STUDIES ON T-2636 ANTIBIOTICS. III

A NEW COMPONENT, T-2636 F

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A new antibiotic T-2636 F was isolated from three different sources, 1) the reaction mixture of T-2636 D with the esterase of *Streptomyces rochei* var. *volubilis* (No. T-2636), 2) the filtered broth of the *Streptomyces* strain, and 3) the bile and urine samples of rabbits, which was pre-administered T-2636 C parenterally. T-2636 F has the following chemical properties: colorless needles, m. p. 178~179°C (decomp.), λ_{max} 228 mμ (EtOH), $[\alpha]_D^{24}$ −210° (*c* 1.0, DMF), molecular formula $C_{25}H_{35}NO_7 \cdot CH_3OH$. T-2636 F shows moderate activities against Gram-positive bacteria. The preparation of the enzyme of *Streptomyces* and application of the enzyme for bioassay of T-2636 antibiotics are described.

In the previous paper[1,2], it was reported that T-2636 antibiotics A, B, C, D, E and M were found in the fermented broth of the *Streptomyces rochei* var. *volubilis*, and T-2636 A was hydrolyzed to T-2636 C by the *Streptomyces* esterase.

A new component, T-2636 F was isolated from the hydrolysate of T-2636 D with the *Streptomyces* enzyme or several fungal enzymes. This component was also isolated from the filtered broth of the *Streptomyces*, and was found as a metabolite of T-2636 C in rabbit. Isolation, chemical and microbiological properties and sensitive assay method of T-2636 F are reported in this paper.

Isolation of T-2636 F

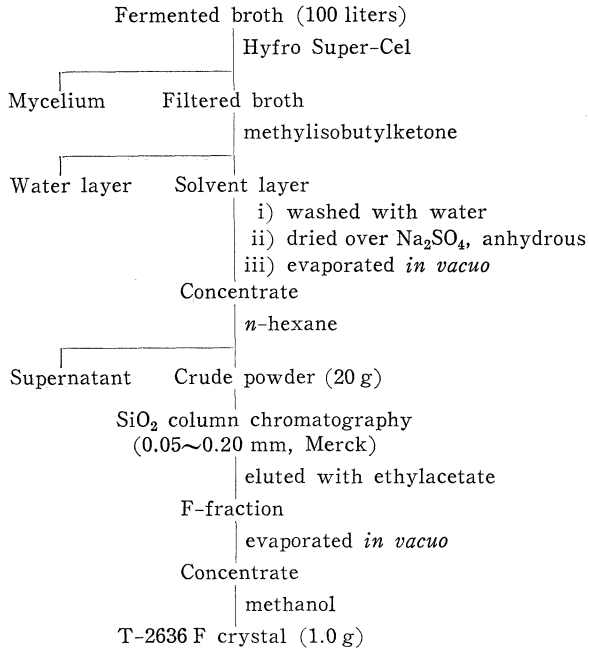
T-2636 D was incubated with *Streptomyces* enzyme I[1]. The reaction mixture was extracted with ethylacetate, and the solvent layer was evaporated *in vacuo*. The residue was crystallized from methanol to obtain T-2636 F. Enzyme preparations of *Trametes sanguinea*[3] and *Aspergillus sojae*[4] can be also used for the conversion of T-2636 D to F.

Submerged culture of *Streptomyces rochei* var. *volubilis* was performed in a medium containing glucose 5%, glycerol 0.5%, peptone 1.0%, meat extract 0.5%, soybean flour 1.0%, magnesium sulfate 0.1%, calcium carbonate 0.5% (pH 7.0), and soybean oil 0.3%. Fermented broth was harvested after 43 hours. Form the filtered broth T-2636 F was isolated as a minor component of T-2636 antibiotics by the procedure summarized in Fig. 1.

After intravenous or intraperitoneal administration of T-2636 C into rabbits, the

bile and urine were collected for 6 hours (TSUCHIYA, K., unpublished). The bile was extracted with ethylacetate and the solvent layer was evaporated *in vacuo*. The residue was crystallized from methanol to obtain T-2636 F. From the urine, the substances having same Rf value for T-2636 C and F were also obtained.

Fig. 1. Isolation of T-2636 F from fermented broth of *Streptomyces rochei* var. *volubilis*



Chemical Properties of T-2636 F

T-2636 F is neutral colorless needles melting at 178~179°C with decomposition. It has a specific rotation of $[\alpha]_D^{24}$ −210° (*c* 1.0, dimethylformamide). It is soluble in tetrahydrofuran, dimethylformamide and pyridine, and is moderately soluble in acetone, methanol and ethanol. This antibiotic gives violet-blue color in carbomycin test, but is negative in erythromycin test[5].

The molecular weight is assumed to be 461, because vapor pressure osmometry in acetone gives a value of 503, and the mass spectrum shows the highest mass number of m/e 443 ($M^+ - H_2O$). Elemental analysis was found: C 63.06, 62.97, H 7.94, 7.91,

Fig. 2. IR spectrum of T-2636 F (KBr)

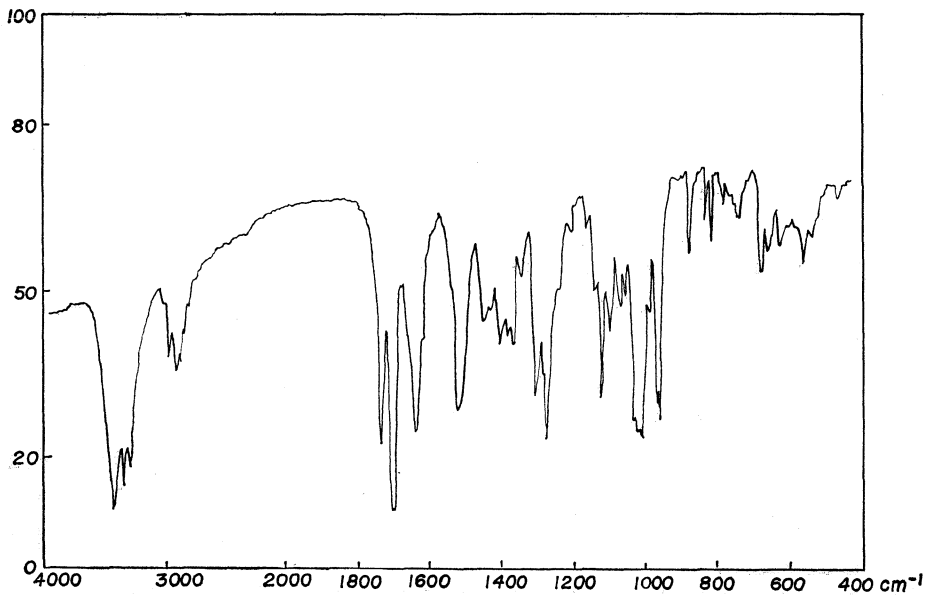
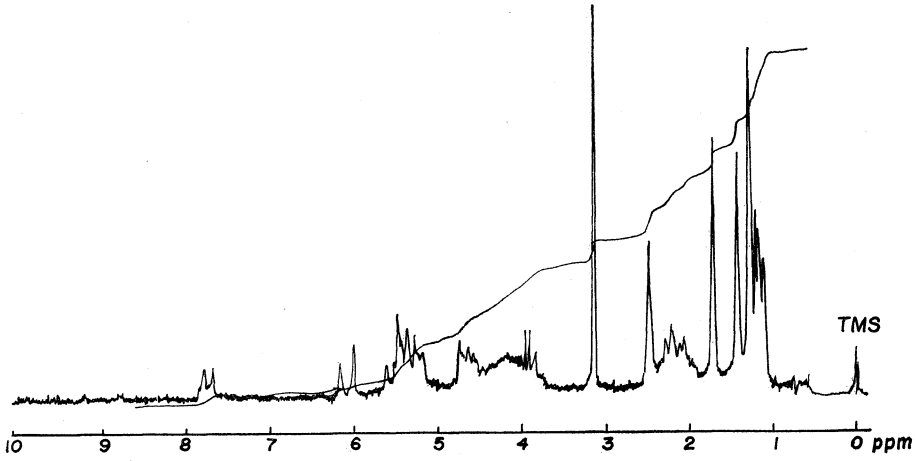


Fig. 3. NMR spectrum of T-2636 F (100 Mc d_6-DMSO)



N 2.89, 2.90 (%). Calculated for $C_{25}H_{35}NO_7 \cdot CH_3OH$: C 63.27, H 7.96, N 2.84 (%), (one mole of methanol was observed in the nuclear magnetic resonance spectrum of the crystal).

The ultraviolet spectrum in ethanol shows a maximum at 228 mμ (ε=48,400). The infrared spectrum indicates the presence of lactone (ν_{max}^{KBr}=1730 cm^{-1}) and ketone (1705 cm^{-1}) groups (Fig. 2). There is no absorption corresponding to -OAc in T-2636 D or the methyl ketone in T-2636 C. In the nuclear magnetic resonance spectrum lack of -OAc group was observed (Fig. 3).

Thin-layer chromatography of T-2636 F on silica gel gives a single spot, and Rf-values are as follows: 0.22 with chloroform - methanol (93 : 7), 0.27 with ethylacetate - acetone (95 : 5), 0.25 with methylethylketone - ethylether (1 : 3), 0.40 with benzene - acetone (1 : 1).

The structural relationship between T-2636 F and D was

Table 1. Antimicrobial activity* of T-2636 F and A

Test organisms	MIC (μg/ml)		A/F
	T-2636 F	T-2636 A	
Staphylococcus aureus 209P-JC	12.5	6.25	0.5
S. aureus 209P-IFO 3061	50	25	0.5
S. aureus Pc-R, Mc-R	50	25	0.5
S. epidermidis IFO 12993	12.5	12.5	1
Micrococcus subflavus IFO 3062	100	100	1
M. caseolyticus IFO 3760	25	25	1
M. aurantiacus IFO 12422	0.78	6.25	8
M. flavus IFO 3242	3.13	6.25	2
Sarcina lutea PCI 1001	0.20	0.78	4
S. albida IFO 3063	3.13	3.13	1
S. valiabilis IFO 3067	1.56	3.13	2
S. subflava IFO 12992	0.78	1.56	2
Bacillus subtilis PCI 219	>100	>100	—
B. brevis IFO 3331	100	100	1
B. cereus IFO 3466	>100	>100	—
Escherichia coli IFO 3044	>100	>100	—
Proteus vulgaris IFO 3045	100	100	1
Mycobacterium sp. ATCC 607	>100	>100	—
Aspergillus niger IFO 4066	>100	>100	—
Candida albicans IFO 0583	>100	>100	—

* Activity of antibiotics was determined by agar dilution method (nutrient agar, pH 7.0).

Table 2. Assay of T-2636 antibiotics

Antibiotic tested	Assay without enzymatic conversion	Assay with enzymatic conversion
T-2636 A	4.1%	97.5%
T-2636 C	100	100
T-2636 D	0.4	101
T-2636 F	3.9	97.5

Reaction mixture for enzymatic conversion: Antibiotic solution (20 μg/ml) 0.5 ml, 0.2% enzyme solution 1.0 ml, MICHAELIS buffer (pH 3.0) 0.5 ml.
Incubation: 37℃, 2 hours.
Assay: the cup method (*S. lutea*).
Standard: T-2636 C (10 μg/ml) as 100% potency.

established by the comparison of their derivatives. T-2636 F was treated with acetic anhydride in pyridine to give its triacetate, which was in complete accordance with T-2636 D diacetate[2] by m.p., IR, NMR and MS spectra.

Antimicrobial Spectrum of T-2636 F

Antimicrobial activities of T-2636 F were examined by the agar dilution method. The results are shown in Table 1. T-2636 F shows the antibacterial spectrum similar to that of T-2636 A. T-2636 F is active against Gram-positive bacteria, and its activity against some of *Sarcina* and *Micrococcus* species is more potent than that of T-2636 A.

Enzyme Preparation for the Assay of T-2636 Antibiotics

The *Streptomyces* enzyme I[1] was partially purified by means of fractional precipitation with ammonium sulfate in the range of 0.26~0.53 saturation. After dialysis against water, the fraction was freeze-dried. This preparation had not only an esterase activity (T-2636 A→C or T-2636 D→F), but also a dehydrogenase activity (T-2636 F→C). This enzyme preparation can be used for the sensitive assay of T-2636 F or other T-2636 antibiotics (Table 2).

Discussion

On the basis of chemical and microbiological properties, T-2636 F is found to be a new component in T-2636 antibiotics. From their structural relationships[6], it is obvious that one of the *Streptomyces* extracellular enzyme (an esterase) deacetylates the acetyl group at C_{14} of T-2636 D, and also another enzyme of the *Streptomyces* (a dehydrogenase) dehydrogenates the hydroxyl group at C_2' of T-2636 F. An enzyme in rabbit also reduces T-2636 C to T-2636 F which is more polar than the former, and then excretion of the antibiotic is facilitated.

Experimental

Isolation of T-2636 F

(1) From the reaction mixture of T-2636 D with esterase: Four grams of T-2636 D was dissolved in 600 ml of methanol. One gram of the *Streptomyces* enzyme I[1] was dissolved in 2.5 liters of water. The both solutions were mixed and the mixture was incubated for 3 hours at 34~36°C. From the reaction mixture methanol was eliminated *in vacuo*, and the concentrate was extracted twice with ethylacetate (1.0 liter and 0.5 liter). The extract was washed with 800 ml of 3% sodium bicarbonate and with water subsequently. The washed ethylacetate solution was concentrated *in vacuo* to dryness. The residue was crystallized from methanol to yield 1.4 g of T-2636 F as colorless needles.

(2) From the fermented broth: A 100-liter portion of 47-hour fermented broth of *Streptomyces rochei* var. *volubilis* (No. T-2636) was filtered with 2 kg of Hyflo Super-Cel to give 92 liters of filtered broth. The filtered broth was extracted twice with each 46 liters of methylisobuthylketone. The organic solvent layer was washed with water, dried over anhydrous sodium sulfate, and concentrated *in vacuo* to 1 liter. One liter of *n*-hexane was added to the concentrate and the yellow precipitate appeared was collected by filtration. Twenty grams of the precipitate dissolved in 300 ml of ethylacetate and chromatographed on the column of 500 g of silica gel (0.05~0.20 mm, Merck). T-2636 antibiotics were fractionated by developing the column with 9 liters of ethylacetate. Each fraction (100 ml size) was examined by thin-layer chromatography, and T-2636 F fractions were combined. The combined fractions were concentrated *in vacuo* to dryness. The residue

was crystallized from methanol to yield 1 g of T-2636 F. Elemental analysis was found; C 63.06, H 7.94, N 2.89 (%). The crystal was in good accord with T-2636 F obtained from D in the data of $[\alpha]_D^{24}$, UV, IR and NMR spectra.

(3) From bile of rabbits: Each 50 mg of T-2636 C was administerd intravenously into two rabbits, weighing about 3 kg. Bile was collected for six hours via a fine polyethylene tube from the biliary tract of animal surgically (TSUCHIYA, K., unpublished). T-2636 antibiotic containing in bile was estimated by UV absorption at 227 mμ (9.0 mg) or the cup method after enzymatic conversion to T-2636 C (6.4 mg). One hundred and five milliliters of bile was extracted twice with ethylacetate (each 70 ml). The organic solvent layer was washed with water and concentrated *in vacuo*. Seven milligrams of crude material was crystallized from methanol to yield 4.5 mg of T-2636 F as colorless needles, which was in good accord with T-2636 F obtained from D in the data of m. p., IR and MS spectra.

Preparation of enzyme

A 80-liter portion of filtered broth of the *Streptomyces* was extracted with ethylacetate to remove T-2636 antibiotics and the water layer was concentrated *in vacuo* to 15 liters. Eighty liters of cooled ethanol was added to the cooled concentrate and precipitate was collected by filtration through Hyflo Super-Cel layer. Wet precipitate was dissolved in water and the solution was made up to 4 liters. Eight hundred grams of ammonium sulfate was added to the solution. The supernatant was separated centrifugally and to which 800 g of ammonium sulfate was added again. The precipitate occurred was collected by centrifuge and dissolved in 100 ml of 0.1 M phosphate buffer (pH 6.0). The solution was dialyzed against 2 liters of water for 18 hours, and inner solution was freeze-dried to yield 11.5 g of enzyme preparation.

Bioautography of T-2636 antibiotics after thin-layer chromatography

Ten microliters of solution containing 0.025~0.25 μg of T-2636 antibiotics was applied on silica gel plate (Tokyo Chemical Industry Co.). After developing with ethylacetate-acetone (95 : 5), the plate was spraied with 1 % methanolic solution of enzyme preparation obtained from the *Streptomyces*. Then T-2636 A, D, F were converted to T-2636 C rapidly, and detection of each spot was carried out by usual bioautographic method with *Sarcina lutea* PCI 1001 as test organism.

Sensitive assay of T-2636 F

The mixture composed 1.0 ml of T-2636 F solution (0.6~20 μg/ml), 0.5 ml of 0.2 % enzyme solution and 0.5 ml of MICHAELIS buffer (pH 3.0), was incubated at 37°C for 2 hours. Antibiotic activity of the reaction mixture was estimated by the cup method with *Sarcina lutea* PCI 1001 as test organism. Trypto-soy agar (Eiken Chemical Co.) (pH 6.5) was used as assay medium, and T-2636 C was used for standard as 100 % potency.

T-2636 F triacetate

A solution of 100 mg of T-2636 F in 2 ml of pyridine and 1 ml of acetic acid anhydride was held at room temperature overnight, then poured into ice water. The crude crystal was filtered and crystallized from diethyl ether. Colorless prisms, 90 mg, m. p. 211~215°C (decomp.), $[\alpha]_D^{23}$ −208° (*c* 1, MeOH), elemental analysis calculated for $C_{31}H_{41}NO_{10}$: C 63.36, H 7.03, N 2.38 (%); found, C 63.39, H 6.78, N 2.58 (%). UV spectrum; λ_{max}^{EtOH} =229 mμ (ε =50,900), MS spectrum, m/e 587 (M^+).

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