Note

Bioassay of Ansamitocin P-3,
an Antitumor AntibioticKazunori HATANO, Eiji HIGASHIDE
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Ansamitocins,¹⁾ produced by *Actinosynnema pretiosum* subsp. *pretiosum* No. C-15003 (Synonym: *Nocardia* sp. No. C-15003),^{2,3)} have antileukemic activity,⁴⁾ and inhibit the growth of eukaryotic microorganisms, but not that of prokaryotes.²⁾ In ansamitocin fermentation, *A. pretiosum* subsp. *pretiosum* No. C-15003 mainly produces ansamitocin P-2, P-3 and P-4 in the ratio of about 15 : 55 : 30,⁵⁾ but, in the presence of valine, selectively produces ansamitocin P-3.⁶⁾ To establish a process for the fermentative production of ansamitocin P-3, it is necessary to determine the antibiotic in a large number of fermentation samples. In an early study on the production of ansamitocin P-3, we tried to assay ansamitocin P-3 by the method of Hanka and Barnett⁷⁾ using *Penicillium avellaneum*. This assay method, however, could not be applied to our present study because the susceptibility to ansamitocin P-3 was low and the edge of the zone of inhibition was obscure. Therefore, we established a more favorable method for the assay of ansamitocin P-3.

To select the test organism, 396 strains of yeast from the Institute for Fermentation, Osaka (IFO), were employed. Among them, *Filobasidium uniguttulatum* IFO 0699 was selected as the most suitable test organism, based on the highest susceptibility to ansamitocin P-3 and the formation of a clear edge of the inhibition zone. The test organism was inoculated on a slant medium (Trypticase soy agar (BBL), supplemented with 1% glucose) and incubated at 28°C for 66 hr. A loopful of the culture was inoculated into 5 ml of Trypticase soy broth (BBL) in a test tube and the inoculant was incubated at 28°C for 18 hr on a reciprocal shaker. The resultant culture (about 5×10^8 cells/ml) was diluted 10-fold with 0.9% NaCl solution and 0.5 ml of the diluent was seeded into 100 ml of an assay medium (Trypticase soy agar supplemented with 1% glucose and 0.5% NaCl) at about 42°C. The seeded medium (10 ml) was poured into plastic Petri-dishes (9-cm diameter) to make plates. The assay was carried out by the agar-well method: Four wells were cut in each seeded agar plate with a cylinder punch (8-mm diameter) and then filled with 50- μ l sample solutions. Plates were incubated at

28°C for 42 hr. Each component, ansamitocin P-2, P-3 and P-4, was dissolved in methanol and the solutions were serially diluted with 0.1M phosphate buffer (pH 7.0) to final concentrations of 20, 10, 5, 2.5, 1.25 and 0.63 μ g/ml, before use. Dose-response curves of P-2, P-3 and P-4 were constructed by plotting the average zone diameter (mm) against the concentration (μ g/ml) on semi-logarithmic section paper (Fig. 1). The relative susceptibilities of ansamitocin P-2 and P-4 to that of P-3 were 44 and 152%, respectively. Concentrations of ansamitocin P-3 in unknown samples were calculated from the curve of P-3.

In order to evaluate the accuracy of the assay method, error and reproducibility of the assay results were exam-

FIG. 1. Dose-response Curves of Ansamitocin P-2, P-3 and P-4.

The regression lines of P-2 (○), P-3 (●) and P-4 (○) are $Y = 18.1 \log X + 10.1$, $Y = 18.1 \log X + 16.9$ and $Y = 18.3 \log X + 20.2$, respectively. Y , diameter of inhibition zone (mm); X , concentration of each ansamitocin component (μ g/ml).

TABLE I. INFLUENCE OF ANSAMITOCIN P-2 AND P-4 ON DETERMINATION OF ANSAMITOCIN P-3

The test was carried out by the agar-well method using *Filobasidium uniguttulatum* IFO 0699. The plates were incubated at 28°C for 42 hr.

Concentration of ansamitocin (μ g/ml)			Detection as P-3 (μ g/ml)
P-3	P-2	P-4	
5	—	—	5.0
5	0.5	—	5.0
5	1.0	—	5.1
5	1.5	—	5.4
5	2.0	—	5.9
5	—	0.5	5.1
5	—	0.75	5.4
5	—	1.0	6.0
5	0.25	0.25	5.1
5	0.5	0.5	5.4
5	0.75	0.75	5.6

TABLE II. REPRODUCIBILITY TEST

Sample		Detection ($\mu\text{g/ml}$)				Average ($\mu\text{g/ml}$)	Standard deviation
1	4.8	4.4	4.7	4.6	4.8	4.7	0.17
2	6.2	6.4	6.2	6.4	6.9	6.4	0.29
3	10.5	9.2	9.9	9.6	9.3	9.7	0.52
4	12.0	13.2	12.8	13.0	13.0	12.8	0.47
5	20.0	19.0	19.8	19.3	19.2	19.5	0.42

ined. Table I shows that the error of the assay of P-3 was less than 10% even if the sample contained 30% P-2, 15% P-4 or 10% of each them in relation to the concentration of P-3. Table II shows the high reproducibility of the test results, based on the standard deviation. In addition, the recovery yield of a known amount of ansamitocin P-3 added to the fermentation broth was in the range of 95.8 to 105% (data not shown).

These results indicate that the bioassay method described above is useful for determining ansamitocin P-3 in broth samples.

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