CHEMICAL STUDIES ON TUBERACTINOMYCIN. VIII[1)]

ISOLATION OF TUBERACTINAMINE N, THE CYCLIC PEPTIDE MOIETY OF TUBERACTINOMYCIN N, AND CONVERSION OF TUBERACTINOMYCIN N TO O

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Tuberactinamine N, the cyclic peptide moiety of tuberactinomycin N, was obtained in a crystalline state through liberation of γ-hydroxy-β-lysine from tuberactinomycin N by acid treatment. Tuberactinamine N possesses an intramolecular hydrogen bond in its molecule and showed antibacterial activities comparable to those of the original antibiotics. Conversion of tuberactinomycin N to O was achieved through coupling of diacyl-β-lysine with tuberactinamine N followed by removal of the protecting groups.

Peptide antibiotics tuberactinomycins isolated from *Streptomyces griseoverticillatus* var. *tuberacticus* comprise four congeners, tuberactinomycins A, B, N, and O, all of which are effective against tubercule bacilli in comparable concentrations.[2~4)] Their chemical structures had been already established by X-ray analysis and chemical investigations as shown in Fig. 1.[5~10)] An intramolecular hydrogen bond fixes the cyclic peptide moiety in a definite conformation for each antibiotic molecule.* The cyclic moiety of tuberactinomycins A and B without the amino acid in the branched chain is now called tuberactinamine A and that of tuberactinomycins N and O named tuberactinamine N. Isolation of these tuberactinamines seems to be significantly important from the viewpoint on relationship between antimicrobial activities and their conformations, and particularly for exploration of new semi-synthetic antibiotics with improved or prominent biological activities.

Fig. 1. Chemical structures of tuberactinomycins.

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

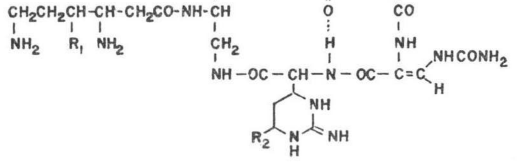
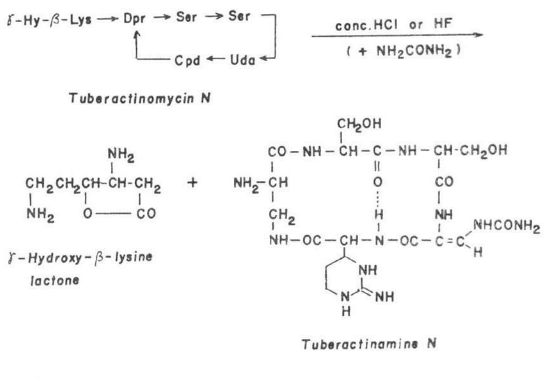


Fig. 2. Isolation scheme of tuberactinamine N.

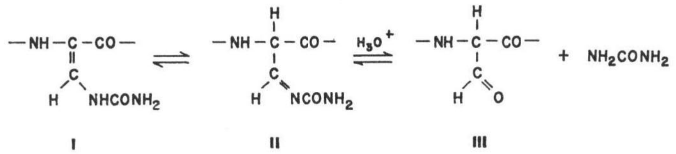


* Presence of the intramolecular hydrogen bond of β-turn type in crystalline state of tuberactinomycin O was first found by X-ray analysis.[6)] The same conformational situation in solutions of all congeners was identified by NMR analysis which will be reported soon elsewhere.

Of the four congeners of the antibiotics, tuberactinomycins A and N involve γ-hydroxy-β-lysine as the branched side chain. This amino acid was readily liberated in fact from the cyclic part accompanying with a lactonization of the hydroxy amino acid, when these antibiotics were treated with strong acid like concentrated sulfuric acid, concentrated hydrochloric acid or anhydrous hydrogen fluoride.[10] The selective cleavage of the peptide bond in the branched side chain from the cyclopeptide moiety is presumably assisted by the formation of γ-lactone. Consequently, optimal conditions were worked out for the isolation of tuberactinamine N (Fig. 2).

In order to achieve the above objective, it is needed to introduce some devise to prevent a possible degradation of 3-ureidodehydroalanine residue at the acid treatment. The safe presence of 3-ureidodehydroalanine residue in an aqueous solution may be influenced by an equilibrium between the ureido (**I**) and formyl (**III**) forms as shown in Fig. 3. Once the aldehyde **III** is formed, it may be further changed by oxidation irreversibly. In our experiment, on addition of excess urea in the procedure of the acid treatment, tuberactinamine N with the intact 3-ureidodehydroalanine form was prepared in a pure state in 35~55 % yield.

Fig. 3. Equilibrium in aqueous solution of 3-ureidodehydroalanine residue.



Tuberactinamine N thus obtained was purified by column chromatography on cation exchange resin and isolated as crystalline dihydrochloride. Elemental analysis, dissociation constants, UV and NMR of this compound ascertained the structure, cyclo (L-α-amino-β-alanyl-L-seryl-L-seryl-3-ureidodehydroalanyl-L-capreomycidyl).*

The NMR study of tuberactinamine N confirmed the presence of the same intramolecular hydrogen bond between α-NH of capreomycidine** residue and CO of serine residue adjacent to α,β-diaminopropionic acid residue to those found in all of tuberactinomycins.*** Since a ring conformation plays an important role for an exhibition of biological activities in peptide antibiotics, such similarity in conformations of tuberactinamine N and tuberactinomycins may suggest an antibiotic effect in the former. Actually, tuberactinamine N showed some antimicrobial activities for *Corynebacterium diphtheriae* P.W.8 and *Mycobacterium* ATCC 607 (Table 1), and also almost the same strength of antitubercular activities for several strains of human tubercule bacilli to those of tuberactinomycin N (Table 2). Furthermore, tuberactinamine N was found to have less toxicity compared with the original antibiotic tuberactinomycin N, in lethal amount for mice.

* In the nomenclature of tuberactinamine N, α-amino-β-alanyl was used to a residue name of α,β-diaminopropionic acid to make clear the peptide linkage.

** In the cases of tuberactinomycins A and B, α-NH group of tuberactidine in place of capreomycidine was concerned in the intramolecular hydrogen bond.

*** The summarized results of NMR studies for tuberactinamine and tuberactinomycins will be reported soon. Confirmation of the hydrogen bonds was done by temperature dependence and deuterium exchange experiments.

Table 1. Comparison of minimum inhibitory concentrations of tuberactinamine N with those of tuberactinomycin N

Test organisms	MIC (mcg/ml)	
	Tuberactinamine N	Tuberactinomycin N
Corynebacterium diphtheriae P.W.8	25	6.3
Bacillus subtilis ATCC 6633	>100	25
Escherichia coli NIHJ	>100	25
Salmonella typhosa H 901	>100	50
Klebsiella pneumoniae ATCC 10031	100	25
Proteus vulgaris OX 19	>100	50
Mycobacterium ATCC 607	12.5	6.3

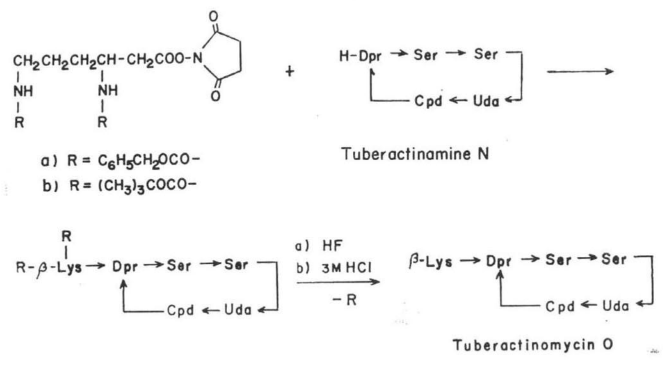
Table 2. Susceptibility of human tubercule bacilli isolated from patients to tuberactinamine N (Tua N) and tuberactinomycin N (Tum N)*

		Effect on growth**				
Drug concentration (mcg/ml)		0	12.5	25	50	100
Wild strain I	Tua N	++	++	+	$+_5$	−
	Tum N	++	++	+	$+_6$	−
Wild strain II	Tua N	++	++	++	−	−
	Tum N	++	++	++	−	−
BCG	Tua N	++	++	+	−	−
	Tum N	++	++	++	−	−

* 1% OGAWA egg medium
** Criteria of visual growth: ++: more than 200 colonies, +: less than 200 colonies, $+_5$: 5 colonies, −: no growth.

The isolation of tuberactinamine N opened a way for an interconversion in tuberactinomycin family. Thus, the known tuberactinomycin O was obtained by introduction of β-lysine at free α-amino group of α,β-diaminopropionic acid residue in tuberactinamine N which was derived from tuberactinomycin N. The introduction reaction was carried out as follows (Fig. 4). N^{β},N^{ε}-Dibenzyloxycarbonyl-L-β-lysine 1-succinimidyl ester was coupled with tuberactinamine

Fig. 4. Preparation of tuberactinomycin O from tuberactinamine N.



N in dimethylformamide. The coupling product was debenzyloxycarbonylated with anhydrous hydrogen fluoride to give tuberactinomycin O which was isolated as hydrochloride. The synthetic sample was completely identical with the natural antibiotic in all respects. Alternatively, use of N^{β},N^{ε}-di-*t*-butyloxycarbonyl-L-β-lysine 1-succinimidyl ester also gave tuberactinomycin O in a similar manner, thus providing a direct chemical evidence in favor of the proposed structure for tuberactinomycin N.

Furthermore, introductions of other acyl groups such as another amino acids, fatty acids, peptides into free amino group of tuberactinamine N may lead to production of new interesting antibiotics. In other words, tuberactinamine will become possibly an important key intermediate as well as 6-aminopenicillanic acid (6-APA) in penicillin chemistry.

Experimental

The NMR spectra were recorded on a Varian Associates XL-100-15 Spectrometer in deuterium oxide, and 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal reference. The specific rotation was obtained with a Perkin-Elmer 141 Polarimeter. UV spectra were recorded on a Hitachi 124 Spectrophotometer. Thin-layer chromatography was carried out by the ascending method on silica gel G using developing solvents of acetone - 10 % ammonium acetate - 10 % ammonium hydroxide (10 : 9 : 1) and *n*-butanol - acetic acid - water (4 : 1 : 2). Paper electrophoresis was carried out at 750 volt and 10 mA for 1.5 hours on Toyo Roshi No. 51 paper using buffer solution of pyridine - acetic acid - water (30 : 4 : 966).

Isolation of Tuberactinamine N.

(**a**) Use of concentrated hydrochloric acid. The mixture of tuberactinomycin N hydrochloride (50.0 g, 0.063 mol) and urea (50.0 g, 0.83 mol) was dissolved in 300 ml of concentrated hydrochloric acid. After allowing to stand at room temperature for 14 days, the solution was concentrated *in vacuo*. To the residue ethanol was added and precipitate was filtered off. Crude product (67.5 g) thus obtained was purified on Dowex 50 W$\times$2 (200$\sim$400 mesh, 2.7$\times$75 cm) column previously buffered with 0.2 M pyridine-acetate buffer (pH 3.1). Elution was carried out gradiently with 1,000 ml of pH 3.1 buffer to 2,000 ml of pH 5.0 buffer (2 M pyridine-acetate) and completed with additional 1,500 ml of the latter. Effluents next to fractions of γ-hydroxy-β-lysine, containing only tuberactinamine N, were collected and concentrated *in vacuo*. To a solution of the residue in 6 M hydrochloric acid, ethanol was added to precipitate tuberactinamine N hydrochloride, yield 21.1 g (55 %). Recrystallization from aqueous ethanol gave fine needles, mp 268$\sim$269°C (decomp.), $[\alpha]_D^{27} -6.3°$ (*c* 1, H_2O).

Found:	C, 36.63; H, 5.65; N, 24.67; Cl, 11.36 %.
Calcd. for $C_{19}H_{31}O_8N_{11}\cdot 2HCl\cdot 1/2\ H_2O$:	C, 36.60; H, 5.50; N, 24.71; Cl, 11.37 %.

UV spectrum: λ_{max} 268 nm (H_2O, ε 22,000); 268 nm (0.1 M HCl, ε 22,000); 286 nm (0.1 M NaOH, ε 14,000).

NMR spectrum (δ ppm): 1.77 (1H, m) and 2.10 (1H, m); 3.32 (3H, m); 3.90$\sim$4.30 (6H, m); 4.30$\sim$4.50 (2H, m); 4.84 (1H, t, J=5 Hz); 5.00 (1H, d, J=2.5 Hz); 8.04 (1H, s).

Unreacted tuberactinomycin N was recovered in 10$\sim$25 % yield from the other elution fraction. Some of tuberactinamine N was lost in the precipitation of the crude product and in the column purification. Therefore a true yield of tuberactinamine N could exceed 60 or 70 %. Only minor spot of positive ninhydrin reaction and undetectable by UV lamp being presumably ascribed to a desureido peptide was recognized besides tuberactinamine N and tuberactinomycin N on the paper electropherogram of the reaction mixture.

(**b**) Use of anhydrous hydrogen fluoride. Tuberactinomycin N hydrochloride (1.00 g, 1.26 mmol) and urea (1.00 g, 22.7 mmol) were dissolved in about 10 ml of anhydrous hydrogen fluoride in HF-reaction apparatus. After allowing to stand at room temperature for 5 days, hydrogen fluoride was completely evaporated *in vacuo*. The residue was dissolved in a small amount of 6 M hydrochloric acid and allowed to stand for several hours. Insoluble material was filtered off and ethanol was added to the filtrate. Precipitate (780 mg) thus obtained was treated as described under (a) using Dowex 50 W×2 (200~400 mesh, 1.2×45 cm) column and gradient buffer from pH 3.1 (150 ml) to pH 5.0 (300 ml). The overall yield of tuberactinamine N hydrochloride was 281 mg (36 %).

N^{β},N^{ε}-Dibenzyloxycarbonyl-L-β-lysine 1-Succinimidyl Ester.

To a solution of N^{β}, N^{ε}-dibenzyloxycarbonyl-L-β-lysine* (414 mg, 1 mmol) in 10 ml of tetrahydrofuran, dicyclohexylcarbodiimide (247 mg, 1.2 mmol) in 5 ml of tetrahydrofuran was added. After stirring at 0°C for 1 hour, N-hydroxysuccinimide (138 mg, 1.2 mmol) was added on cooling and then stirring was continued at room temperature overnight. Acetic acid (12 mg, 0.2 mmol) was added in order to decompose excess carbodiimide. Dicyclohexylurea precipitated was filtered off and the filtrate was concentrated *in vacuo*. When the residue was triturated with *n*-hexane and ether, crystalline product was obtained, yield 430 mg (84 %). Recrystallization from ethyl acetate-ether and *n*-hexane gave needles, mp 81~82°C.

Found: C, 61.01; H, 5.67; N, 8.32 %.
Calcd. for $C_{26}H_{29}O_8N_3$: C, 61.05; H, 5.71; N, 8.22 %.

N^{β},N^{ε}-Di-*t*-butyloxycarbonyl-L-β-lysine 1-Succinimidyl Ester.

To a suspension of N^{β},N^{ε}-di-*t*-butyloxycarbonyl-L-β-lysine dicyclohexylammonium salt* (528 mg, 1 mmol) in 10 ml of water, citric acid (210 mg, 1 mmol) was added and extraction with ethyl acetate was repeated three times. The extract was washed with saturated sodium chloride solution, dried over anhydrous sodium sulfate, and then concentrated *in vacuo*. Preparation of the active ester using the oily residue obtained above was carried out in a similar way to that of benzyloxycarbonyl derivative. Recrystallization from ether gave needles, yield 400 mg (90 %), mp 125~126°C.

Found: C, 53.91; H, 7.44; N, 9.50 %.
Calcd. for $C_{20}H_{33}O_8N_3$: C, 54.16; H, 7.50; N, 9.48 %.

Preparation of Tuberactinomycin O.

(a) To a suspension of tuberactinamine N hydrochloride (177 mg, 0.288 mmol) in 6 ml of dimethylformamide, N^{β},N^{ε}-dibenzyloxycarbonyl-L-β-lysine 1-succinimidyl ester (221 mg, 0.432 mmol) and triethylamine (35.0 mg, 0.346 mmol) were added. After stirring at room temperature for 17 hours, ether was added to the reaction mixture. Precipitate thus obtained was filtered off and debenzyloxycarbonylated with anhydrous hydrogen fluoride in the presence of 0.2 ml of anisole in HF-reaction apparatus. Removal of the protecting group was completed at 0°C for 30 minutes. After immediate evaporation of hydrogen fluoride, the residue was washed with benzene and dissolved in a small amount of water. Extractions with benzene and ethyl acetate were repeated and an aqueous layer was concentrated *in vacuo*. The residue was dissolved in 1 ml of 6 M HCl and ethanol was added. Precipitate thus obtained was recrystallized from aqueous ethanol, yield 206 mg (92 %), mp 240~245°C (decomp.) [natural compound; ~240°C (decomp.)], $[\alpha]_D^{23}$ −21.3° (*c* 1, H_2O), [natural compound; −19.6° (*c* 1, H_2O)]. Thin-layer chromatogram, paper electropherogram, and NMR spectrum of the synthetic compound were completely identical with those of natural tuberactinomycin O hydrochloride.

(b) N^{β},N^{ε}-Di-*t*-butyloxycarbonyl-L-β-lysine 1-succinimidyl ester (288 mg, 0.650 mmol) was condensed with tuberactinamine N hydrochloride (266 mg, 0.433 mmol) in dimethylformamide in the presence of triethylamine (52.6 mg, 0.520 mmol). After stirring at room temperature for 24 hours, ether was added. Gelatinous precipitate filtered was dissolved in water and extracted with ethyl acetate. An aqueous layer was concentrated *in vacuo* and the residue was dissolved in 2 ml of 3 M hydrochloric acid. After allowing to stand at room temperature for 1 hour, ethanol was added. Precipitate thus obtained was recrystallized from aqueous ethanol, yield 276 mg (82 %), mp 240~245°C (decomp.), $[\alpha]_D^{23}$ −21.3° (*c* 1, H_2O).

* A convenient method for preparation of diacyl β-lysine will be reported soon in this series of study.

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References

1) WAKAMIYA, T.; T. TESHIMA, I. KUBOTA, T. SHIBA & T. KANEKO: Chemical studies on tuberactinomycin. VII. Synthesis of γ-hydroxy-β-lysine. Bull. Chem. Soc. Jap. 47: 2292~2296, 1974
2) NAGATA, A.; T. ANDO, R. IZUMI, H. SAKAKIBARA, T. TAKE, K. HAYANO & J. ABE: Studies on tuberactinomycin (tuberactin), a new antibiotic. I. Taxonomy of producing strain, isolation and characterization. J. Antibiotics 21: 681~687, 1968
3) ANDO, T.; K. MATSUURA, R. IZUMI, T. NODA, T. TAKE, A. NAGATA & J. ABE: Studies on tuberactinomycin. II. Isolation and properties of tuberactinomycin N, a new tuberactinomycin group antibiotic. J. Antibiotics 24: 680~686, 1971
4) IZUMI, R.; T. NODA, T. ANDO, T. TAKE & A. NAGATA: Studies on tuberactinomycin. III. Isolation and characterization of two minor components, tuberactinomycin B and tuberactinomycin O. J. Antibiotics 25: 201~207, 1972
5) WAKAMIYA, T.; T. SHIBA, T. KANEKO, H. SAKAKIBARA, T. TAKE & J. ABE: Chemical studies on tuberactinomycin. I. The structure of tuberactidine, guanidino amino acid component. Tetrahedron Letters 1970-40: 3497~3500, 1970
6) YOSHIOKA, H.; T. AOKI, H. GOKO, K. NAKATSU, T. NODA, H. SAKAKIBARA, T. TAKE, A. NAGATA, J. ABE, T. WAKAMIYA, T. SHIBA & T. KANEKO: Chemical studies on tuberactinomycin. II. The structure of tuberactinomycin O. Tetrahedron Letters 1971-23: 2043~2046, 1971
7) NODA, T.; T. TAKE, A. NAGATA, T. WAKAMIYA & T. SHIBA: Chemical studies on tuberactinomycin. III. The chemical structure of viomycin (tuberactinomycin B). J. Antibiotics 25: 427~428, 1972
8) WAKAMIYA, T.; T. SHIBA & T. KANEKO: Chemical studies on tuberactinomycin. IV. The chemical structure of γ-hydroxy-β-lysine, a new amino acid isolated from tuberactinomycin A and N. Bull. Chem. Soc. Jap. 45: 3668~3672, 1972
9) WAKAMIYA, T.; T. SHIBA, T. KANEKO, H. SAKAKIBARA, T. NODA & T. TAKE: Chemical studies on tuberactinomycin. V. Structures of guanidino amino acids in tuberactinomycins. Bull. Chem. Soc. Jap. 46: 949~954, 1973
10) WAKAMIYA, T. & T. SHIBA: Chemical studies on tuberactinomycin. VI. The absolute configuration of γ-hydroxy-β-lysine in tuberactinomycins A and N. J. Antibiotics 27: 900~902, 1974