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

III. BIOLOGICAL ACTIVITIES

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The six major components of M-92, a new antibiotic complex produced by *Micromonospora verruculosa* MCRL 0404 showed a similar type of antimicrobial spectrum. Among these components, VA-2 exhibited the most potent antimicrobial activity, particularly significantly against some Gram-positive bacteria and *Neisseria*. VA-2 and BN-1 also exhibited marked inhibitory effects against L-forms of *Staphylococcus aureus* 209P and *Mycoplasma*. The MICs of VA-2, BA-4 and BN-1 were remarkably affected by the pH of the test medium, the inoculum size and the amount of horse serum added in the medium. By intraperitoneal administration, these components showed good protective effects in mice infected intraperitoneally with *Staphylococcus aureus* Smith. However, the protective effect decreased remarkably by other administration routes. In addition, components such as VA-2 and BN-1 exhibited cytotoxicity against HeLa S-3 cells *in vitro* and excellent *in vivo* antitumor activity against Ehrlich carcinoma. VA-2 possessed a high order of acute toxicity to mice [LD_{50}: 1.9 mg/kg (i.p.); 1.8 mg/kg (i.v.)], but others were relatively less toxic.

M-92, a new antibiotic complex, was obtained from culture broth and mycelia of *Micromonospora verruculosa*[1]. As reported in the foregoing paper, this complex was separated into the six major components by silica gel column chromatography, respectively designated VA-2, BA-4, BA-5, BN-1, BN-2 and BN-3[2]. Those components obtained as reddish violet to blue amorphous powder are acidic or weakly acidic in nature and are considered to possess a quinoid structure judging from their physicochemical properties. The present paper is concerned with *in vitro* antimicrobial spectra, influences of medium pH, inoculum size and serum on the antibacterial activity, and protective effects against experimental bacterial infection by these components. In addition, cytotoxity on HeLa S-3 cells (*in vitro*), antitumor activity against Ehrlich carcinoma (*in vivo*) and acute toxicity in mice are also involved.

Materials and Methods

Antibiotics

M-92 components were prepared as described in the preceding papers[1,2]. Antibiotic solutions for *in vitro* or *in vivo* test were prepared by dissolving the antibiotics in a small quantity of dimethylsulfoxide followed by dilution with sterile deionized water.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of each antibiotic component was determined by the two-fold serial agar dilution method. The test organisms are those maintained in the stock culture collection of our laboratory.

Antibacterial and Antifungal Activity: One loopful of the culture suspension (10^6 cells/ml) of test organisms, except for *Mycobacterium*, was streaked on an assay plate containing the antibiotic, and the plates were incubated as follows: bacteria, at 37°C for 18 hours; fungi, at 27°C for 2~7 days. MIC

was determined as the lowest concentration at which the visible growth of the test organism was completely inhibited.

Anti-L-form Activity: The L-forms were induced from *Staphylococcus aureus* 209P JC-1 by the penicillin-disc method on L-form agar[3] (3.7% brain heart infusion broth, 5% NaCl, 1% agar and 10% horse serum). The stable L-form strain L-PG-412 was obtained by 10 transfers on the L-form agar supplemented with 5 units/ml of benzyl penicillin. The strain did not revert to the parent bacterial form during about 10 transfers on L-form agar containing no benzyl penicillin. On the contrary, the unstable L-form L-PG-212 reverted to the parent form on the L-form agar containing no benzyl penicillin. These L-forms were positive for coagulase production and resistant to penicillins. MICs of benzyl penicillin against these L-forms were 25~100 units/ml. The stable L-form was cultured in the L-liquid culture medium[4] (3.7% brain heart infusion broth, 5% NaCl and 10% horse serum), while the unstable L-form was cultured in the L-liquid culture medium supplemented with 5 units/ml of benzyl penicillin. For the source of inoculum, these organisms were incubated for 3 days at 37°C and 0.01 ml of the cell culture (10^6 cells/ml) was dropped on the assay plate. The plates were sealed with vinyl-tape and kept standing at 37°C for 3 days. L-Form colonies grown on the plate were observed under a light microscope. The lowest concentration at which an antibiotic completely inhibited the growth of colonies was regarded as the MIC of this antibiotic.

Antimycoplasmal Activity: Mycoplasmas were cultured in the liquid medium[5] [2.1% PPLO broth (Difco) 70 ml, fresh horse serum 20 ml, 25% fresh yeast extract 10 ml, thallium acetate 50 mg and benzyl penicillin (Na) 50,000 units in 100 ml (pH 7.6~7.8)]. After incubation at 37°C for about 3 days, the culture was used as the inoculum source. The antibiotic was diluted with the solid medium[6] [3.7% PPLO agar (Difco) 70 ml, fresh horse serum 20 ml and 25% fresh yeast extract 10 ml]. A small quantity (0.01 ml) of the cell culture (10^6~10^7 cells/ml) was dropped on the assay plate. Plates were sealed with vinyl-tape and incubated at 37°C for 7 days. Colonies grown on the plates were observed under a light microscope.

Influence of Various Factors on MIC

The influence of various factors on the MIC of an antibiotic was examined by the two-fold serial broth dilution method. To assess the influence of medium pH, pH values of the medium were adjusted by 1 N HCl or 1 N NaOH. The influence of serum on MIC was examined by the volume of horse serum added in the medium. A small quantity (0.1 ml) of the culture suspension was added into 2 ml of the assay broth containing an antibiotic and the broth was incubated at 37°C for 18 hours.

Mouse Protection Test from Experimental Bacterial Infection

Male *dd*Y mice aged 4 weeks, weighing 19~21 g were used. An overnight culture of *Staphylococcus aureus* Smith in Trypto-soy broth (Eiken) at 37°C was suspended in 5% bacteriological mucin (ICN Pharmaceuticals, Inc.) solution. The volume of $100 \times LD_{50}$ of the above suspension was used to infect the mice intraperitoneally. The protective effect (ED_{50}) was estimated from the survival ratio for 7 days.

Cytotoxic Test on HeLa S-3 Cells

About 3×10^5 cells of HeLa S-3 were incubated in the culture tube with 1 ml of the medium. After incubation for 24 hours at 37°C, the old medium was replaced by fresh medium containing the antibiotic and the incubation was continued for 3 days. Cytological changes were determined by the microscopic observation of the cell stained with May-Gruenwald Giemsa solution.

Anti-Ehrlich Activity Test in Mice

Female ICR mice weighing 19~22 g were used. For Ehrlich ascites tumor, antitumor activity was evaluated by comparing the mean survival day of the treated mice with that of non-treated mice, *i.e.* by percentage increase in life span (ILS%). For Ehrlich solid tumor, antitumor activity was evaluated in terms of the inhibitory ratio of the tumor growth at 10 days after implantation by comparing the mean tumor weight in the treated mice with that of non-treated mice, *i.e.* by percentage of inhibitory ratio.

Acute Toxicity Test in Mice

Male *dd*Y mice aged 4 weeks, weighing 19~21 g were used. LD_{50} values were estimated from the lethal ratio for 7 days after a single intraperitoneal or intravenous administration.

Table 1. Antimicrobial spectra of M-92 components.

Test organism	Medium[b]	MIC (μg/ml)					
		VA-2	BA-4	BA-5	BN-1	BN-2	BN-3
Staphylococcus aureus 209P JC-1	I	0.000008	0.125	0.78	0.00625	0.0313	0.0313
Staphylococcus aureus 199R (TC, Mac)^r	I	0.000016	0.25	0.78	0.00625	0.0313	0.0313
Staphylococcus epidermidis Kawamura	I	0.000031	0.25	0.78	0.00625	0.0313	0.0313
Streptococcus faecalis CN-478	I	0.000125	0.5	1.56	0.05	0.78	0.125
Streptococcus pyogenes	II	0.0001	0.078	1.25	0.019	1.56	0.39
Streptococcus pneumoniae type-1	II	0.00001	0.039	0.625	0.009	0.78	0.019
Corynebacterium diphtheriae P.W. 8	II	0.00001	0.019	0.156	0.009	0.78	0.019
Bacillus subtilis ATCC 6633	I	0.000001	0.004	0.156	0.001	0.0313	0.004
Mycobacterium tuberculosis $H_{37}RV$[a]	III	0.01	5	10	1.0	5.0	5.0
Escherichia coli JC-2	I	0.0156	>100	>100	100	>100	>100
E. coli ML-1410 RGN-823 (TC, CM, SM, SA, ABPC, KM)^r	I	0.0313	>100	>100	100	>100	>100
Salmonella typhi T-58	I	0.0156	25	>100	100	>100	>100
Klebsiella pneumoniae PCI-602	I	0.0313	50	>100	>100	>100	>100
Proteus mirabilis TU-1698	I	0.0156	100	>100	100	>100	>100
Proteus vulgaris IID-874	I	0.0156	100	>100	100	>100	>100
Proteus morganii Kono	I	0.0156	50	>100	100	>100	>100
Citrobacter freundii GN-346	I	0.0156	>100	>100	100	>100	>100
Serratia marcescens 7006 (TC)^r	I	0.0156	>100	>100	100	>100	>100
Pseudomonas aeruginosa PI-67 (GM)^r	I	0.0313	>100	>100	100	>100	>100
Pseudomonas aeruginosa No. 12	I	0.0313	>100	>100	100	>100	>100
Neisseria meningitidis 13090	IV	0.001	1.25	10	0.625	0.625	0.625
Neisseria gonorrhoeae Yoshioka	IV	0.00001	0.039	0.625	0.039	0.039	0.039
Bordetella pertussis Tohama	IV	0.001	1.25	10	1.25	1.25	1.25
Xanthomonas citri	V	3.12	>50	>50	25	25	50
Candida albicans 3147	V	25	>50	50	>50	>50	>50
Penicillium chrysogenum 48–132	VI	25	>50	>50	>50	50	>50
Cochliobolus miyabeanus	VI	6.25	>50	>50	25	>50	12.5
Piricularia oryzae	VI	1.56	>50	>50	12.5	50	>50
Fusarium nivale	VI	50	>50	>50	>50	>50	>50
Microsporium gypseum	VI	50	>50	>50	>50	>50	>50
Trichophyton mentagrophytes	VI	>50	>50	>50	>50	>50	>50

a) The MIC was determined by broth dilution method.

b) Medium I: Heart infusion agar. II: Brain heart infusion agar. III: Kirchner medium. IV: Brain heart infusion agar supplemented with 10% horse serum. V: Heart infusion agar supplemented with 1% glycerin. VI: Sabouraud agar.

Results and Discussion

Antimicrobial Spectrum and Activity

Antimicrobial spectra and MICs of antibiotic M-92 components are shown in Tables 1 and 2. All components showed similar antimicrobial spectra *in vitro* and exhibited marked activities particularly against some Gram-positive bacteria and *Neisseria*. VA-2 and BN-1 exhibited remarkable activities against L-forms of *S. aureus* and *Mycoplasma* and these activities were higher than those against the parent *Staphylococcus aureus* 209P. Among M-92 components, VA-2 exhibited the most potent antimicrobial activity against all test organisms. VA-2 inhibited most of the Gram-positive bacteria at a concentration of 0.000001~0.0001 μg/ml, *Neisseria* at 0.00001~0.001 μg/ml, L-forms of *S. aureus* at 0.000156 μg/ml and *Mycoplasma* at 0.0000313~0.002 μg/ml. Moreover, VA-2 inhibited the growth of a number of drug resistant strains as well as those of the sensitive strains. VA-2 showed weak antifungal activity as well.

Table 2. Anti-L-form and antimycoplasmal activity of VA-2 and BN-1.

Test organism	Medium[c]	MIC (μg/ml)	
		VA-2	BN-1
Staphylococcus aureus 209P JC-1	VII	0.000625	0.0625
Staphylococcus aureus L-PG-412[a]	VII	0.000156	0.0313
Staphylococcus aureus L-PG-412	VIII	0.000156	0.0313
Staphylococcus aureus L-PG-212[b]	VIII	0.000156	0.0313
Mycoplasma pneumoniae Mac	IX	0.0005	0.0625
Mycoplasma gallisepticum Kp-13	IX	0.000125	0.0156
Mycoplasma gallisepticum PG-31	IX	0.00025	0.0313
Mycoplasma pulmonis PG-22	IX	0.0000313	0.0039
Mycoplasma mycoides	IX	0.0078	1.0
Acholeplasma laidlawii	IX	0.001	0.125
Acholeplasma laidlawii PG-10	IX	0.002	0.25

a) Stable L-form strain.
b) Unstable L-form strain.
c) Medium VII: Brain heart infusion broth supplemented with 5% NaCl, 10% horse serum and 1% agar. VIII: Medium VII supplemented with 5 units/ml of PC-G(Na). IX: PPLO agar supplemented with 20% horse serum and 10% yeast extract solution (25%).

Table 3. Influence of various factors on the antibacterial activity of M-92 components.

Factor		MIC (μg/ml)		
		VA-2	BA-4	BN-1
pH	5.5	0.0000156	0.01	0.001
	6.0	0.0000312	0.02	0.001
	7.0	0.0000312	0.078	0.002
	8.0	0.00025	0.625	0.0078
Inoculum size	10^8	0.001	1.25	0.25
	10^7	0.00025	0.625	0.0312
	10^6	0.0000312	0.078	0.002
	10^5	0.0000156	0.039	0.001
	10^4	0.0000156	0.039	0.0005
Serum[a] (%)	0	0.0000312	0.078	0.002
	10	0.0005	0.312	0.0156
	25	0.0005	0.625	0.0312
	50	0.001	2.5	0.0625

a) Horse serum.
Test organism: *Staphylococcus aureus* Smith.
Inoculum size except inoculum effect test: 10^6 cells/ml.
Medium: Heart infusion broth.

Influence of Medium pH, Inoculum Size and Serum on Antibacterial Activity

The influence of medium pH, inoculum size and serum on the antibacterial activities of VA-2, BA-4 and BN-1 are shown in Table 3. The MIC of the component was dependent upon the pH of

Table 4. Protective effect of M-92 components in mice intraperitoneally infected with *Staphylococcus aureus* Smith.

Component	MIC (μg/ml)	ED_{50} (mg/kg)[a]			
		i.p.	i.v.	i.m.	p.o.
VA-2	0.000016	<0.00035	0.013	0.7	>4
BA-4	0.125	<0.125	1.14	>20	>20
BA-5	0.39	<0.125	>20	>20	>20
BN-1	0.00625	<0.125	4.4	17.6	>20
BN-2	0.0313	<0.125	>20	>20	>20
BN-3	0.0313	<0.125	>20	>20	>20

[a] The antibiotic was given in a single dose at 1 hour after infection.

Table 5. Cytotoxic activity of M-92 components on HeLa S-3 cells.

Component	Minimum degenerating concentration (μg/ml)	Component	Minimum degenerating concentration (μg/ml)
VA-2	0.001	BN-1	0.25
BA-4	4.0	BN-2	1.0
BA-5	4.0	BN-3	1.0

Medium: Eagle's balanced salt solution containing 0.1% yeast extract, 0.5% lactalbumin hydrolysate and bicarbonate, supplemented with 20% fetal calf serum and kanamycin (100 μg/ml).

Table 6. Effect of M-92 components on Ehrlich carcinoma (ascites form).

Component	Dose[a] (mg/kg/day)	Mean survival day	ILS[b] (%)	60-day survivors
VA-2	1.0	17.3	21.0	0 / 5
	0.5	>57.0	>298.6	4 / 5
	0.25	>44.2	>209.1	1 / 5
	0.125	>43.8	>206.3	1 / 5
	0.063	>47.0	>228.7	1 / 5
	0.031	34.2	139.2	0 / 5
	0.016	30.2	111.2	0 / 5
	0.008	17.6	23.1	0 / 5
BA-4	10	>44.4	>210.5	2 / 5
	5	35.2	146.2	0 / 5
	2.5	21.6	51.0	0 / 5
BA-5	10	19.2	34.3	0 / 5
	5	22.4	56.6	0 / 5
BN-1	10	30.0	109.8	0 / 5
	5	>51.8	>262.2	3 / 5
	2.5	>48.8	>241.3	2 / 5
	1.25	28.6	100.0	0 / 5
	0.63	21.6	51.0	0 / 5
BN-2	10	>48.6	>239.9	2 / 5
	5	26.0	81.8	0 / 5
	2.5	16.6	16.1	0 / 5
BN-3	10	>48.8	>241.3	2 / 5
	5	37.5	162.2	0 / 5
	2.5	20.4	42.7	0 / 5
Non-treated		14.3	0.0	0 / 5

Ascites cells (10^6) of Ehrlich carcinoma were intraperitoneally inoculated into female ICR mice.

[a] The antibiotic was intraperitoneally administered once daily for 5 days, starting 24 hours after the inoculation.

[b] ILS(%)=(mean survival day in the treated group/mean survival day in the non-treated group — 1)×100.

the test medium and the components were substantially less active at an alkaline pH. The influence of alkalization was especially remarkable on VA-2 and BA-4 of the acidic type. The MICs of the components were raised significantly when the inoculum size was increased. The presence of horse serum at a final concentration of 10~50% in the medium negatively affected the activity of the components.

Protective Effect from Experimental Infection in Mice

Table 4 shows comparative activities of M-92 components on mice infected intraperitoneally with *S. aureus* Smith. By intraperitoneal administration, these components showed a good protective effect but by intravenous, intramuscular or oral administration, the effects decreased remarkably. The reduction of the antibacterial activities of M-92 components in the presence of horse serum may be related to the low protective effects of these components when given by intravenous, intramuscular or oral route.

Cytotoxic Activity

The cytotoxic activities of M-92 components on HeLa cells are shown in Table 5. The cytological changes of HeLa S-3 cells were observed at concentrations higher than 4 μg/ml of all components of M-92. VA-2 exhibited a significant cytotoxic activity and the minimum degenerating concentration was 0.001 μg/ml.

Table 7. Effect of M-92 components on Ehrlich carcinoma (solid form).

Component	Dose[a] (mg/kg/day)	Mean of tumor weight (g)	Inhibition ratio[b] (%)
VA-2	1.0	—	Toxic (5/5)[c]
	0.5	0.03	98.7
	0.25	0.11	95.3
	0.125	0.55	76.6
	0.063	1.37	41.7
	0.031	1.81	23.0
BA-4	20	—	Toxic (5/5)
	10	2.28	3.0
	5	2.32	1.3
BA-5	20	1.61	31.5
	10	1.87	20.4
	5	1.91	18.7
BN-1	20	—	Toxic (5/5)
	10	0.27	88.5
	5	0.71	69.8
	2.5	0.96	59.1
	1.25	1.78	24.3
BN-2	20	1.38	41.3
	10	0.84	64.3
	5	1.98	15.7
BN-3	20	1.59	32.3
	10	1.98	15.7
	5	1.93	17.9
Non-treated		2.35	0.0

Ascites cells (2×10^6) of Ehrlich carcinoma were subcutaneously implanted into female ICR mice.

a) The antibiotic was intraveneously administered once daily for 5 days, starting 24 hours after the implantation.

b) Inhibition ratio (%)=(1−mean tumor weight of the treated group/mean tumor weight of the non-treated group)×100.

c) The figures in the parenthesis=number of died mice/number of mice used.

Anti-Ehrlich Activity in Mice

The effect of M-92 components on Ehrlich carcinoma (ascites form) are shown in Table 6. By intraperitoneal administration once daily for 5 days, VA-2, BA-4, BN-1, BN-2 and BN-3 increased the life span of the tumor bearing mice. Among these components, VA-2 and BN-1 exhibited the most potent antitumor activities. The ILS of VA-2 was higher than 200% at the dose of 0.5~0.063 mg/kg/day, but a dose of 1.0 mg/kg/day was toxic for mice. The ILS of BN-1 was higher than 200% at 5 and 2.5 mg/kg/day, but 10 mg/kg/day was toxic.

Table 8. Acute toxicity of M-92 components in mice.

Component	LD_{50} (mg/kg)	
	i.p.	i. v.
VA-2	1.9	1.8
BA-4	>100	43.5
BA-5	>100	>100
BN-1	>100	91
BN-2	>100	>100
BN-3	>100	>100

Observation period: 7 days
Calculation: LITCHFIELD-WILCOXON's method

Table 7 shows the inhibitory effect of M-92 components on the growth of Ehrlich carcinoma (solid form) implanted in mice. By intravenous administration once daily for 5 days, VA-2 and BN-1 inhibited tumor growth. VA-2 at a dose of 0.5 and 0.25 mg/kg/day, and BN-1 at 10 mg/kg/day inhibited growth with the inhibitory ratio of greater than 80%.

Acute Toxicity

The acute toxicities of M-92 components are shown in Table 8. VA-2 possessed a high order of toxicity in mice and the LD_{50}s were 1.9 mg/kg and 1.8 mg/kg by i.p. and i.v. administration, respectively. LD_{50}s of BA-4 and BN-1 by intravenous injection were found to be 43.5 and 91 mg/kg, respectively, but those of BA-5, BN-2 and BN-3 were >100 mg/kg, suggesting the lower toxicities of these components than VA-2.

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