TOLYPOMYCIN, A NEW ANTIBIOTIC. VII
ASSAY METHOD FOR TOLYPOMYCIN Y

TŌRU HASEGAWA

Microbiological Research Laboratories,
Research and Development Division,
Takeda Chemical Industries, Ltd., Osaka, Japan

(Received for publication June 19, 1971)

A differential assay method has been investigated in order to distinguish tolypomycin Y from the other components which were produced simultaneously by *Streptomyces tolypophorus*. About 600 strains were tested for sensitivity to tolypomycin Y and the other components. It was found that the growth of *Streptococcus alcalophilus* IFO 3531 was inhibited by tolypomycin Y, but not by the other components. The assay method for the differentiation of tolypomycin Y from the other components was established using the paper disc method with *Streptococcus alcalophilus* IFO 3531.

As reported in a previous paper, *Streptomyces tolypophorus*[1] was found to produce tolypomycin Y[2] together with other components with antibacterial activity (hereafter referred to as F-A). Both tolypomycin Y and F-A have similar antibacterial spectra, and tolypomycin Y could not be distinguished from F-A by the usual bioassay method. For the determination of tolypomycin Y a method using thin-layer chromatography[3] was useful, but it was not suitable for assaying many samples.

In this report a differential assay method of tolypomycin Y is described, using *Streptococcus alcalophilus* IFO 3531.

Materials and Methods

I. Antibiotics used

1. Tolypomycin Y: Crystalline tolypomycin Y described in previous paper[2].

2. F-A: F-A was prepared as follows: The culture filtrate was extracted with ethyl acetate at pH 4.0 and the extract was concentrated *in vacuo*. Petroleum ether was added to the concentrate to yield a crude powder. This crude powder was dissolved in a small amount of ethyl acetate, and the solution was applied on a thin-layer silica gel plate, previously impregnated with ethyl acetate containing 1 % oxalic acid and the plate developed with the same solvent. All active fractions except tolypomycin Y were collected and extracted with ethyl acetate. The extracts were washed with water and dried to give F-A as yellowish brown powder.

II. Selection of the test organism

1. Organisms used: About 600 strains of bacteria in the type cultures of the Institute for Fermentation, Osaka, were tested: Including both Gram-positive and negative bacteria.

2. Screening method: Paper strips were soaked with an acetone solution of tolypomycin Y (3.125 μg/ml and 25 μg/ml) and F-A (31.25 μg/ml and 250 μg/ml) and were put on nutrient agar. The plates were left in a cold room for 18 hours to allow for

sufficient diffusion. Each organism was then cross-streaked on the agar plates and incubated at 37°C for 18 hours, then the inhibition was measured.

III. Assay method

After the test organism was cultured on glucose nutrient agar at 37°C for 18 hours, the organism was suspended in a solution of 0.5 % skimmed milk (2 ml/slant) and the suspension was preserved in a deep-freezer (~−20°C). The agar plate was prepared by using 5 ml of the basal layer and 4 ml of the seed layer which contained 0.5 ml of the suspension of the test organism per 100 ml of the medium.

The assay was performed by the paper disc method using acetone solutions of tolypomycin Y (6.25 μg/ml and 25 μg/ml) as standard.

Results and Discussion

I. Screening of Test Organisms

One hundred and forty bacilli, 7 bacteria, 16 brevibacteria, 8 mycobacteria, 23 micrococci, 21 corynebacteria, 22 sarcina, 2 streptococci, 9 staphylococci and 370 strains of other Gram-positive and Gram-negative bacteria were tested for sensitivity to tolypomycin Y and F-A, respectively. Almost all these organisms showed similar sensitivities to both tolypomycin Y and F-A but only *Streptococcus alcalophilus* was sensitive to tolypomycin Y and resistant to F-A. A comparison of the activities of tolypomycin Y with those of F-A on representative test organisms is shown in Table 1.

II. Assay Medium

1. Composition of the medium

The following nitrogen sources for the assay medium for growth of *Streptococcus alcalophilus* were investigated: meat extract, bonito extract, peptone, chrysalis extract and Casamino acids. Carbon sources were also tested and the following was selected as the most suitable composition for the assay medium: 1 % glucose, 1 % bonito extract, 0.5 % meat extract, 0.5 % peptone, 0.5 % NaCl and 1.5 % agar.

2. Effect of pH

The growth of *Streptococcus alcalophilus* is favored in alkaline medium. On the other hand, tolypomycin Y exhibits a larger inhibition zone in acidic medium than in alkaline medium[8]. The effect of pH of the medium on the inhibition zone was investigated at pH 6.0, 6.5 and 6.8. A clear inhibition zone was produced on the media at pH 6.5 or 6.8 (Fig. 1), and no inhibition zone was shown by F-A under these conditions.

Table 1. Comparison of the inhibition lengths of tolypomycin Y with those of the other components

Test organisms	Diameter of inhibition zone (mm)			
	Tolyomycin Y		The other components (F-A)	
	3.125 μg/ml	25 μg/ml	31.25 μg/ml	250 μg/ml
Streptococcus alcalophilus	10	15	0	0
Corynebacterium sepedonicum	8	14	13	18
Brevibacterium roseum	15	20	12	17
Mycobacterium avium	12	17	7	14
Staphylococcus aureus Terajima	12	18	10	19
Staphylococcus aureus FDA 209P	12	17	8	18
Micrococcus glutamicus	14	18	11	20

Fig. 1. Effect of pH on the inhibition zone.

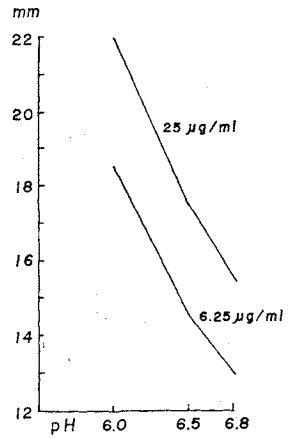


Fig. 2. Correlation between tolypomycin Y and the other components (F-A).

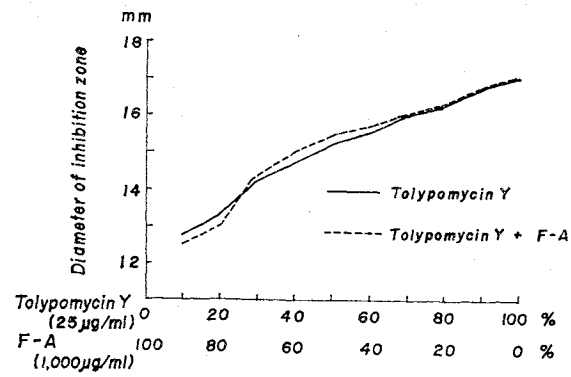


Fig. 3. Effect on the other components (F-A) on the inhibition zone.

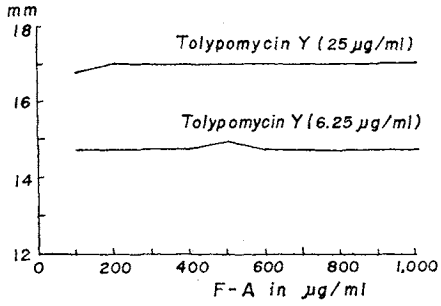
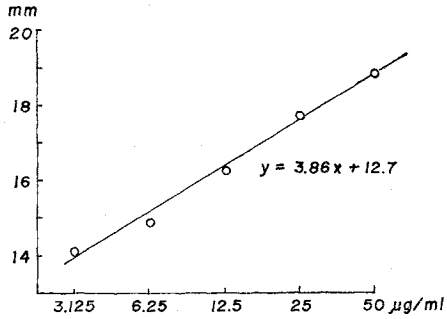


Fig. 4. Correlation between potency and inhibition diameter of tolypomycin Y.



It was established that the assay medium at pH 6.5 was the most favorable for the differential assay with *Streptococcus alcalophilus*.

3. Effect of F-A on the inhibition zone

The interaction of the antibacterial activity between tolypomycin Y and F-A was investigated. As shown in Fig. 2, a dose-response curve of tolypomycin Y was not influenced by the addition of F-A. The inhibition zone diameter shown by tolypomycin Y (6.25 μg/ml or 25 μg/ml) was not influenced even if 40-times as much F-A was added as tolypomycin Y (Fig. 3). Rifamycins B[4] and O[5], which chemically resemble tolypomycin Y[6,7], do not show the activity against *Streptococcus alcalophilus*. From the above-mentioned results, the following conditions were chosen for the selective assay of tolypomycin Y using the paper disc method:

Test organism: *Streptococcus alcalophilus* IFO 3531.
Assay medium: 1 % glucose, 1 % bonito extract, 0.5 % meat extract, 0.5 % peptone, 0.5 % NaCl and 1.5 % agar.
pH of medium: 6.5
Incubation time: 18 hours.

III. Accuracy of the Assay Method

The linear regression and the correlation between the diameter of the inhibition

Table 2. Comparison of the potencies between microbiological assay method and thin-layer chromatographic method

Hours after incubation	Total potency* (μg/ml)	Tolypomycin Y** (μg/ml)	Tolypomycin Y*** (μg/ml)
24	29	19	12
30	64	58	50
36	100	94	88
42	120	94	86
48	42	36	38
54	36	29	25
60	42	15	12
66	36	12	8
72	42	11	< 5
78	18	7	< 5
84	18	6	< 5
90	14	3	< 5

* Test organism: *Staphylococcus aureus* FDA 209 P.
** Test organism: *Staphylococcus alcalophylus* IFO 3531.
*** The potency estimated by using TLC-method.

zone and the logarithm of the potency obtained by the above-mentioned method was studied (Fig. 4). Between the logarithmic values (x) of the concentration of tolypomycin Y and the diameter (y) of the inhibition zone, the correlation coefficient was 0.98 and regression coefficient was 3.86. The regression linear line was calculated for $y=3.86x+12.7$ in the range of 3.125 to 25 μg/ml of tolypomycin Y. The confidence limits for the regression coefficient were 3.44 and 4.28, respectively. From these results, the potency of tolypomycin Y could be assayed by this method with 6.25 μg/ml and 25 μg/ml solutions of tolopomycin Y as the standard.

Table 3. Recovery of added tolypomycin Y

Samples		Tolypomycin Y (μg/ml)	Tolypomycin Y added (μg/ml)	Total tolypomycin Y found (μg/ml)	Recovery of tolypomycin Y (%)
Culture filtrate	1	82	0	82	—
〃	2	82	20	110	107.8
〃	3	82	40	125	102.4
Ethyl acetate extract	1	54	0	54	—
〃	2	54	20	78	105.4
〃	3	54	40	94	100
〃	4	68	0	68	—
〃	5	68	10	75	96.1
〃	6	68	20	80	90.9
〃	7	68	30	102	112.4

The potency of tolypomycin Y in the culture broth can be determined directly by this method or indirectly by assay using a thin-layer chromatographic method and the two procedures are compared in Table 2.

The recovery percentage of tolypomycin Y in various samples was examined. As shown in Table 3, it was 90.9~112.4%.

Conclusions

Attempts to differentiate each component in an antibiotic complex have been already reported for the determination of penicillin[8,9]. Mikamycins A and B were also determined by a method using their characteristic synergistic action[10]. However, for these it was necessary to use 2 or 3 kinds of test organisms and to calculate the difference of the potencies. This method, however, is simple and effective for estimating selectively the potency of tolypomycin Y without interference by other components of the fermentation broths such as rifamycins B and O.

Acknowledgements

The author wishes to express his gratitude to Dr. S. TATSUOKA for his encouragement and to Drs. R. TAKEDA and A. MIYAKE for their advice.

References

1) SHIBATA, M.; T. HASEGAWA & E. HIGASHIDE: Tolypomycin, a new antibiotic. I. *Streptomyces tolypophorus* nov. sp., a new antibiotic, tolypomycin-producer. J. Antibiotics 24: 810~816, 1971
2) KISHI, T.; H. YAMANA, M. MUROI, S. HARADA, M. ASAI, T. HASEGAWA & K. MIZUNO: Tolypomycin, a new antibiotic. III. Isolation and characterization of tolypomycin Y. J. Antibiotics 25: 11~15, 1972
3) HASEGAWA, T.; E. HIGASHIDE & M. SHIBATA: Tolypomycin, a new antibiotic. II. Production and preliminary identification of tolypomycin Y. J. Antibiotics 24: 817~822, 1971
4) SENSI, P.; A. M. GRECO & R. BALLOTTA: Rifomycin. I. Isolation and properties of rifomycin B and rifomycin complex. Antibiot. Annual 1959/1960: 262~270, 1960
5) SENSI, P.; R. BALLOTTA & A. M. GRECO: Rifomycin. V. Rifomycin O, a new antibiotic of rifomycin family. Farmaco (Ed. Sci.) 15: 228~234, 1960
6) KISHI, T.; M. ASAI, M. MUROI, S. HARADA, E. MIZUTA, S. TERAO, T. MIKI & K. MIZUNO: Tolypomycin. I. Structure of tolypomycinone. Tetrahedron Letters 1969(2): 91~95, 1969
7) KISHI, T.; S. HARADA, M. ASAI, M. MUROI & K. MIZUNO: Tolypomycin. II. Structures of tolyposamine and tolypomycin Y. Tetrahedron Letters 1969(2): 97~100, 1969
8) HIGUCHI, K. & W. H. PETERSON: Estimation of types of penicillin in broths and finished products. A microbiological method. Anal. Chem. 19: 68~71, 1947
9) HOBBY, G. L.; B. BURKHART & B. HYMAN: Chemotherapeutic action of various forms of penicillin on hemolytic streptococcal infections in mice. Proc. Soc. Exp. Biol. & Med. 63: 296~301, 1946
10) WATANABE, K.: Studies on mikamycin. V. *In vitro* synergistic action and differential assay of mikamycin components. J. Antibiotics, Ser. A 13: 62~69, 1960