TOLYPOMYCIN, A NEW ANTIBIOTIC. III
ISOLATION AND CHARACTERIZATION OF TOLYPOMYCIN Y

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Tolypomycin Y, $C_{43}H_{54}N_2O_{14}$, is a new ansamycin antibiotic produced by *Streptomyces tolypophorus*. It is a lipophylic neutral substance that crystallizes as yellow needles and has λ_{max}^{EtOH} 232, 290, 337, 370~430 (shoulder) mμ and $[\alpha]_D^{22}+326°$ (in EtOH). Tolypomycin Y shows strong *in vitro* and *in vivo* antimicrobial activities against Gram-positive bacteria and a low acute toxicity.

In the course of screening for new antibiotics which are orally effective against mice infected with *Staphylococcus aureus*, a new antibiotic tolypomycin Y has been isolated from the fermentation broth of *Streptomyces tolypophorus*[1,2].

Taxonomy[1] of *S. tolypophorus* and production[2] of the antibiotic are described in the preceding papers. This paper deals with the isolation and physico-chemical properties of tolypomycin Y, which was shown to be a new ansamycin antibiotic. Two other ansamycin antibiotics were isolated from the fermentation broth and identified as rifamycins B and O.

Isolation of Tolypomycin Y

As detailed in the experimental section, to obtain tolypomycin Y the culture filtrate[2] of *S. tolypophorus* was extracted with ethyl acetate at pH 8 and concentrated *in vacuo*. *n*-Hexane or a mixture of ether-petroleum ether was added to the concentrate to obtain a crude powder. The crude powder was dissolved in ether and the solution was concentrated and the residue purified by chromatography on silica gel or on activated charcoal. From the concentrate of the active fractions yellow needles of tolypomycin Y were crystallized and recrystallized from ethyl acetate.

Physico-chemical Properties of Tolypomycin Y

The physico-chemical properties of tolypomycin Y are as follows:

1. Crystalline form and melting point: From ethyl acetate as yellow needles which lose their anisotropy at 120~125°C but do not show a definite melting point below 300°C.

2. Character: Lipophilic almost neutral substance.

3. Elemental analysis: Found C 61.40; H 6.49; N 3.14; O 28.51; OCH_3 3.31; H_2O 2.15 % (Karl Fischer method).

4. Molecular weight; 820~853 (measured by V.P.O. method in ethyl acetate).

5. Molecular formula: $C_{43}H_{54}N_2O_{14}\cdot H_2O$. Calcd.: C 61.41; H 6.71; N 3.33; O 28.55;

OCH_3 3.69; H_2O 2.14; MW 822.

6. Specific rotation: $[\alpha]_D^{22}+326°$ (*c* 1, ethanol); $[\alpha]_D^{21}+376°$ (*c* 0.5, acetone); $[\alpha]_D^{22}+325°$ (*c* 0.35, chloroform).

7. Color reaction: Positive to TOLLENS' reagent and magnesium acetate reagent. Negative to BARTON's ($FeCl_3+K_3Fe(CN)_6$) reagent, MOLISCH reaction and EHRLICH reagent.

8. Solubility: Very soluble in methanol, ethanol, *n*-butanol, acetone, chloroform, ethyl acetate or benzene. Insoluble or sparingly soluble in water, petroleum ether or *n*-hexane.

9. Rf values: The Rf values which were obtained by paper chromatography using the ascending method on Whatman filter paper No. 1 and observed as visible colored spots and as inhibition zones on bioautograms employing *Staphylococcus aureus* are as follows;

n-Hexane – benzene – ethanol – water (1 : 3 : 1 : 3)	Rf 0.78
n-Hexane – benzene – acetone – water (30 : 10 : 18 : 22)	Rf 0.68
n-Hexane – ether – acetone – water (15 : 5 : 8 : 12)	Rf 0.27

The Rf values obtained by thin-layer chromatography on silica gel containing 2% of oxalic acid are as follows:

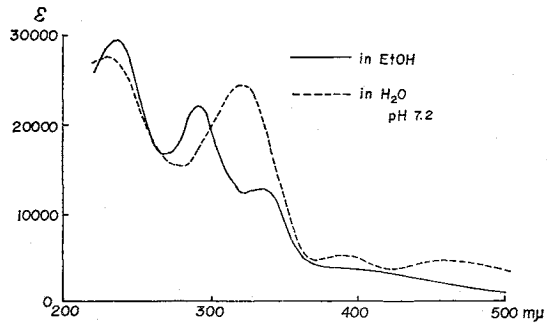
Ethyl acetate and acetone (1 : 1) mixture containing 1% oxalic acid	Rf 0.05
Ethyl acetate solution containing 1% oxalic acid	Rf 0.00
Acetone solution containing 1% oxalic acid	Rf 0.2

10. Absorption spectrum: The ultraviolet and visible absorption spectra in ethanol and phosphate buffer at pH 7.2 are shown in Fig. 1 and the significant absorption maxima observed are as follows:

λ_{max}^{EtOH} 232 mμ (ε=29,000), 290 mμ (23,800), 337 mμ (12,700) and 370 to 430 mμ (shoulder).

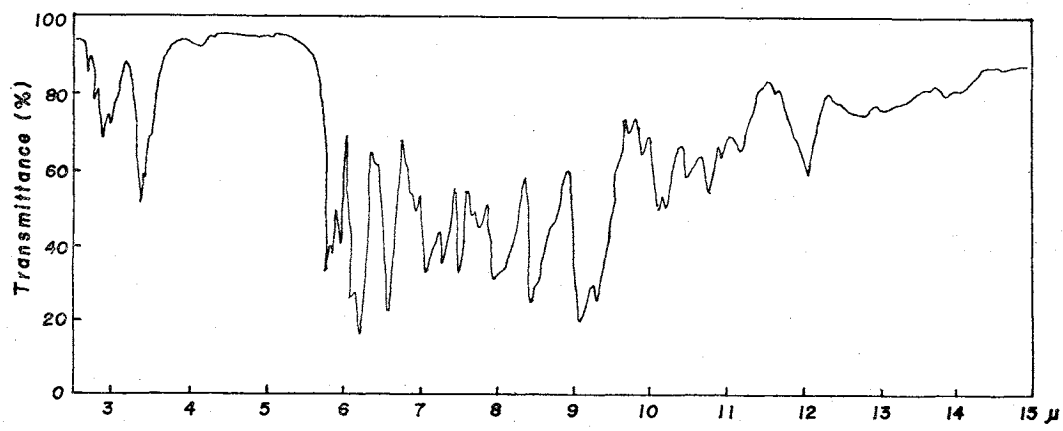
$\lambda_{max}^{pH\ 7.2}$ 234.5 mμ (27,900), 319 mμ (25,500), 387 mμ (3,000), 465 mμ (3,300), 488 mμ (3,200)

Fig. 1. Ultraviolet and visible spectra of tolypomycin Y



The infrared absorption spectrum in chloroform solution is shown in Fig. 2. The presence of -OH (3565, 3500 cm^{-1}), -OH or -NH (3400 cm^{-1}), ester carbonyl (1715 cm^{-1}), carbonyl (1684, 1665 cm^{-1}) and amide (1620 cm^{-1}) groups

Fig. 2. Infrared spectrum of tolypomycin Y ($CHCl_3$)



is indicated.

An aqueous solution of tolypomycin Y is unstable, but the powder kept at 20°C for 9 months in dry state presserved initial activity.

Biological Properties of Tolypomycin Y

Tolypomycin Y shows a strong *in vitro* antimicrobial activity against Gram-positive bacteria and also, to some extent, inhibits the growth of Gram-negative bacteria and acid-fast bacteria (Table 1). Moreover, a comparison of the antimicrobial spectrum with those of wide-spectrum antibiotics available, shows that tolypomycin Y had a wide spectrum differing from those of the other antibiotics (Table 2). In the medium with or without horse serum, the same activities of tolypomycin Y were observed as shown in Table 3.

Tolypomycin Y, at the similar concentrations, is as effective against staphylococci isolated from patients, as against the standard laboratory staphylococci[5]. It is active against experimental infections in mice by Gram-positive bacteria *via* subcutaneous, intraperitoneal, intravenous and oral administration[5]. The acute toxicities of tolypomycin Y in mice observed by subcutaneous, intraperitoneal and oral route are: LD_{50} 2,200 mg/kg (s.c.), 330 mg/kg (i.p.), 5,700 mg/kg (p.o.), respectively.

Table 1. Antimicrobial spectra of tolypomycin Y (Agar dilution method)

Test organisms	M.I.C. (mcg/ml)
Staphylococcus aureus FDA 209 P	0.001
S. aureus FDA 209 P SM-R*	0.0001
S. aureus FDA 209 P CTC-R	0.001
S. aureus FDA 209 P CP-R	0.0001
S. aureus FDA 209 P OE-R	0.001
S. aureus FDA 209 P XM-R	0.02~0.05
S. aureus FDA 209 P GM-R	0.005
S. aureus FDA 209 P CM-R	0.001~0.002
S. aureus FDA 209 P MM-R	0.005
S. aureus FDA 209 P RM-R	>50
S. aureus Terajima	0.005
S. aureus Heatley	0.005
S. aureus ATCC 4012	0.005
Bacillus subtilis PCI 219	0.1
Bacillus cereus IFO 3466	0.1
Bacillus brevis IFO 3331	0.005
Sarcina lutea IFO 3232	0.001
Micrococcus flavus IFO 3242	0.001
Escherichia coli IFO 3044	50
Escherichia coli IFO 3045	10
Pseudomonas aeruginosa IFO 3080	50
Mycobacterium avium IFO 3153	>50
Mycobacterium smegmatis IFO 3083	10~20
Mycobacterium phlei IFO 3158	50
Mycobacterium sp. 607	>50
Candida albicans IFO 0583	>50
Saccharomyces cerevisiae IFO 0209	>50
Penicillium chrysogenum IFO 4626	>50
Aspergillus niger IFO 4066	>50

* SM: Streptomycin, CTC: Chlortetracycline, CP: Chloramphenicol, OE: Oleandomycin and Erythromycin, XM: Xanthomycin, GM: Glumamycin, CM: Chromomycin, MM: Mikamycin, RM: Rifamycin, R: Resistant.

Table 2. Comparison of antibacterial activities of tolypomycin Y with those of other known antibiotics (Agar dilution method)

Antibiotics	M.I.C. (mcg/ml)				
	Escherichia coli	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Bacillus subtilis*
Tetracycline	5	10	50	0.5	0.5
Chloramphenicol	10	1	50	5	2
Streptomycin	5	2	>100	5	5
Colistin	2	>100	5	50	50
Cephaloridine	5	100	100	0.01	0.05
Rifamycin SV	> 50	10~20	> 50	0.01	0.2
Rifamycin B	>200	200	200	0.1~0.025	2.5
Tolypomycin Y	50	10	50	0.001	0.1

The above reported physicochemical and biological properties show that tolypomycin Y is different from other known antibiotics.

Table 3. Effect of horse serum on the antibacterial activities of tolypomycin Y (Agar dilution method)

Test organisms	M.I.C. (mcg/ml)			
	0 %	10 %	20 %	30 %
Staphylococcus aureus	0.001~0.0005	0.001	0.001	0.001
Bacillus subtilis	0.05	0.05	0.05	0.05
Bacillus brevis	0.005	0.01	0.01	0.01

Isolation and Identification of Rifamycins B and O

S. tolypophorus was cultured under conditions reported previously[2]. The culture filtrate at pH 8 was extracted with ethyl acetate, acidified to pH 2, and reextracted with ethylacetate. Yellow needles obtained after chromatography on a column with silica gel were identical with rifamycin B obtained from *Streptomyces mediterranei*[3] in specific rotation, ultraviolet, visible and infrared spectra.

Oxidation of this compound gave a product which was identical with rifamycin O[4] in specific rotation, elemental analysis, ultraviolet, visible, infrared and nuclear magnetic resonance spectra. Oxidative hydrolysis of this product (rifamycin B) gave a product, which was identified with rifamycin S[6] by elemental analysis, ultraviolet, visible, infrared and nuclear magnetic resonance spectroscopy.

From the ethyl acetate extract of the culture filtrate at pH 8, pale yellow prisms were isolated, besides tolypomycin Y, by chromatography on a column with silica gel. This product was identical with rifamycin O[4] in all respects.

Experimental

Tolypomycin Y

(1) The culture filtrate[2] (840 liters) of *S. tolypophorus* was extracted with ethyl acetate (280 liters) at pH 8. After concentration of the organic solvent layer, the concentrate was added to ether - petroleum ether or ether - *n*-hexane (1 : 10) to give 70 g of crude powder. The crude powder (300 mg) was dissolved in 18 ml of ether and the solution was purified by chromatography on a column of silica gel (12 g) (0.05~0.02 mm), eluted with ethyl acetate containing 1 % oxalic acid. The antibiotic remained at the top part of the silica gel column, and was extracted with ethyl acetate from the silica gel which had been extruded from the column. The ethyl acetate solution was concentrated under reduced pressure and the residue was crystallized from ethyl acetate, to obtain 11 mg of tolypomycin Y as yellow needles.

(2) The culture filtrate[2] (12 liters) was washed with 4 liters of *n*-hexane, adjusted to pH 3.0 with diluted H_2SO_4 and extracted with 6.4 liters of ethyl acetate. The ethyl acetate solution was washed twice with 2 liters of SÖRENSEN phosphate buffer solution (pH 8.0) and then with water. After concentration of the ethyl acetate solution, the active fraction was separated by thin-layer chromatography on silica gel (Merck, 0.08 mm) impregnated with oxalic acid using acetone containing 1 % of oxalic acid. Tolypomycin Y was observed at Rf 0.2 and extracted with ethyl acetate from the silica gel. The extract was concentrated under reduced pressure and 45 mg of yellow needles crystallized from the concentrate after addition of *n*-hexane or petroleum ether. It was recrystallized from the mixture of *n*-hexane - ethyl acetate (3 : 1) to give 25 mg of pure crystals.

(3) The crude powder (30 g) described in (1) was extracted with 1 liter of ether. After concentration of the ethereal extract, the concentrate was dissolved in 30 ml of ethyl acetate and precipitated with 200 ml of *n*-hexane to give 11.6 g of powder. The powder (10 g) was dissolved in 50 ml of ethyl acetate - ether (1 : 1) and purified by

chromatography on a column of activated charcoal (100 g) using ether, ethyl acetate, chloroform, in order. The active component was eluted with ethyl acetate and purified by chromatography on a column of silica gel as described above, to obtain 1.54 g of tolypomycin Y as yellow needles.

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