TOLYPOMYCIN, A NEW ANTIBIOTIC. II

PRODUCTION AND PRELIMINARY IDENTIFICATION OF TOLYPOMYCIN Y

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The culture broth of *Streptomyces tolypophorus* was shown to contain a new antibiotic, tolypomycin Y, as a main component, from the difference in the loss of antibiotic potency caused by heating or preservation of broths at various culture ages was assumed to contain other antibiotic components. By means of microbiological assay and thin-layer chromatography, the cultural conditions for enrichment of tolypomycin Y were investigated. As a result, combinations of glucose with glycerol, and peptone with soybean flour were found to be good carbon and nitrogen sources, respectively, and the production of tolypomycin Y was increased by the addition of iron-containing salts to such a medium. On the basis of these studies, a suitable medium for the enrichment of tolypomycin Y was established. Furthermore, preliminary studies on the antibacterial activity and chemical properties of crude tolypomycin Y in comparison with those of known antibiotics showed that tolypomycin Y resembles the rifamycins.

In the course of studies on the production of antibiotic by *Streptomyces tolypophorus*[1], it was noticed that the loss of the antibiotic activity by heating or preservation of the culture broth changed with the culture age of the organism. Therefore, it was assumed that the organism produces at least two kinds of antibiotics which have different stabilities, and in fact the organism was found to produce other components together with the main antibiotic, tolypomycin Y[2]. It was recognized that the production of these antibiotics varies with the fermentation medium and the age of the fermentation, and a suitable medium for the selective production of tolypomycin Y was looked for. Tolypomycin Y was produced by suitable cultural conditions. This antibiotic was found to be similar to the rifamycins in their various properties.

The present paper deals with the change of the potency in the fermentation broth, the selection of a suitable medium for the production of tolypomycin Y and the preliminary identification of the antibiotic.

Materials and Methods

1. Fermentation.

Shake culture was carried out on a rotary shaker (220 rpm, 5 cm radius). Medium (50 ml) in a 200 ml Erlenmyer flask was sterilized in an autoclave at 121°C for 15 minutes. The media for the seed and the basal main cultures were as follows: seed culture medium, 1% glucose, 1% soybean flour, 1% corn steep liquor and 0.3% $CaCO_3$ (pH 7.0); and basal main culture medium, 5% glucose, 2% glycerol, 1.5% soybean flour, 0.2% peptone and

0.5 % $CaCO_3$ (pH 7.0). The seed and main cultures were incubated at 28°C for 48 and 66 hours, respectively.

In the tank fermentation, spores of *S. tolypophorus* were inoculated into a 2-liter flask containing 500 ml of the seed culture medium and incubated at 28°C for 48 hours on a reciprocal shaker (120 stokes per minute). The resulting seed culture (500 ml) was inoculated into 30 liters of the basal main culture medium and incubated at 28°C for 66 hours (stirring : 280 rpm ; aeration : 30 liters/min.).

2. Antimicrobial activity.

(1) Total potency : The total potency of the samples was estimated by the paper disc method with either *Staphylococcus aureus* 209 P or *Sarcina variabilis* as the test organisms, using tolypomycin Y as the standard. The assay medium used for *S. aureus* 209 P consisted of 1 % glucose, 0.5 % meat extract, 0.5 % yeast extract and 1.5 % agar (pH 7.0). For *S. variabilis* it consisted of 0.5 % peptone, 0.5 % meat extract, 0.5 % K_2HPO_4 and 1.5 % agar (pH 7.0).

(2) Potencies of tolypomycin Y and other components : The filtered broth (100 ml) was extracted with ethyl acetate at pH 8.0 and the extract was concentrated to 1 ml *in vacuo*. The concentrate (0.02 ml) was applied to thin-layer chromatography on Silica gel G (Merck Co.), which had been previously impregnated with ethyl acetate containing 1 % oxalic acid and dried, and it was developed with the same solvent.

The orange-colored band (Rf 0.05~0.1) corresponding to tolypomycin Y and the yellowish brown-colored bands corresponding to other components were extracted with acetone (1 ml), and the potencies of these acetone extracts were assayed as described later. The potencies of acetone extracts containing tolypomycin Y or other components were estimated by the paper disc method with *S. aureus* 209 P, using tolypomycin Y as the standard.

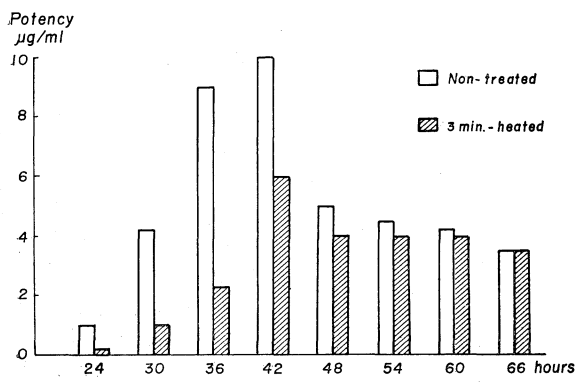
(3) Antibacterial spectrum : The antibacterial spectrum was examined by a serial agar dilution method.

Results and Discussion

1. Antibiotic Potency in the Culture Broth

The tank fermentation was carried out with the basal main culture medium at 28°C for 66 hours, and the total potencies before and after heating of the culture broth at various ages of the fermentation were assayed (Fig. 1). Up to 48 hours of the fermentation, the potency of broths decreased by 30~60% on heating, but the potency of broths from 48 to 66 hours culture was rather stable to heating.

Fig. 1. Antibacterial activity in culture broth at various ages by heating.



The loss of potency of the antibiotic during preservation at 4°C for 24 hours was examined, using either culture broth or filtrate at various ages of the fermentation. It was found that the potency of the culture broth did not change during conservation at 4°C, but that of the filtered broth decreased up to about 42 hours. As shown in Fig. 2, the total potencies against *S. aureus* 209 P run parallel to those against *S. variabilis* up to 42 hours, but after 48 hours of culture

Fig. 2. Antibacterial activity in culture broth at various ages preserved in cold room.

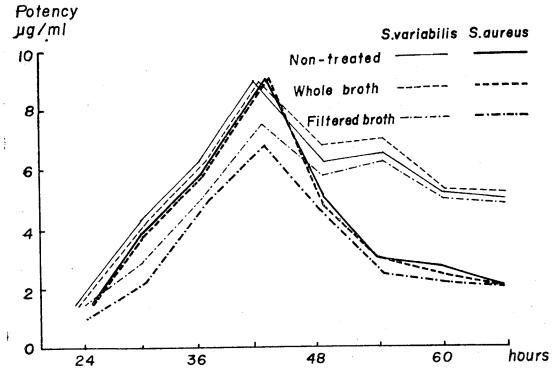
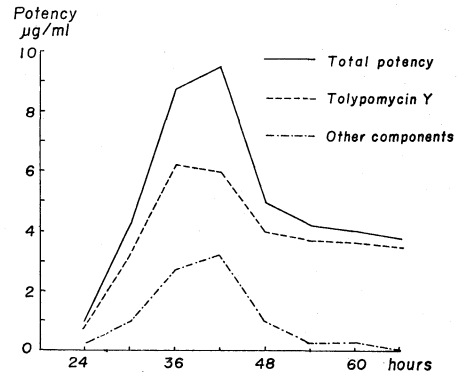


Fig. 3. Potencies of tolypomycin Y and other components at various culture ages.



the potencies against these two test organisms were different.

It was assumed that the streptomyces produced simultaneously tolypomycin Y, and other components having different biological properties.

2. Production of Tolypomycin Y

The interrelationship of the production of tolypomycin Y and the other components was examined. The time course of the production of tolypomycin Y and other components in the tank fermentation was investigated as described under Materials and Methods section II-2 (Fig. 3). The maximum titers of tolypomycin Y and the other components were observed in the fermentation broths at 36 and 42 hours, respectively. It was found that up to the 36 hours the total potency of the culture broth corresponded to that of tolypomycin Y, but beeween 48 th and 66 th hours the other components were markedly produced which influenced the total potency.

3. Cultural Conditions for Producing Tolypomycin Y

Preliminary experiments using shake cultures were performed to find a suitable medium for the production of tolypomycin Y. The effects of various carbon sources on the growth of the organism and the production of tolypomycin Y were investigated. In conclusion, glucose, glycerol, lactose and fructose were found to be suitable source of carbon for the production of tolypomycin Y and the growth of *S. tolypophorus*. Although maltose and dextrin supported moderate growth, they were not effective for the production of tolypomycin Y when used as the sole carbon source. On the other hand, a combination of peptone with soybean flour was found to be a suitable source of nitrogen for the production of tolypomycin Y. From the results of preliminary experiments, the following basal medium was selected: 5% glucose, 2% glycerol, 1.5% soybean flour, 0.2% peptone and 0.5% $CaCO_3$ (pH 7.0).

Furthermore, the effect of inorganic salts on the total potency was investigated and it was found that the iron salts were effective in stimulating the antibiotic production (Tables 1 and 2).

(1) Effects of iron salts: The results in Table 2 show that the production of tolypomycin Y increased by two to five times when iron salts were added. In particular the medium containing 0.05% of ferrous sulfate gave the maximum titer of tolypomycin Y. When more than 0.05% of ferrous sulfate was present, rifamycin B[8] and

Table 1. Effect of inorganic salts on the production of the antibiotic

%	36 hours		66 hours		%	36 hours		66 hours	
	pH	Total potency (μg/ml)	pH	Total potency (μg/ml)		pH	Total potency (μg/ml)	pH	Total potency (μg/ml)
$Fe_2(SO_4)_3$ 0.01	7.20	16.8	6.60	14.8	$MnCl_2$ 0.01	6.60	14.8	6.20	14.8
$Fe_2(SO_4)_3$ 0.05	7.00	50.0	6.20	6.2	$MnCl_2$ 0.05	6.20	26.4	6.20	13.2
$MgSO_4$ 0.01	7.20	14.0	6.60	5.8	$CuCl_2$ 0.01	7.00	4.8	6.60	2.8
$MgSO_4$ 0.05	7.20	16.2	6.80	12.6	$CuCl_2$ 0.05	7.20	0.8	6.60	<0.8
$ZnSO_4$ 0.01	7.00	7.8	7.20	7.0	$CaCl_2$ 0.01	6.00	19.6	6.40	9.4
$ZnSO_4$ 0.05	7.20	9.2	6.40	14.8	$CaCl_2$ 0.05	6.40	16.0	6.40	10.4
$CuSO_4$ 0.01	7.20	16.0	7.20	6.2	NaCl 0.1	7.20	2.8	6.60	10.4
$CuSO_4$ 0.05	7.20	<0.8	7.40	<0.8	NaCl 0.05	7.20	24.4	6.80	10.4
$FeCl_3$ 0.01	7.20	25.6	6.60	11.0	K_2HPO_4 0.1	7.00	5.6	6.40	3.2
$FeCl_3$ 0.05	7.00	42.0	6.20	14.8	K_2HPO_4 0.05	7.00	6.2	7.00	3.2
$ZnCl_2$ 0.01	7.00	16.0	7.00	7.0	Control	7.20	17.6	6.80	11.8
$ZnCl_2$ 0.05	7.00	4.0	7.60	3.4					

Table 2. Effect of iron salts on the production of tolypomycin Y

%	36 hours			66 hours		
	pH	Tolypomycin Y (μg/ml)	Other components (μg/ml)	pH	Tolypomycin Y (μg/ml)	Other components (μg/ml)
$FeCl_2$ 0.01	6.80	25.4	40.2	7.00	<1.0	9.6
$FeCl_2$ 0.05	6.80	41.0	12.0	6.80	<1.0	10.2
$FeCl_3$ 0.01	6.80	27.4	26.6	7.00	<1.0	9.6
$FeCl_3$ 0.05	6.60	41.4	15.8	6.80	<1.0	4.6
$FeSO_4$ 0.01	6.80	21.8	35.4	6.40	<1.0	9.4
$FeSO_4$ 0.05	6.60	48.6	26.6	6.00	<1.0	9.8
$Fe_2(SO_4)_3$ 0.01	6.80	32.6	16.2	7.00	<1.0	9.4
$Fe_2(SO_4)_3$ 0.05	6.40	42.4	23.6	6.00	<1.0	9.6
Control	7.00	9.6	19.8	7.00	<1.0	7.0

O[4] were produced in the culture[2].

(2) Combined effect of ferrous sulfate with alkaline earth metal salts or alkali metal salts: As shown in Table 3, the addition of K-, Ca- and Ba-salts promotes the production of antibiotics. Consequently, the following medium for the production of tolypomycin Y was selected: 5% glucose, 2% glycerol, 1.5% soybean flour, 0.2% peptone, 0.05% $FeSO_4$, 0.5% $CaCO_3$ and 0.005% $Ba(OH)_2$.

4. Preliminary Studies for Identification of Tolypomycin Y

The crude tolypomycin Y was extracted with ethyl acetate from the culture filtrate.

The antibiotic was active against Gram-positive and some of acid-fast bacteria, it was especially active against *S. aureus* and moreover maintained its activity against the strains resistant to chloramphenicol, tetracylines and macrolide antibiotics. Also the antibiotic showed weak activity against Gram-negative bacteria.

On the other hand, as shown in Table 4 and Fig. 4, crude tolypomycin Y was a physiologically acidic substance[5], because it showed stronger activity in acidic medium than in alkaline medium.

The minimum inhibitory concentration of crude tolypomycin Y against *S. aureus* was lower than that against *B. subtilis*. And the color of the antibiotic solution changed from yellow in acidic to orange in alkaline solution. These properties of

Table 3. Effect of alkaline earth metal salts and alkali metal salts on the production of tolypomycin Y

(%)		(%)	36 hours pH	36 hours Tolypomycin Y (μg/ml)	36 hours Other components (μg/ml)	66 hours pH	66 hours Tolypomycin Y (μg/ml)	66 hours Other components (μg/ml)
$FeSO_4$	0.05+K_2HPO_4	0.05	6.40	<1.0	<1.0	6.60	<1.0	2.0
〃	KCl	0.05	6.40	42.0	45.6	6.40	<1.0	16.2
〃	KNO_3	0.05	7.00	48.3	44.0	7.00	<1.0	16.8
〃	K_2SO_4	0.05	6.60	55.8	52.6	6.40	<1.0	25.8
〃	$Ca(OH)_2$	0.05	6.60	33.3	21.6	6.40	<1.0	9.6
〃	$CaCl_2$	0.05	6.40	49.3	34.0	6.40	<1.0	19.6
〃	$Ca(NO_3)_2$	0.05	6.40	27.5	55.0	6.40	<1.0	13.6
〃	$BaCl_2$	0.01	6.80	7.8	27.4	6.20	<1.0	24.2
〃	$BaCl_2$	0.05	7.20	29.8	31.2	6.40	<1.0	27.0
〃	$BaCO_3$	0.01	7.00	19.4	15.6	6.40	<1.0	25.2
〃	$BaCO_3$	0.05	7.00	23.5	17.6	6.40	<1.0	27.0
〃	$Ba(OH)_2$	0.01	7.00	39.3	20.4	6.40	<1.0	27.0
〃	$Ba(OH)_2$	0.005	7.00	64.8	21.4	6.20	<1.0	19.2
〃	$Ba(CH_3CO_2)_2$	0.01	6.80	56.0	12.8	6.40	<1.0	20.2
〃	$Ba(CH_3CO_2)_2$	0.005	6.80	55.5	12.6	6.20	<1.0	29.6
〃	Control		7.00	45.0	20.0	5.80	<1.0	14.2

Fig. 4. Effect of pH on activity of tolypomycin Y by paper disc method.

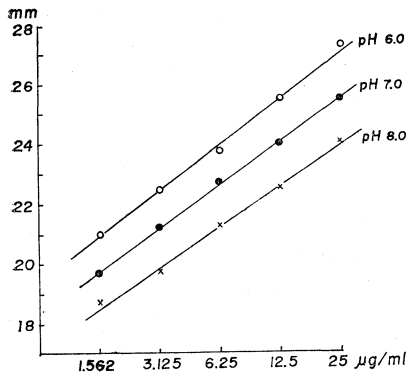


Table 4. Effect of pH on the activity of crude tolypomycin Y by agar dilution method

	Minimum inhibitory concentration (μg/ml) pH 6	pH 7	pH 8
Crude tolypomycin Y	0.005	0.01~0.005	0.025

Table 5. Comparison between crude tolypomycin Y and known pH-indicator antibiotics

Antibiotics	Minimum inhibitory concentration (μg/ml) *S. aureus*	*B. subtilis*	Color change in acidic solution	in alkaline solution
Cyanomycin	6	10	red	blue
Ericamycin	0.25	0.75	red	purple
Litmocidin	0.25	—	red	blue
Mycorhodin	0.015	0.03	red	blue
Rifamycin B	0.025~0.1	2.5	yellow	orange
Xanthomycin	0.002	0.0005	yellow	purple
Crude tolypomycin Y	0.01~0.025	0.25	yellow	orange

crude tolypomycin Y were compared with the known antibiotics which have similar characteristics (Table 5).

By this comparison, crude tolypomycin Y was shown to resemble rifamycin B, so further comparison with the rifamycins was performed. The antimicrobial spectrum of crude tolypomycin Y was compared with those of rifamycins B, O and SV[6,7] (Table 6). The antibiotic activity of crude tolypomycin Y was generally greater than rifamycins B, O and its antibacterial spectrum was rather similar to that of rifamycin SV. However, a distinct difference betwen crude tolopymycin Y and rifamycin SV was found in the activity against *Bacillus brevis*. The antibiotic also did not inhibit the growth of a strain resistant to rifamycin even at a concentration of 50 μg/ml.

From the above-mentioned results, it was concluded that tolypomycin Y is

Table 6. Antimicrobial spectra of crude tolypomycin Y and rifamycins

Test organisms	M.I.C. (μg/ml)			
	Crude tolypomycin Y	Rifamycin B	Rifamycin O	Rifamycin SV
Escherichia coli	50～>50	>50	>50	>50
Proteus vulgaris	10～20	>50	>50	10～20
Pseudomonas aeruginosa	20～50	>50	>50	>50
Staphylococcus aureus	0.002～0.005	0.5	0.2	0.01
Staphylococcus aureus R*	>50	>50	>50	>50
Bacillus subtilis	0.2	5	1	0.2
Bacillus cereus	0.2	20	10	2
Bacillus brevis	0.01～0.005	10	1	0.2
Mycobacterium avium	>50	>50	>50	50
Candida albicans	>50	>50	>50	>50

R*: Rifamycin SV (Rifocin ®) resistant strain.

expected to be a novel antibiotic belonging to the rifamycin group.

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