorption at 268 mμ using a Hitachi model 101 spectrophotometer. As shown in Fig. 1, the elution pattern revealed the presence of two peaks in the crude tuberactinomycin A.

The minor component was further purified by treating the appropriate eluate fraction as indicated in Fig. 2 yielding tuberactinomycin B.

(2) Isolation of tuberactinomycin O from crude tuberactinomycin N

As reported in our previous paper[2], a new antibiotic of the tuberactinomycin family designated as tuberactinomycin N was produced by a mutant streptomycete. An active minor component was also isolated from the crude tuberactinomycin N by the procedure described in Fig. 2 and given the name of tuberactinomycin O.

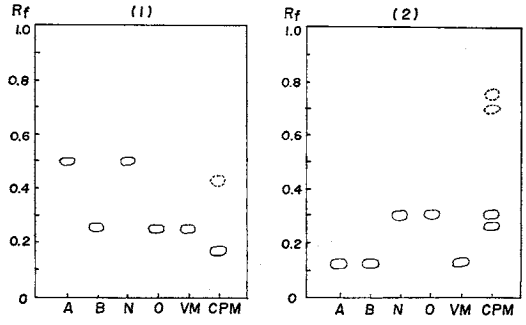
The elution patterns of crude tuberactinomycins and viomycin are given in Fig. 1.

Fig. 2. Isolation scheme of tuberactinomycins B and O

Culture broth of *St. griseoverticillatus* var. *tuberacticus* or N 6-130 strain
↓
Crude tuberactinomycins A and B complex or tuberactinomycins N and O complex
↓
CG-50 (NH_4 form) column
↓ buffered and developed with 0.4 M ammonium acetate buffer (pH 9.0)
I Active fraction Tuberactinomycin A or N
II Active fraction
↓ neutralized with HCl
↓ diluted with H_2O
IRC-50 (Na form) column
↓ eluted with 1 N HCl
Active fraction
↓ neutralized with NaOH
↓ decolorated with active carbon
↓ evaporated *in vacuo*
↓ precipitated with MeOH
↓ filtered
↓ dried *in vacuo*
Tuberactinomycin B or O

Fig. 3. Thin-layer chromatogram of tuberactinomycins, viomycin and capreomycins (Kiesel-gel G, Merck)

10% CH_3COONH_4-acetone 10% NH_4OH (9 : 10 : 1) Phenole-H_2O-28% ammonia water (30 : 10 : 0.6)
CPM ; Capreomycin



(3) Thin-layer chromatogram of tuberactinomycins

The four tuberactinomycin components were differentiated from each other also by thin-layer chromatography on silica-gel plates (Kiesel-gel G, Merck) with two solvent systems. The results are shown in Fig. 3. In these two systems, tuberactinomycin B and viomycin had identical Rf values and thus were presumed identical.

2. Physico-chemical Properties of Tuberactinomycins B and O

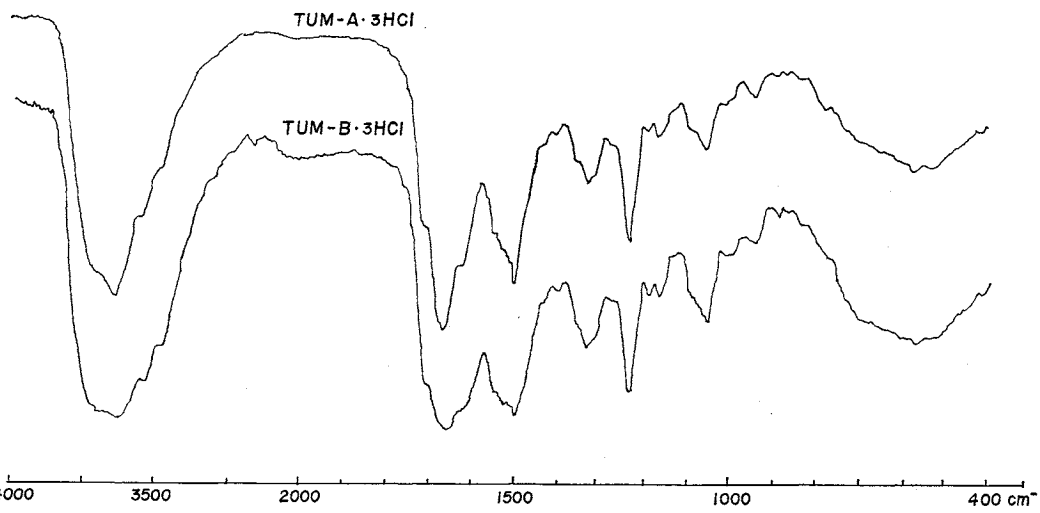
The physico-chemical properties of hydrochlorides of tuberactinomycins B and O are compared with those of tuberactinomycins A and N (Table 1).

Infrared spectra and results of the amino acid analysis are presented in Figs. 4, 5

Table 1. Physico-chemical properties of tuberactinomycins B, O, A and N hydrochlorides.

Properties	Tuberactinomycin-B	Tuberactinomycin-O	Tuberactinomycin-A	Tuberactinomycin-N
Solubility	very soluble in water, slightly soluble in MeOH and EtOH, insoluble in common organic solvents	very soluble in water, soluble in MeOH, slightly soluble in EtOH, insoluble in common organic solvents	very soluble in water, slightly soluble in MeOH and EtOH, insoluble in common organic solvents	very soluble in water, slightly soluble in MeOH and EtOH, insoluble in common organic solvents
pK	$pKa_1=7.30$, $pKa_2=10.10$, $pKa_3>11$	$pKa_1=7.35$, $pKa_2=9.80$, $pKa_3>11.5$	$pKa_1=7.2$, $pKa_2=10.3$, $pKa_3>10$	$pKa_1=7.25$, $pKa_2=10.05$, $pKa_3>11$
Melting point	m.p. >250°C (decomp.)	m.p. >240°C (decomp.)	m.p. ≧244°C (decomp.)	m.p. ≧245°C (decomp.)
Optical rotation	$[\alpha]_D^{21}$ −16.0 (*c* 1.0, H_2O)	$[\alpha]_D^{21}$ −19.6 (*c* 1.0, H_2O)	$[\alpha]_D^{21}$ −31.5 (*c* 1.0, H_2O)	$[\alpha]_D^{21}$ −19.1 (*c* 1.0, H_2O)
Molecular formula	$C_{25}H_{43}N_{13}O_{10}\cdot 3HCl$	$C_{25}H_{43}N_{13}O_9\cdot 3HCl$	$C_{25}H_{43}N_{13}O_{11}\cdot 3HCl$	$C_{25}H_{43}N_{13}O_{10}\cdot 3HCl$
U.V. spectrum	269 mμ ($E_{1cm}^{1\%}$ 315) in H_2O	268.5 mμ ($E_{1cm}^{1\%}$ 305) in H_2O	268 mμ ($E_{1cm}^{1\%}$ 330) in H_2O	268 mμ ($E_{1cm}^{1\%}$ 342) in H_2O
	and in N/10 HCl	269 mμ ($E_{1cm}^{1\%}$ 320) in N/10 HCl	268.5 mμ ($E_{1cm}^{1\%}$ 313) in N/10 HCl	and N/10 HCl
	286 mμ ($E_{1cm}^{1\%}$ 205) in N/10 NaOH	288 mμ ($E_{1cm}^{1\%}$ 170) in N/10 NaOH	285.5 mμ ($E_{1cm}^{1\%}$ 206.5) in N/10 NaOH	288 mμ ($E_{1cm}^{1\%}$ 215) in N/10 NaOH
Color reaction	positive : ninhydrin SAKAGUCHI	positive : ninhydrin	positive : ninhydrin SAKAGUCHI	positive: ninhydrin, biuret
	negative : isatin,	negative : SAKAGUCHI, isatin	negative : isatin	negative : SAKAGUCHI, isatin
I.R. spectrum	Fig. 4	Fig. 5	Fig. 4	Fig. 5

Fig. 4. Infrared absorption spectra of tuberactinomycins A and B hydrochlorides (KBr)



and 6, respectively.

(1) Identification of tuberactinomycin B with viomycin

From the properties of tuberactinomycin B described above and especially from the chromatographic patterns, the amino acid analysis and infrared spectra, we could not differentiate the antibiotic from viomycin. Moreover, the biological activity of tuberactinomycin B was in good agreement with that of viomycin. Consequently it

Fig. 5. Infrared absorption spectra of tuberactinomycins N and O hydrochlorides (KBr)

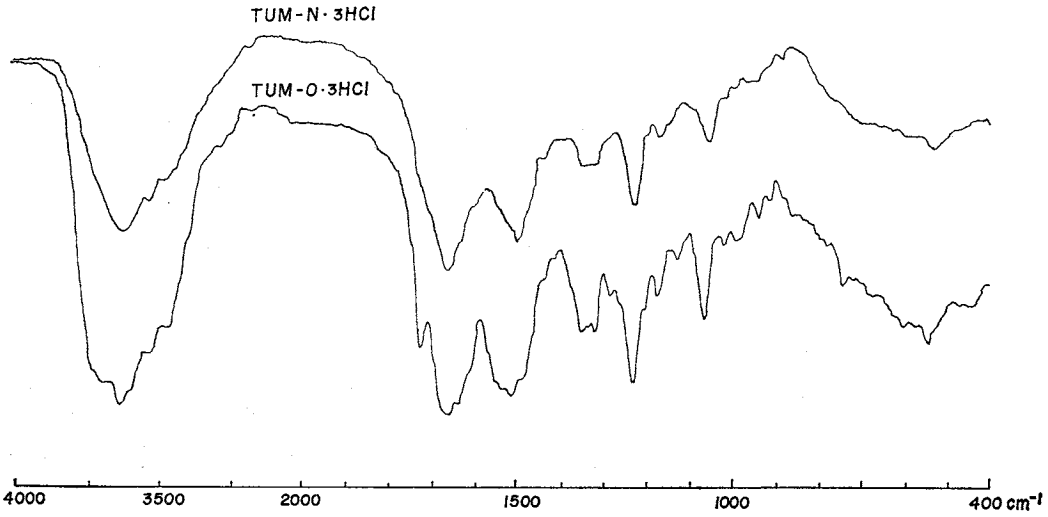
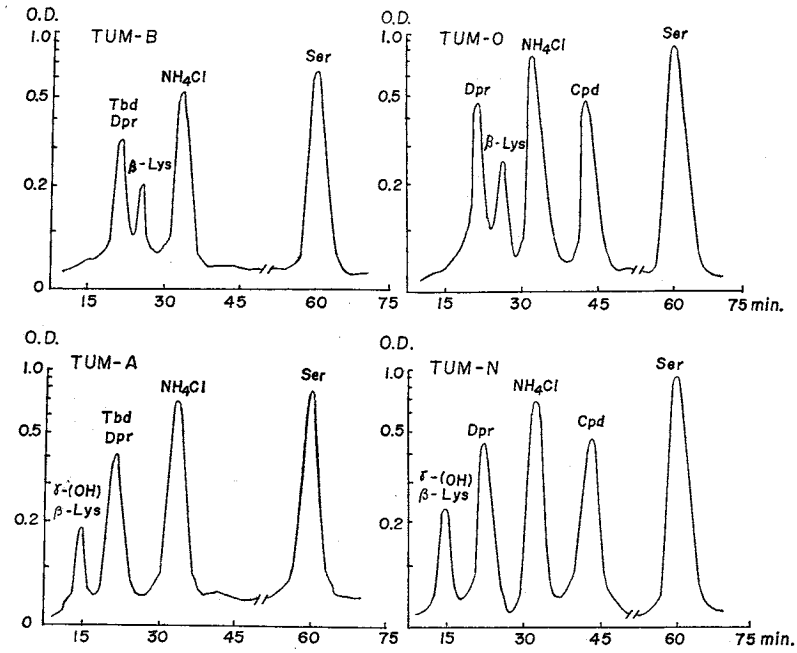


Fig. 6. Amino acid analysis of tuberactinomycins B, O, A and N (JEOL-JLC-5 AH)
Tbd ; L-tuberactidine, Dpr ; L-α,β-diamino propionic acid, β-Lys ; L-β-lysine, Ser ; L-serine, Cpd ; L-capreomycidine, γ-(OH)-β-Lys ; γ-hydroxy-L-β-lysine.



was concluded that tuberactinomycin B, the minor component of crude tuberactinomycin A, was identical with viomycin.

(2) Differentiation of tuberactinomycin O from other related antibiotics

On amino acid analysis, tuberactinomycin O gave capreomycidine which is the same guanidino-amino acid found in the capreomycins. However, the chromatographic patterns were different from those of viomycin and the capreomycins.

As shown in Fig. 6, the four tuberactinomycins differ each other in their L-β-lysine

Table 2. Amino acid composition of tuberactinomycins

	A	B	N	O
Molecular formula	$C_{25}H_{43}N_{13}O_{11}$	$C_{25}H_{43}N_{13}O_{10}$	$C_{25}H_{43}N_{13}O_{10}$	$C_{25}H_{43}N_{13}O_9$
Amino acid composition (mol.)	Ser (2) Dpr (1) Uda (1) γ-(OH)-β-Lys (1) Tbd (1)	Ser (2) Dpr (1) Uda (1) β-Lys (1) Tbd (1)	Ser (2) Dpr (1) Uda (1) γ-(OH)-β-Lys (1) Cpd (1)	Ser (2) Dpr (1) Uda (1) β-Lys (1) Cpd (1)

Ser : L-serine ; Dpr : L-α,β-diaminopropionic acid ; Uda : 3-ureidodehydro-alanine ; γ-(OH)-β-Lys : γ-hydroxy-L-β-lysine ; β-Lys : L-β-lysine ; Tbd : L-tuberactidine ; Cpd : L-capreomycidine.

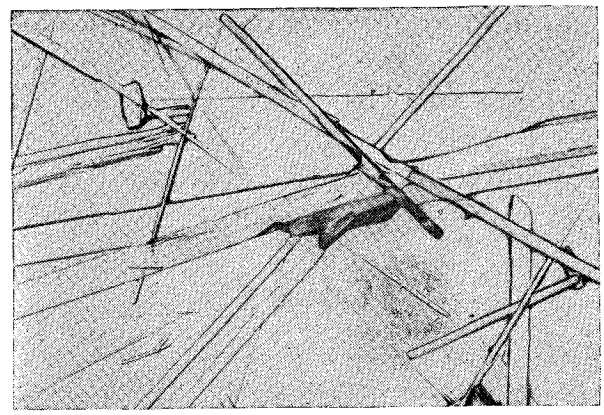
Table 3. Minimum inhibitory concentrations of tuberactinomycins

Test organisms	Media	M.I.C. (mcg/ml)			
		Tuberactinomycin B	Tuberactinomycin O	Tuberactinomycin A	Tuberactinomycin N
Staphylococcus aureus FDA 209 P	A	50	100	50	>100
Staphyloccoccus citreus		50	50	50	50
Bacillus subtilis PCI 219		3.2	3.2	12.5	12.5
Micrococcus flavus		50	50	25	>100
Sarcina lutea ATCC 1001		100	100	100	100
Nocardia asteroides		12.5	12.5	3.2	3.2
Escherichia coli NIHJ		25	50	25	50
Salmonella enteritidis Gaertner		25	50	100	—
Pseudomonas aeruginosa A_3		6.3	12.5	12.5	>100
Shigella flexneri		50	50	12.5	100
Klebsiella pneumoniae PCI 602		50	50	—	—
Mycobacterium ATCC 607	B	3.2	6.3	12.5	6.3

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.

and guanidino amino acid moieties. Tuberactinomycins A and N both contain γ-hydroxy-L-β-lysine rather than L-β-lysine. In addition, tuberactinomycins A and B contain L-tuberactidine while tuberactinomycins N and O both contain L-capreomycidine. All contain serine, L-α,β-diaminopropionic acid and 3-ureidodehydro-alanine.

Plate 1



(3) Chemical structure of tuberactinomycins

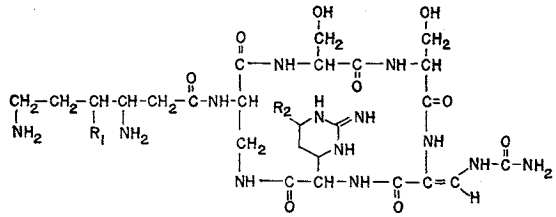
On the basis of the extreme similarity of amino acid compositions and properties of tuberactinomycins and viomycin, it is suspected that all these peptides may have the same amino acid sequence. However, the chemical studies on partial hydrolyzates of these antibiotics[3] suggested the amino acid sequence different from the structure for viomycin proposed was proposed by other workers[4].

The X-ray crystallographic analysis on the crystal of tuberactinomycin O

HCl·2 HBr·3 H_2O (Plate 1) was carried out by the co-operative work with Prof. NAKATSU of Kwansei-Gakuin University and his colleagues.

Thus the total chemical structure of tuberactinomycins were finally determined as shown in Fig. 7[5]. The detail of chemical studies mentioned above will be published in another paper.

Fig. 7. Chemical structure of tuberactinomycins



TUM	A	B	N	O
R_1	OH	H	OH	H
R_2	OH	OH	H	H

3. Biological Properties of Tuberactinomycins B and O

(1) Antimicrobial spectrum

The minimum inhibitory concentrations of tuberactinomycins B and O against various microorganisms as determined by serial agar dilution is summarized in Table 3.

These activities are similar to those of main components, tuberactinomycins A and N.

(2) Antituberculous activity

The antituberculous activities of the tuberactinomycins and viomycin against the human tubercule bacilli $H_{37}Rv$ in KIRCHNER's semiliquid medium and OGAWA's egg medium are shown in Table 4.

Table 4. Susceptibility of human tubercule bacilli $H_{37}Rv$ to tuberactinomycins and viomycin (VM)

Medium	Drug conc.	Effect on growth***				
		Tuberactinomycin				VM
	mcg/ml	A	B	N	O	
KIRCHNER*	16	—	—	—	—	—
	8	—	—	—	—	—
	4	‡	—	0.5	—	—
	2	‡	‡	‡	‡	‡
	1					‡
	0	‡	‡	‡	‡	‡
OGAWA**	50	—	—	—	—	—
	25	—	—	—	—	—
	10	‡	‡	‡	‡	‡
	5	‡	‡	‡	‡	‡
	0	‡	‡	‡	‡	‡

* KIRCHNER semi-liquid medium with 10% horse serum
** OGAWA egg medium
Inoculum: 10^{-3} mg, Incubation: 37℃, 21 days
*** Criteria of visual growth:
‡~‡: confluent to discrete colonies according to the amount of growth
+~−: or actual figures: less than 200 colonies or no growth
(Tested by Dr. Y. KOSEKI, National Inst. of Health, Tokyo)

(3) Toxicity

The acute toxicities (LD_{50}) of tuberactinomycins B and O in male mice by intravenous administration were 400 mg/kg and 370 mg/kg, respectively.

Discussion

Two minor biologically active components have been isolated from crude tuberactinomycin preparations by resin column chromatography using Amberlite CG-50. One, tuberactinomycin B, was identified as viomycin, and the another, tuberactinomycin O, proved to be a new member of the antibiotic family.

Thus, four components of this peptide family, tuberactinomycins A, B, N and O, have been isolated from two different producing strains. Two of them, tuberactinomycins A and B (viomycin) were products of the original soil isolate, whereas the other two components, tuberactinomycins N and O, were produced by an artificially-derived mutant of the parent streptomycete.

All four antibiotics contain L-serine, L-α,β-diaminopropionic acid and 3-ureidodehydroalanine. They differ as follows:

A : γ-Hydroxy-L-β-lysine, L-tuberactidine
N : γ-Hydroxy-L-β-lysine, L-capreomycidine (deoxytuberactidine)
B : L-β-Lysine, L-tuberactidine
O : L-β-Lysine, L-capreomycidine (deoxytuberactidine)

Therefore, the two minor components, tuberactinomycins B and O, are the deoxy-forms of the corresponding main components, tuberactinomycins A and N, respectively.

The details of the chemical studies on tuberactinomycins will be reported in another paper.

Acknowledgement

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