# THE MECHANISM OF ARISTEROMYCIN
## I. GROWTH INHIBITION OF *XANTHOMONAS ORYZAE* BY ARISTEROMYCIN

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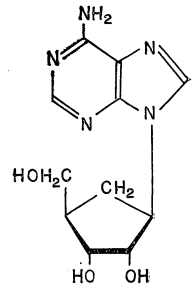
Five micrograms per milliliter of aristeromycin, a nucleoside antibiotic, completely inhibited the growth of *Xanthomonas oryzae* at all growing stages. When cells of *Xanthomonas oryzae* were treated with the antibiotic at the beginning of cultivation and transferred to a growth medium without antibiotic, the lag phase was prolonged without the change in the length of the log phase. Incubation of *Xanthomonas oryzae* with the antibiotic, reduces the viable cell count without affecting the optical density of the cell suspension. It was assumed that aristeromycin inhibited the growth of *Xanthomonas oryzae* by its bacteriocidal action. Ten to a hundred-fold amounts of either of adenosine, adenine, deoxyadenosine and inosine reversed the growth by aristeromycin. Complete reversal of the cell growth was not seen in the presence of these compounds because of their own growth inhibitory activity. Adenosine exhibited the strongest competitive activity with the antibiotic. It was suggested that the antibiotic acted on the metabolic pathway of adenylic acid, adenosine and adenine.

Aristeromycin is a new antibiotic which was isolated from culture filtrates of *Streptomyces citricolor*[1]. Its chemical structure was elucidated in a previous report[2] as shown in Fig. 1. Of many microorganisms tested, aristeromycin specifically inhibited the growth of *Xanthomonas oryzae*. Although the antibiotic did not exhibit any toxic effect against animals even at high dosages, it repressed or inhibited the growth of plants at fairly low concentrations[3].

To explain these characteristics of aristeromycin, the mode of action of aristeromycin against *X. oryzae* was investigated.

Fig. 1.
The structure of aristeromycin




### Materials and Methods

Microorganisms and media: *X. oryzae* (Uyeda et Ishiyama) DOWSON N5801 was used throughout the present experiments. Potato-sucrose medium containing 20 % boiled potato extract, 3 % sucrose and 2.5 % agar was used as slant medium. Culture medium (XO medium) contained 0.5 % sucrose, 0.1 % Na-glutamate, 0.001 % L-cysteine-HCl, 0.01 % $MgCl_2 \cdot 6H_2O$, 0.001 % $FeSO_4 \cdot 7H_2O$, 0.001 % $MnSO_4 \cdot H_2O$, 0.005 % $KH_2PO_4$ and 0.005 % $K_2HPO_4$. XO medium supplemented with 1 % of peptone and 0.0001 % of EDTA-Fe and 2 % of Bacto-Agar (Difco) (modified SUWA medium[4]) was used for counting viable cells.

Measurement of cell growth: Cells on stock slant were transferred to a fresh slant and incubated at 27°C for 2~3 days. This new growth was suspended in XO medium and propagated with reciprocal shaking at 27°C for 50~90 hours, cells were collected by centrifugation (8,000×*g*, 10 minutes) and washed twice with saline and resuspended in XO medium to make an optical density at 660 mμ 0.46. One tenth ml of the seed suspension was used to inoculate each unit consisting of 4.5 ml of XO medium, 0.25 ml of solution containing test materials and 0.25 ml of various concentrations of aristeromycin. Incubation was as described above. Growth of the cells was followed by measuring the optical density at 660 mμ with a Hitachi Spectrophometer (Type EPO-B)[5].

Measurement of viable cell number: Appropriate aliquots of cell suspensions were plated on modified SUWA medium and colonies were counted after incubation at 27°C for 48 hours.

Chemicals: Crystalline aristeromycin was prepared according to the method described in a previous paper[1]. All other chemicals used in this report were purchased from Wako Pure Chemical Ind., Ltd.

## Results

The effect of aristeromycin on the growth of *X. oryzae* at various growing stages

Fig. 2. Growth inhibition of *Xanthomonas oryzae* by aristeromycin.

One half ml of aristeromycin (final 0, 0.05, 0.1, 1 and 5 μg/ml) was added to a mixture of 4.5 ml of XO medium and 0.1 ml of seed suspension at 0 hour (A), 22 hours (B) or 30 hours (C).

A : aristeromycin 0 mcg/ml C : aristeromycin 0.1 mcg/ml E : aristeromycin 5 mcg/ml
B : aristeromycin 0.05 mcg/ml D : aristeromycin 1 mcg/ml

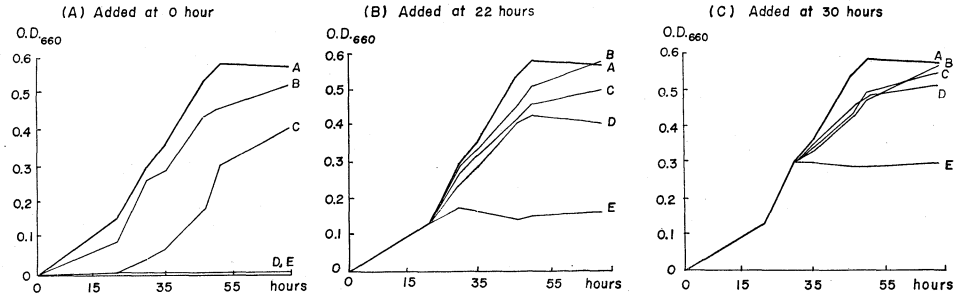



Fig. 3. The relationship between aristeromycin treatment time and growth of *Xanthomonas oryzae*.

One half ml of aristeromycin (final 5 μg/ml), 4.5 ml of XO medium and 0.1 ml of seed suspension was incubated at 27℃. At 3, 10, 21 or 28 hours, the cells were collected from aliquots of culture by centrifugation (1,000×*g*), washed twice with saline, resuspended in 5 ml of XO medium and incubated again without aristeromycin.

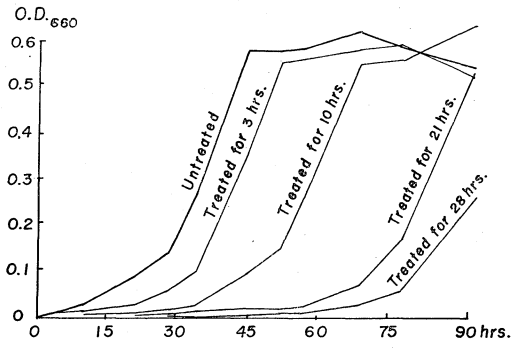
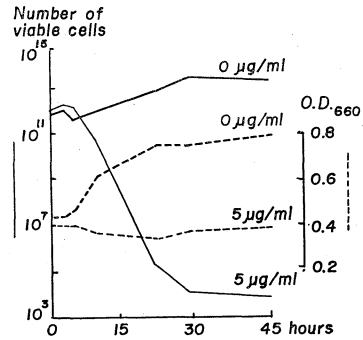



Fig. 4. The change of numbers of viable cell and optical densities of *Xanthomonas oryzae* by aristeromycin.

Four and a half ml of the cell suspension ($10^{12}$/ml) were incubated at 27℃ with or without 0.5 ml of aristeromycin (final 5 μg/ml) and the incubation was stopped at 0, 3, 5, 10, 24 or 48 hours. The cells were collected by centrifugation (1,000×*g*, 10 min.), washed twice with saline, resuspended in 5 ml of XO medium and the viable cells were determined. At the same time, growth was estimated by measuring the optical density.



were examined. As indicated in Fig. 2 (A), growth of *X. oryzae* was completely inhibited by addition of more than 1 μg/ml aristeromycin at 0 hour. When added after 22 hours of incubation, 1 μg/ml of antibiotic caused some inhibition of growth but 5 μg/ml was necessary for complete inhibition (Fig. 2 (B)). As shown in Fig. 2 (C), when aristeromycin was added after 30 hours of cultivation, growth was completely inhibited at concentrations of 5 μg/ml level, and no effects were observed at lower concentrations. In summary, it was concluded that 5 μg/ml of aristeromycin completely inhibited the growth of *X. oryzae* at any stages of the growth.

The relationship between the length of treatment time and growth curve was determined. Replicates containing 4.5 ml of XO medium, 0.5 ml of 5 μg/ml solution of aristeromycin and 0.1 ml of seed culture were incubated for 3, 10, 21 and 28 hours. Cells were collected from each solution, washed with saline, and cultivated in XO medium again. Cultivation and the measurement of growth were carried out as described in "Materials and Methods". As shown in Fig. 3, the induction period was prolonged with longer exposure to aristeromycin. The length of logarithmic phase remained almost the same irrespective of the length of treatment with antibiotic. Changes in the number of viable cells after the treatment with aristeromycin are presented in Fig. 4. The treatment of cells with aristeromycin for 3~5 hours did not markedly change the number of viable cells. Viable cells were, however, significantly decreased by exposure to antibiotic for 20~30 hours. From these results, it may be expected that the prolongation of induction period by treatment for 5 hours was not dependent on the decrease of viable cells. The elongation of the induction period for cells treated 3~5 hours may be due to different mechanisms than those involved when cells were treated with the antibiotic for 20 and 30 hours.

Table 1. Reversal of aristeromycin inhibition by purine and pyrimidide derivatives.

Mixtures of 0.25 ml of solutions of test compounds (final concentration 0.5 mM), 0.25 ml of aristeromycin solution (final concentration 0 or 0.005 mM), 4.5 ml of XO medium and 0.1 ml of seed suspension were incubated under the same conditions as Fig. 2. Figures in the table indicate the optical density at 660 mμ.

| Addition 50 mM | Aristeromycin | Incubation period (hours) 30 | 50 |
|---|---|---|---|
| Adenine | + | 0.19 | 0.18 |
| | − | 0.21 | 0.22 |
| Adenosine | + | 0.23 | 0.26 |
| | − | 0.2 | 0.25 |
| Guanine | + | 0 | 0 |
| | − | 0.2 | 0.58 |
| Guanosine | + | 0 | 0 |
| | − | 0.15 | 0.47 |
| Hypoxanthine | + | 0 | 0 |
| | − | 0.15 | 0.23 |
| Inosine | + | 0.14 | 0.19 |
| | − | 0.2 | 0.24 |
| Xanthine | + | 0 | 0 |
| | − | 0.17 | 0.56 |
| Xanthosine | + | 0 | 0 |
| | − | 0.15 | 0.55 |
| Deoxyadenosine | + | 0.27 | 0.5 |
| | − | 0.27 | 0.5 |
| Cytosine | + | 0 | 0 |
| | − | 0.18 | 0.56 |
| Cytidine | + | 0 | 0 |
| | − | 0.12 | 0.56 |
| Uracil | + | 0 | 0 |
| | − | 0.19 | 0.62 |
| Uridine | + | 0 | 0 |
| | − | 0.16 | 0.6 |
| Thymine | + | 0 | 0 |
| | − | 0.19 | 0.58 |
| Thymidine | + | 0 | 0 |
| | − | 0.15 | 0.51 |
| Deoxyuridine | + | 0 | 0 |
| | − | 0.18 | 0.52 |
| None | + | 0 | 0 |
| | − | 0.27 | 0.6 |

+ : with aristeromycin (0.005 mM)
− : without aristeromycin

Vitamins, amino acids, saccharides and compounds related to nucleic acid metabolism were tested for their effects on aristeromycin activity. As presented in Table 1,

only adenine, adenosine, deoxyadenosine and inosine partially reversed aristeromycin. These compounds, individually, exhibited inhibitory effects on the growth of *X. oryzae*. The effects of different concentrations of these compounds on growth inhibition and reversal of aristeromycin inhibition are summarized in Table 2. The growth curves of *X. oryzae* at various concentrations of adenosine and with or without aristeromycin are also illustrated in Figs. 5 and 6. Adenine, adenosine, hypoxanthine and inosine inhibited the growth of *X. oryzae* at 0.05, 0.05, 0.5 and 0.05 mM, respectively. Reversal of the inhibitory activity of 0.005 mM aristeromycin was observed with 0.05, 0.1, 0.5 and 0.5 mM concentrations of adenosine, adenine, deoxyadenosine and inosine respectively. Finally, the growth inhibition was tested in the presence of various concentrations of a constant ratio of adenine and aristeromycin. As indicated in Fig. 7, almost the same recovery of the growth was observed with constant ratios of adenosine and aristeromycin even though the amounts of aristeromycin varied.

Table 2. Reversal of aristeromycin inhibition by purine derivatives.

Mixtures of 0.25 ml of solutions of test compounds (final concentration 0.005, 0.05, 0.1 or 0.5 mM), 0.25 ml of aristeromycin solution (final concentration 0 or 0.005 mM), 4.5 ml of XO medium and 0.1 ml of seed suspension were incubated. Figures in the table indicate the optical density at 660 mμ.

| Addition | mM | Aristeromycin 0 mM | | Aristeromycin 0.005 mM | |
|---|---|---|---|---|---|
| | | Incubation period (hours) | | Incubation period (hours) | |
| | | 30 | 50 | 30 | 50 |
| Adenine | 0.5 | 0.21 | 0.22 | 0.19 | 0.18 |
| | 0.1 | 0.21 | 0.25 | 0.18 | 0.22 |
| | 0.05 | 0.2 | 0.22 | 0 | 0 |
| | 0.005 | 0.27 | 0.57 | 0 | 0 |
| Adenosine | 0.5 | 0.2 | 0.25 | 0.23 | 0.26 |
| | 0.1 | 0.2 | 0.27 | 0.2 | 0.27 |
| | 0.05 | 0.21 | 0.28 | 0.14 | 0.24 |
| | 0.005 | 0.23 | 0.56 | 0 | 0 |
| Deoxyadenosine | 0.5 | 0.18 | 0.57 | 0.14 | 0.5 |
| | 0.1 | 0.14 | 0.6 | 0 | 0 |
| Hypoxanthine | 0.5 | 0.15 | 0.23 | 0 | 0 |
| | 0.1 | 0.26 | 0.57 | 0 | 0 |
| | 0.05 | 0.2 | 0.6 | 0 | 0 |
| | 0.005 | 0.26 | 0.58 | 0 | 0 |
| Inosine | 0.5 | 0.2 | 0.2 | 0.14 | 0.19 |
| | 0.1 | 0.14 | 0.2 | 0 | 0 |
| | 0.05 | 0.16 | 0.22 | 0 | 0 |
| | 0.005 | 0.26 | 0.56 | 0 | 0 |
| None | | 0.27 | 0.6 | 0 | 0 |

Fig. 5. Growth inhibition of *Xanthomonas oryzae* by adenosine.

Mixtures containing 0.25 ml of adenosine solution (final concentration 0, 0.05, 0.1 or 0.5 mM), 4.75 ml of medium and 0.1 ml of seed suspension were incubated and growth determined.

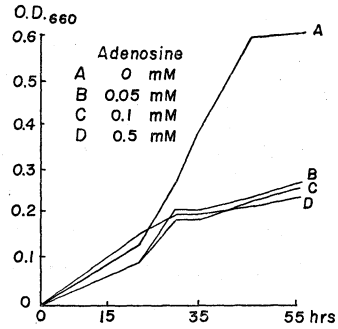
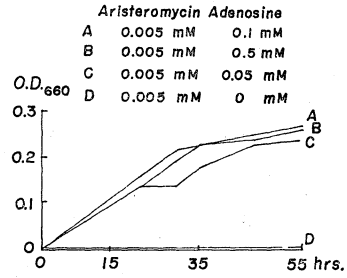



Fig. 6. Reversal of aristeromycin inhibition by adenosine.

Mixtures containing 0.25 ml of adenosine solution (final concentration 0, 0.05, 0.1 or 0.5 mM), 0.25 ml of aristeromycin solution (final concentration 0.005 mM), 4.5 ml of XO medium and 0.1 ml of seed suspension were incubated and O.D. determined.




## Discussion

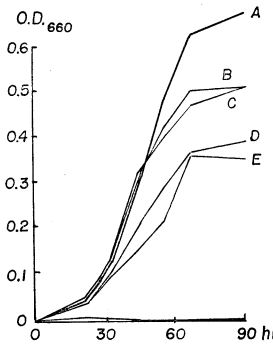
Many antimicrobial agents have been studied for their bacteriostatic and bacteriocidal properties. UMEZAWA[6] differentiates these activities as follows; in the presence of bacterio-

static antimicrobial agents, bacteria can grow but the growth is slower than that in the normal condition, whereas, in the presence of bacteriocidal antimicrobial agents bacteria cannot grow and viable cell numbers decrease. Generally, antibiotics show bacteriostatic activity at low concentrations and bacteriocidal activity at high concentrations, but differences between two activities are not clear.

The action of aristeromycin on *X. oryzae* was found to be bacteriocidal as determined by growth and on changes of viable cell numbers.

Fig. 7. Competitive reversal of aristeromycin inhibition by adenosine.

Mixtures, containing 0.25 ml of adenosine solution (final concentration 0, 0.1, 0.2, 0.5 or 1 mM), 0.25 ml of aristeromycin (final concentration 0.005 or 0.01 mM), 4.5 ml of XO medium and 0.1 ml of seed suspension were incubated, and O.D. determined.




| | Aristromycin (I) | Adenosin (II) | Ratio (II/I) |
|---|---|---|---|
| A : | — mM | — mM | — |
| B : | 0.01 | 1 | 100 |
| C : | 0.005 | 0.5 | 100 |
| D : | 0.005 | 0.1 | 20 |
| E : | 0.01 | 0.2 | 20 |
| F : | 0.005 | — | — |

Adenine, adenosine, hypoxanthine and inosine inhibited the growth of *X. oryzae*. Several reports have demonstrated that the mechanisms of inhibition of these adenine derivatives against microorganisms are as follows: (1) inhibition of biosynthesis of pyrimidine moiety in thiamine[7], (2) repression of the biosynthesis of purine nucleotides[8], and (3) unbalancing of amounts of pyrimidine and purine bases in cells[9]. Growth inhibition of *X. oryzae* by these compounds was not reversed by the addition of guanosine, uridine and cytidine, thus the mechanism of growth inhibition of *X. oryzae* by adenine derivatives remains obscure.

Adenosine and to a lesser degree, deoxyadenosine, adenine and inosine could partially reverse aristeromycin activity. Ribose, AICAR and other compounds tested were ineffective. These observations are interpreted as indicating that the synthetic pathway of purine nucleotides is not inhibited by the presence of aristeromycin. Consequently, it is suggeted that aristeromycin affects the metabolism of adenine derivatives. This suggestion is also supported by the similarity of the chemical structures of the compounds which exhibit interaction. These points will be discussed in detail in a forthcoming paper.

## References

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