STUDIES ON A NEW ANTIBIOTIC M-92 PRODUCED BY *MICROMONOSPORA*

IV. BACTERICIDAL ACTION OF THE COMPONENT VA-2

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The action of VA-2, the most active component of antibiotic M-92, against *S. aureus* is bactericidal but not bacteriolytic. The bactericidal action is markedly affected by incubation temperature, whether bacterial cells are prolific or resting. The bactericidal kinetics of VA-2 is biphasic, since addition of VA-2 caused rapid and straight decrease in viability curve and reached a plateau after several minutes. The bactericidal activity of VA-2 is blocked by 2,4-dinitrophenol. Alike to many membrane-active bacteriocins, VA-2 seems to exert its action through two stages.

As reported previously[1,2,3], antibacterial and anticancer antibiotic M-92 elaborated by *Micromonospora verruculosa* MCRL 0404 is a complex of several structurally related antibiotics having a quinoid nature. Among the six major components, VA-2 exhibited the most potent antibacterial activity, particularly against some Gram-positive bacteria (MICs: 0.000001 ~ 0.0001 μg/ml)[3]. Such a potent activity urged us to examine the mechanism of interaction between VA-2 and bacterial cells, and also the mode of action on the target bacteria.

The present paper concerns the characteristics of the interaction between VA-2 and the susceptible *Staphylococcus aureus* cells which suggest that interaction proceeds through two stages. In some parts of the present experiments, mitomycin C[4] was used for comparison, because mitomycin C, an antitumor quinoid antibiotic, shows biological properties somewhat resembling to VA-2. Mode of action of VA-2 as a DNA synthesis inhibitor will be dealt with in the succeeding paper.

Materials and Methods

Antibiotics and Chemicals

VA-2 was separated and purified as described in the preceding paper[2]. Mitomycin C and other chemicals were obtained from commercial sources. Antibiotic solutions were prepared by dissolving the antibiotic in dimethylsulfoxide and then diluting with an appropriate amount of sterile deionized water.

Microorganisms and Media

Staphylococcus aureus 209P JC-1 maintained in our laboratory was used. Nutrient broth (Difco) and Bacto-agar (Difco) were employed in the present study.

Culture Conditions for Growth and Viability Tests

A test strain was grown in double strength Nutrient broth (2NB) on a reciprocating shaker at 37°C. The bacterial growth was monitored by measuring absorbance at 600 nm. Bacterial cell cultures in an early exponential stage, whose absorbance at 600 nm reached to approximately 0.2, were used for the tests. Viability of cells was determined by counting the colony numbers grown on 2NB agar plates, which were prepared as follows. The cell suspensions were serially diluted with 0.9% physiological

saline, then transferred to Petri dishes and mixed with 2NB agar medium. The incubation was carried out at 37°C for 18 hours.

Preparation of Bacterial Cells

Cultures or suspensions of exponentially growing cells or antibiotic-treated cells were chilled in ice-water, harvested by centrifugation at 4°C and washed with cold phosphate-buffered saline (PBS) consisting of 0.115% Na_2HPO_4, 0.02% KH_2PO_4, 0.8% NaCl and 0.02% KCl (pH 7.2).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MICs and MBCs of antibiotics were determined in 2NB by two-fold serial broth dilution technique. MICs were determined after incubation at 37°C for 18 hours. One loopful of each cultured broth in MIC tests was inoculated onto antibiotic-free 2NB agar plates and after incubation at 37°C for 18 hours. MBCs were recorded as the minimum concentration of an antibiotic permitting no survival of bacteria on these plates.

Bactericidal Activity of VA-2 in the Presence of 2,4-Dinitrophenol

The bactericidal activity of VA-2 and mitomycin C in the presence of 2,4-dinitrophenol (DNP) and the effect of DNP upon susceptible cells previously exposed to VA-2 were examined using exponentially growing cultures under the experimental conditions cited in Table 2 and Fig. 6, respectively.

Results

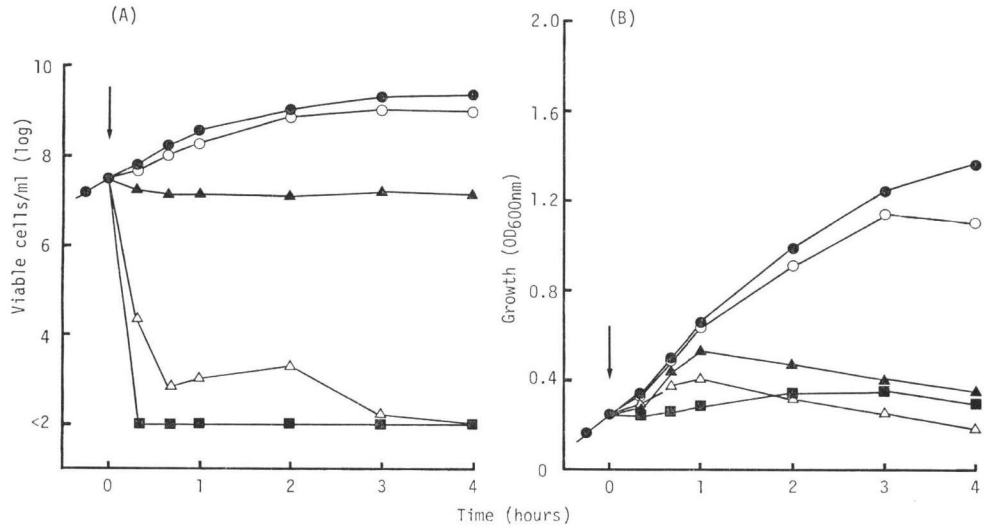
Effects of VA-2 on Viability and Growth of *S. aureus*

The effects of VA-2 on viability of exponentially growing *S. aureus* were examined in 2NB. As shown in Fig. 1 (A), addition of 0.1 μg/ml (final concentration) of VA-2 to *S. aureus* caused a rapid decrease in viability within 20 minutes. The decrease was gradual at 0.01 μg/ml. However, at 0.001 μg/ml, cells did not decrease or increase, suggesting the bacteriostatic action of VA-2 at this concentration, as verified by the fact that cells treated with 0.001 μg/ml of VA-2 for 2 hours showed normal growth under incubation after washing and transferring to the fresh medium. On the other hand, growth of bac-

Fig. 1. Effect of VA-2 on viability (A) and growth (B) of *S. aureus*.

Exponentially growing *S. aureus* 209P JC-1 (OD_{600nm}=about 0.2: $10^7 \sim 10^8$ cells/ml) in double strength Nutrient broth (2NB) were added with different concentrations of VA-2 at the time indicated by an arrow and further incubated at 37°C.

●, Control; ○, 0.0001 μg/ml; ▲, 0.001 μg/ml; △, 0.01 μg/ml; ■, 0.1 μg/ml.



terial cells reflected by turbidity [Fig. 1 (B)] was almost normal at 0.0001 μg/ml, but at 0.001 and 0.01 μg/ml, growth rendered slight inhibition after 1 hour of normal growth. Even at 0.1 μg/ml, turbidity did not decline. The above observations indicate that the action of VA-2 is bactericidal but not bacteriolytic.

Influence of Incubation Medium and Temperature upon Antibacterial Activity of VA-2

Influences of incubation medium and temperature upon killing induced by VA-2 were examined with *S. aureus*. The results are shown in Fig. 2. The drastic decrease in viable cells at 0.1 μg/ml and gradual decrease at 0.01 μg/ml were observed, when cells were incubated at 37°C in 2NB [Fig. 2 (A)] and PBS as well [Fig. 2 (B)] where cells were unable to proliferate. On the other hand, no decrease in viable cells was observed under incubation in 2NB at 0°C [Fig. 2 (C)].

If we can find out a known antibiotic which shows a similar behavior described above, it will be of use to deduce the bactericidal mechanism of VA-2. Thus, twelve commercial antibiotics, actinomycin D, bleomycin, daunomycin, mitomycin C, nalidixic acid, rifampicin, chloramphenicol, gentamicin, tetracycline, colistin, polymyxin B and ampicillin, were examined for this purpose. Among antibiotics tested, mitomycin C and colistin showed the bactericidal profile similar to that of VA-2.

For further investigation on the temperature effect, pulse treatments of *S. aureus* cells with VA-2 were attempted at 37°C and 0°C. The cells treated at each temperature for 5 minutes were washed and incubated in the fresh medium at 37°C, and the resulting damage on the cells was examined. The results are shown in Fig. 3. So long as examined on 0.01 and 0.1 μg/ml of VA-2, the difference of viability of cells due to the temperatures applied was noticed at zero time of subsequent incubation. However, 1 to 4 hours subsequent incubation of treated cells showed that the viability of cells was not affected by temperatures applied in pulse treatments, but only by VA-2 concentration.

Effect of Inoculum Size upon Bactericidal Activity of VA-2

To characterize the bactericidal action of VA-2, experiments described below were carried out in

Fig. 2. Effect of VA-2 on viability of *S. aureus* cells under various incubation conditions.

S. aureus 209P JC-1 cells grown to the exponential phase in 2NB were treated with different concentrations of VA-2 in the following incubation conditions:

(A) Incubation with VA-2 in 2NB, at 37°C.
(B) Exponentially growing cells were harvested, washed once with cold PBS to original volume and further incubated with VA-2 at 37°C.
(C) Cultures were chilled in ice-water, added with VA-2 and kept standing at 0°C.

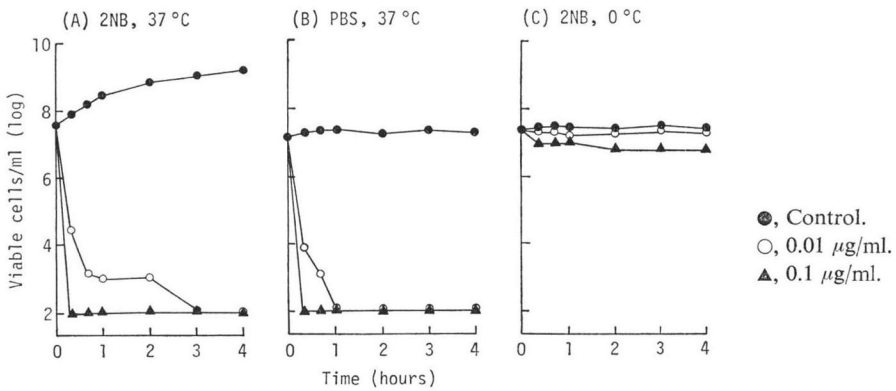


Fig. 3. Influence of temperature in pulse treatment with VA-2.

Twenty ml aliquots were withdrawn from the exponentially growing culture of *S. aureus* 209 P JC-1 (3.6×10^7 cells/ml) in 2NB. The cultures to be treated at 0°C were chilled quickly in an ice-water bath, added with different concentrations of VA-2 and allowed to stand for 5 minutes at 0°C, whilst in other cultures treatments were carried out at 37°C. After 5 minutes of lapse, cells were immediately diluted into 10 volumes of cold PBS and then harvested by centrifugation at 4°C. After 3 times of washing with cold PBS, cells were suspended in the fresh medium and incubated at 37°C. Samples were taken at intervals and plated for survivors.

●, 0.01 μg/ml, 0°C; ○, 0.01 μg/ml, 37°C; ▲, 0.1 μg/ml, 0°C; △, 0.1 μg/ml, 37°C.

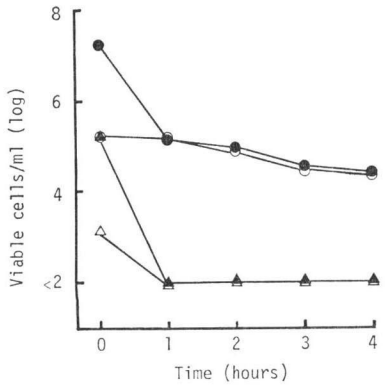


Table 1. Relationship of inoculum size with MICs and MBCs of VA-2 and mitomycin C.

Drug	Inoculum size (cells/ml)	MIC (μg/ml)	MBC (μg/ml)
VA-2	1.2×10^7	0.0025	0.0025
	1.2×10^5	0.000078	0.000078
	1.2×10^3	0.00002	0.00002
Mitomycin C	1.4×10^7	0.2	0.78
	1.4×10^5	0.2	0.78
	1.4×10^3	0.1	0.39

Medium: Double strength Nutrient broth.
Test organism: *S. aureus* 209P JC-1.

parallel with mitomycin C, because mitomycin C resembles VA-2 in chemical and biological properties, and further in bactericidal profile. Table 1 shows MICs and MBCs of VA-2 and mitomycin C against *S. aureus* at different inoculum sizes. MICs and MBCs of VA-2 were markedly affected by inoculum size of test organisms, and at every inoculum size MBC was the same to MIC. Those of mitomycin C were not affected significantly by inoculum size and MBCs were about 4-fold larger than MICs.

The effects of inoculum size were also noticed by comparing killing curves shown by these antibiotics under different cell concentrations of test organisms. As shown in Fig. 4, 0.001 μg/ml of VA-2 had no effect at all on *S. aureus* cells in 2.8×10^8 cells/ml concentration, but decreased by about 1 log in 2.9×10^7 cells/ml, about 3 logs in 4.0×10^6 cells/ml and more than 4 logs in 2.2×10^5 cells/ml. VA-2 was more effective to cells of low concentration than to those of high concentration. However, 0.78 μg/ml of mitomycin C caused decrease in viability of *S. aureus* by 2~3 logs at all cell concentrations ($6.8\times10^8\sim4.8\times10^5$ cells/ml), so that the bactericidal action of mitomycin C did not correlate to the cell concentrations.

Bactericidal Kinetics

Selecting the concentration which could decrease viability at exponential phase (about 10^7 cells/ml) by about 2 logs in 1 hour, we studied the bactericidal kinetics of VA-2 and mitomycin C. As shown in Fig. 5, the killing kinetics of VA-2 was biphasic, since the addition of 0.001 μg/ml (final concentration) of VA-2 immediately caused rapid decrease in viability and the decrease stopped after 20 minutes. Contrast with VA-2, mitomycin C straightly decreased viability after a short lag time (15 to 20 minutes). The action of VA-2 against *S. aureus* also showed single-hit kinetics, as shown by a straight line intercepting the ordinate at 100% survival which was obtained by plotting the percent survival against increasing VA-2 concentration.

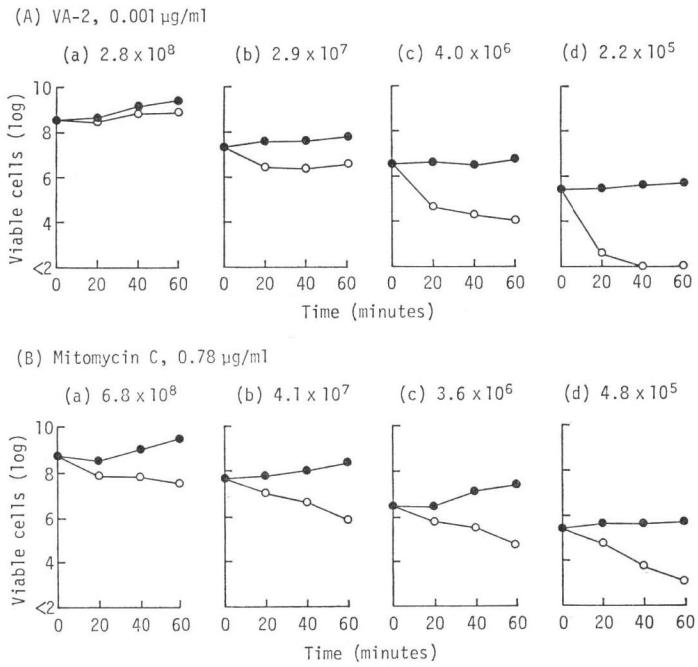
Effect of 2,4-Dinitrophenol on Bactericidal Activity of VA-2

The fact that the temperature effect on bactericidal action of VA-2 was not observed in the 5 minutes

Fig. 4. Correlation of bactericidal activities of VA-2 (A) and mitomycin C (B) with cell concentration in cultures.

Exponentially growing *S. aureus* 209P JC-1 cells (3.3×10^7 cells/ml) in 2NB were harvested by centrifugation. Cells were suspended in the fresh medium to give different cell concentrations. After incubation for 30 minutes at 37°C, 20 ml aliquots were withdrawn from each cell suspension. Then, the cell suspensions were added with 0.001 μg/ml of VA-2 or 0.78 μg/ml of mitomycin C, and duplicate samples without an antibiotic were served as control. After incubation at 37°C, samples were taken at indicated times, and plated for survivors.

●, Control; ○, antibiotic treatment.



pulse treatment suggested that interaction VA-2 with susceptible cells may proceed at least through two stages, temperature-independent and -dependent stages. In order to understand these stages in detail, we examined the effect of DNP, an uncoupler of oxidative phosphorylation, on the bactericidal action of VA-2. The effects of DNP on the bactericidal action of VA-2 and mitomycin C against *S. aureus* are shown in Table 2. DNP alone showed almost no or very weak inhibitory effects upon bacterial growth at 1 and 10 mM concentrations. At these concentrations, DNP prevented the *S. aureus* cells from decrease in viability caused by VA-2. On the contrary, DNP did not protect *S. aureus* cells from killing by mitomycin C. Since the pretreatment of VA-2 with DNP did not decrease its bactericidal activity, it seems unlikely that DNP inactivates VA-2. As shown in Fig. 6, the addition of DNP to VA-2-treated cells immediately stopped the decrease in viability.

Discussion

VA-2, one of the components of a quinoid antibiotic M-92, exhibits a variety of inhibitory effects depending on the concentration: bacteriostatic action at low concentration, morphological change of *Escherichia coli* to long filament at slightly higher but still non-bactericidal concentration (data not shown), and rapid bactericidal action by attacking growing as well as non-growing cells at higher

Fig. 5. Bactericidal kinetics of VA-2 (A) and mitomycin C (B).

Twenty ml aliquots were withdrawn from the exponentially growing culture of *S. aureus* 209P JC-1 (3.6×10^7 cells/ml) in 2NB. Then, the cultures were treated with VA-2 (final concentration: 0.001 μg/ml) or mitomycin C (0.78 μg/ml). After incubation at 37°C, samples were taken at indicated times, and plated for survivors.

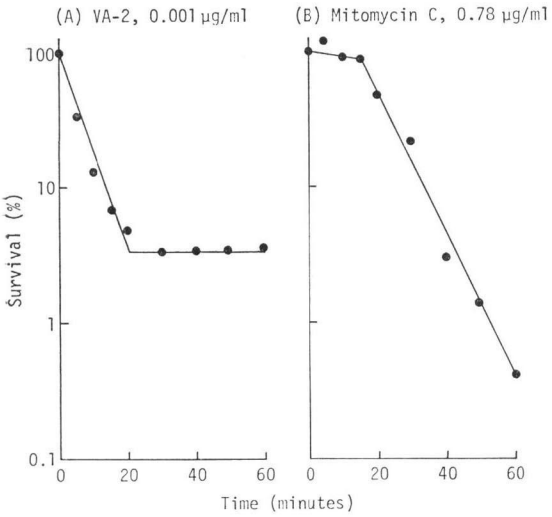


Fig. 6. Effect of the addition of 2,4-dinitrophenol to VA-2-treated cells.

Exponentially growing *S. aureus* 209P JC-1 (6.7×10^7 cells/ml) in 2NB were added with VA-2 (final concentration: 0.01 μg/ml). Duplicate samples were removed and either was added with 2,4-dinitrophenol dissolved in dimethylsulfoxide to give a final concentration of 10 mM at indicated times and further incubated at 37°C. Samples were assayed for survivors on agar plates.

●, Cultures in the presence of VA-2; ○, samples after addition of 2,4-dinitrophenol to the above cultures.

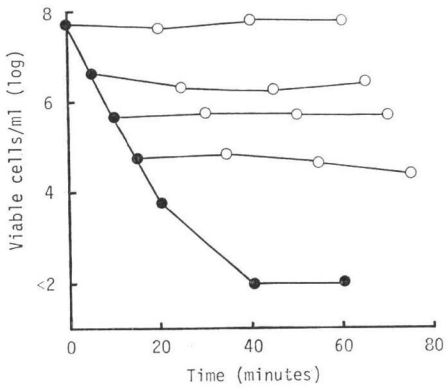


Table 2. Bactericidal activity of VA-2 and mitomycin C in the presence of 2,4-dinitrophenol.

Exponentially growing *S. aureus* 209P JC-1 (2.8×10^7 cells/ml) in 2NB were added with different concentrations of 2,4-dinitrophenol together with VA-2 or mitomycin C. After 1 hour of incubation at 37°C, samples were assayed for survivors on agar plates.

Concentration of 2,4-dinitrophenol (mM)	Viable cells (CFU)		
	VA-2 (0.01 μg/ml)	Mitomycin C (0.78 μg/ml)	No antibiotic
10	1.7×10^7	2.0×10^6	2.9×10^7
1	6.0×10^3	2.2×10^6	2.8×10^7
0.1	$<10^3$	7.4×10^5	7.9×10^7
0	$<10^3$	3.4×10^5	1.1×10^8

concentrations. Elongation of bacterial cells was commonly observed on antibiotics which primarily inhibit cell wall or DNA synthesis[5]. VA-2 further affects several other eucaryotic cells as illustrated by its antitumor activity, cytotoxicity and high order of acute toxicity[3]. These characteristics quite resemble those of a quinoid antibiotic, mitomycin C[4,6] inhibiting DNA synthesis. However, action mechanism of VA-2 differs from that of mitomycin C as described below. The antibacterial effects of mitomycin C was not affected by inoculum size and cell concentration in the cultures, and the bactericidal action initiating after a short lag is uniphasic (Fig. 5). Further the bactericidal action of mitomycin C was not blocked by DNP (Table 2).

Bactericidal action of VA-2 against *S. aureus* was much affected by incubation temperature. However, in the pulse treatment of cells, differences in treating temperatures caused differences in viability (Fig. 3). This finding suggests that at least two distinct stages are involved in the interaction between VA-2 and susceptible bacterial cells. The first (stage I) is the binding or adsorption of VA-2 to the growing or non-proliferating bacterial cells which, as suggested by pulse treatment experiments, happens irrespective of temperature and the second (stage II) is a lethal action at a target to cause an irreversible physiological damage. In addition, the facts that the bacterial killing effect of VA-2 appears at a much slower rate at low temperatures and the bactericidal action is blocked by DNP suggest that VA-2 requires

metabolic energy for the exertion of its inhibitory action, for example, for the transition from stage I to stage II.

Many membrane-active bacteriocins have been recognized to interact with a target cell in two stages and the transition from stage I to stage II is influenced by temperature as referred to in the review articles[7,8,9]. In this respects, interaction of VA-2 with the susceptible cells is quite similar to that of bacteriocin. Moreover, some further resemblances were observed in the action phenomena of VA-2 and proteinous bacteriocins. In certain bacteriocins, stage I interaction is specifically removed by treatment with proteases such as trypsin[10], and the transition from stage I to stage II can be blocked by addition of uncouplers of oxidative phosphorylation such as DNP[11,12]. The bactericidal activity of VA-2 against *S. aureus* was slightly reduced by incubation of 30 minutes at 37°C with trypsin (5 mg/ml, pH 8) (data not shown) and blocked by the addition of DNP as described. Furthermore, observations that VA-2 did biphasically induce killing and its bactericidal action shows a single-hit kinetics (data not shown) are similar to that reported for many bacteriocins[7,8,9]. Though it is too early to adopt the mechanism of bacteriocins to VA-2, the apparent resemblance in bactericidal properties of VA-2 with bacteriocins suggests a way to search for the mechanism of interaction of VA-2 with the cell surface of the target bacteria.

Bactericidal effect of VA-2 against *E. coli* was also examined in parallel with the experiments for *S. aureus*. MBC of VA-2 against *E. coli* was about 100-fold larger than MBC against *S. aureus*. However, the bactericidal action of VA-2 against *E. coli* was temperature dependent, affected by inoculum size in the culture and blocked by DNP, so that the mechanism operating on *E. coli* may be similar to that on *S. aureus*. If so, the difference in bactericidal concentrations to the respective cell may be ascribed to the chemical property of cell surface or the physical circumstances around the binding site on the cells.

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References

1) TANI, K.; N. MATSUZAWA, S. YANO & T. YAMAGUCHI: Studies on a new antibiotic M-92 produced by *Micromonospora*. I. Taxonomy of M-92 producing *Micromonospora* and antibiotic production therefrom. J. Antibiotics 35: 1430~1436, 1982
2) TANI, K. & T. TAKAISHI: Studies on a new antibiotic M-92 produced by *Micromonospora*. II. Isolation and physicochemical properties of M-92 and its components. J. Antibiotics 35: 1437~1440, 1982
3) TANI, K.; Y. ARAI & T. YAMAGUCHI: Studies on a new antibiotic M-92 produced by *Micromonospora*. III. Biological activities. J. Antibiotics 35: 1441~1447, 1982
4) WAKAKI, S.; H. MARUMO, K. TOMIOKA, G. SHIMIZU, E. KATO, H. KAMADA, S. KUDO & Y. FUJIMOTO: Isolation of new fractions of antitumor mitomycins. Antibiot. Chemother. 8: 228~240, 1958
5) SAUGENT, M. G.: Surface extension and the cell cycle in prokaryotes. Adv. Microb. Physiol. 18: 105~176, 1978
6) REICH, E.; A. J. SHATKIN & E. L. TATUM: Bacteriocidal action mitomycin C. Biochim. Biophys. Acta 53: 132~149, 1961
7) REEVES, P.: The bacteriocins. Bacteriol. Rev. 29: 24~45, 1965
8) NOMURA, M.: Colicins and related bacteriocins. Annu. Rev. Microbiol. 21: 257~287, 1967
9) HARDY, K. G.: Colicinogeny and related phenomena. Bacteriol. Rev. 39: 464~515, 1975
10) PLATE, C. A. & S. E. LURIA: Stages in colicin K action as revealed by the action of trypsin. Proc. Nat. Acad. Sci. 69: 2030~2034, 1972
11) OKAMOTO, K.: Requirement of heat and metabolic energy for the expression of inhibitory action of colicin K. Biochim. Biophys. Acta 389: 370~379, 1975
12) STREKER, R. G.; R. A. VENEQIA & R. G. TOBERTSON: Interaction of hemocin with *Escherichia coli*. Antimicrob. Agents Chemother. 19: 668~671, 1981