NOCARDICIN A, A NEW MONOCYCLIC β-LACTAM ANTIBIOTIC. IV

FACTORS INFLUENCING THE *IN VITRO* ACTIVITY OF NOCARDICIN A

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Factors influencing the *in vitro* antimicrobial activity of nocardicin A against *Pseudomonas aeruginosa* and *Proteus mirabilis* were investigated. Sodium chloride was identified as a major inhibitor. Some of the amino acids, sugars and divalent cations were found to be minor inhibitors. The presence of potassium phosphates enhanced nocardicin A activity against *P. aeruginosa*, but antagonized the activity against *P. mirabilis*.

Nocardicin A is a newly developed monocyclic β-lactam antibiotic which was isolated from culture filtrate of *Nocardia uniformis* subsp. *tsuyamanensis* ATCC-21806[1,2]. Nocardicin A shows a potent therapeutic efficacy against mice experimentally infected with *Pseudomonas aeruginosa*, the indole-positive and indole-negative *Proteus* species (except *Proteus morganii*), and *Serratia marcescens*[3,4,5]. However, nocardicin A is inactive *in vitro* against certain organisms, namely *Staphylococcus aureus* and *E. coli* and its activity is reduced against *P. aeruginosa* and *P. mirabilis* in conventional heart infusion medium. The *in vitro* antimicrobial activity of nocardicin A was shown to be markedly influenced by the kinds of media employed.

Of the commercially available media, the suitable media for the assay of antimicrobial activity of this antibiotic against *P. aeruginosa* and *P. mirabilis* were found to be antibiotic medium No. 3 and antibiotic medium No. 5, respectively. This paper deals with the characterization of inhibitors on the activity of nocardicin A in conventional media[3].

Materials and Methods

1. Test strains and test media

All twenty strains tested were isolated from clinical specimens. *P. aeruginosa* strain 1101-5 and *P. mirabilis* strain 1432-49 were used as the test organism for the assay of inhibitors. The growth media used were heart infusion broth (HI, Difco), antibiotic medium No. 3 (A.No3, Difco), antibiotic medium No. 5 (A.No5, Difco), trypticase soy broth (Difco) and minimal salts medium M9. Modification of HI, A.No3 and A.No5 were made in the experiments designed to study the influence of addition or removal of inhibitory substances on the *in vitro* activity of nocardicin A.

2. Test antibiotic and chemicals

Nocardicin A was prepared by the Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. All chemicals used were of reagent grade. Amino acids were purchased from Ajinomoto Co., Ltd.

3. Determination of the minimal inhibitory concentrations

The minimal inhibitory concentrations (MIC) of the test antibiotics were determined by the

agar dilution method with the use of the multipoint inoculator. The inocula used were original and 10^{-2} dilution of an overnight culture in a trypticase soy broth. After incubation at 37°C for 20 hours the lowest concentration that inhibited macroscopic colonial growth was regarded as the MIC.

4. Assay of inhibitors influencing the *in vitro* activity of nocardicin A

Three different methods were used for the assay of inhibitors. In each method, the basal media employed for *P. aeruginosa* and *P. mirabilis* were A.No3 and A.No5, respectively.

(1) MIC methods: MICs were determined using HI medium and the basal medium supplemented with test substances. This method was used mainly for confirmation of inhibitors.

(2) Disc method: A few drops of overnight broth culture of a test organism were spread on the agar plates containing MIC of nocardicin A. Paper discs dipped in the solution of test substances were placed on the plates. When test substances possessed inhibitory activity against nocardicin A, a growth zone of the test organism appears around the discs after overnight incubation.

(3) Colony formation method: One-tenth milliliter of overnight broth culture of a test organism was mixed with 10 ml of basal agar media containing certain concentration of nocardicin A and test substances. As a control, basal and HI agars containing antibiotic were inoculated similarly. After incubation at 37°C for 20 hours, the number of colonies appeared on each plate were counted. Test substances were also examined for their influence on the viability of test organisms in the antibiotic-free medium.

5. Analysis of ingredients of media

Mono- and di-valent cations were determined by emission spectrographic analysis.

6. Cell lysis of *P. aeruginosa* by EDTA and lysozyme

P. aeruginosa strain 1101-5 was inoculated into HI broth, sodium chloride deficient-HI broth or A.No3 and incubated overnight at 37°C. The cells were harvested by centrifugation, washed twice with saline solution, and suspended in 50 mM Tris-HCl buffer, pH 8.0, to give an optical density of 0.600 at 660 nm. To 2.4 ml of the cell suspension, 0.3 ml of EDTA (1 mg/ml) and 0.3 ml of lysozyme (100 μg/ml) were added and the mixture was incubated at 37°C in a cuvette. The decrease in optical density at 660 nm was followed for 5 minutes.

7. Isolation of *P. aeruginosa* mutants altering penetrability of drugs

After *P. aeruginosa* strain 1101-5 was treated with N-methyl-N′-nitro-N-nitrosoguanidine by the procedure of SEKIGUCHI *et al*[6], mutants were selected by virtue of their increased sensitivity to various antibiotics such as nalidixic acid, kanamycin, gentamicin, erythromycin and tetracycline.

Results

1. Inhibitors for Nocardicin A in HI Medium

Preliminary studies indicated that major inhibitors present in HI medium were heat-stable, small molecules, and partially extractable with ethanol, methanol and acidic acetone, and not metabolized by any microbe. From those experiments, it was suggested that sodium chloride was the major substance which interfered with the *in vitro* activity of nocardicin A. Furthermore, it was demonstrated that about twice and four times as much of sodium chloride is contained in HI medium comparing with A. No3 and A. No5 respectively (Table 1). The addition of sodium chloride to the sodium chloride-

Table 1. Mono- and di-valent cation contents in various media

Element	HI-broth	A. No3-broth	A. No5-broth
Na^+	3125*	1575	630
(NaCl)	(0.79%)	(0.40%)	(0.16%)
K^+	435	2765	225
Ca^{++}	2.55	1.10	15.6
Mg^{++}	6.95	5.18	23.6
Zn^{++}	0.70	0.32	0.43
Cu^{++}	0.087	0.021	0.029
Mn^{++}	trace	trace	trace
Cd^{++}	trace	trace	trace

* mg/liter, HI-broth (Difco), A.No3-broth (Difco), A. No5-broth (Difco)
Emission spectrographic analysis

Table 2. Influence of sodium chloride on the MICs of nocardicin A

MIC: μg/ml, 37°C, 20 hours.

Organism		HI		HI-NaCl*		A. No3		A. No5		A. No5+ NaCl**	
		10^8	10^6	10^8	10^6	10^8	10^6	10^8	10^6	10^8	10^6
P. aeruginosa	1101–63	>800	>800	200	50	200	25				
	1101–66	>800	>800	200	50	100	25				
	1101–77	>800	>800	400	100	400	25				
P. mirabilis	1432–58	100	25	12.5	3.13			12.5	6.25	200	12.5
	1432–59	200	100	50	12.5			25	12.5	200	50
	1432–72	200	12.5	12.5	6.25			12.5	6.25	200	12.5

* 0.5% NaCl omitted.

** 1.0% NaCl added

Fig. 1. Influence of sodium chloride on the *in vitro* activity of nocardicin A (I).

Colony formation method used for the assay of the inhibitory activity was performed as described in the text. Selective concentration of nocardicin A for both of *P. aeruginosa* and *P. mirabilis* was 50 μg/ml.

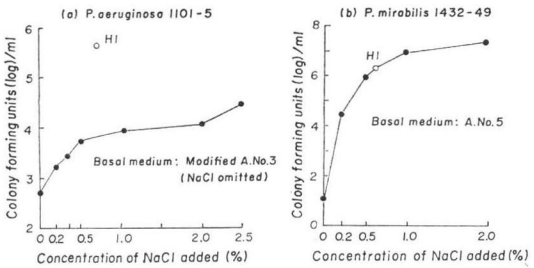


Fig. 2. Influence of sodium chloride on the *in vitro* activity of nocardicin A (II).

Selective concentrations of nocardicin A for *P. aeruginosa* and *P. mirabilis* were 100 and 50 μg/ml, respectively. Sodium chloride-deficient heart infusion agar medium was prepared by combining each of the constituents except sodium chloride in accordance with the formula specified by the Difco Manual. Beef extract used for the above preparation was purchased from Difco Laboratories.

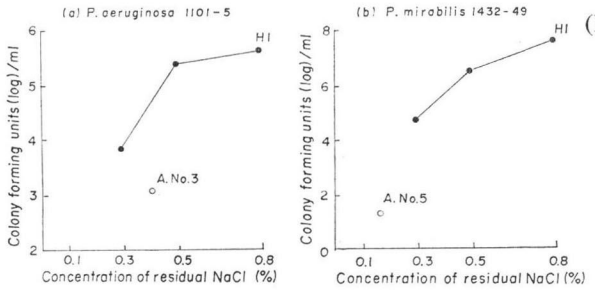
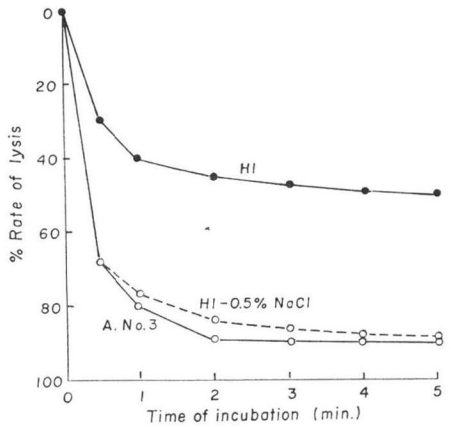


Fig. 3. Influence of culture media on the sensitivity of *P. aeruginosa* 1101-5 to EDTA and lysozyme.

Cells were treated with EDTA and lysozyme as described in the text.



deficient A.No3 or A.No5 resulted in a marked decrease of nocardicin A activity, especially against *P. mirabilis* (Fig. 1 and Table 2). The removal of the sodium chloride from HI medium resulted in an increase of nocardicin A activity (Fig. 2 and Table 2). The mechanism of sodium chloride inhibition of nocardicin A activity was examined. Inhibitors in HI medium did not directly inactivate nocardicin A. While it was found that *P. aeruginosa* grown in HI medium was less lysed by the treatment with EDTA and lysozyme than when cultured in A. No3 or sodium chloride-deficient HI medium

Table 3. Influence of medium on the *in vitro* activity of nocardicin A against antibiotic sensitive mutants of *P. aeruginosa*

MIC: μg/ml, 37°C, 20 hours.

Strain	A. No3		HI	
	10^8	10^6	10^8	10^6
Parent				
1101-5	12.5	6.25	>800	400
Mutants*				
PAS-5	12.5	6.25	25	12.5
PAS-6	12.5	6.25	25	12.5
PAS-7	12.5	3.13	25	6.25
PAS-8	12.5	6.25	25	12.5

* PAS-5~8 derived from *P. aeruginosa* 1101-5 by the treatment with N-methyl-N'-nitro-N-nitrosoguanidine

Table 4. Influence of potassium phosphates on MICs of nocardicin A

MIC: μg/ml, 37°C, 20 hours.

Organism	A. No3		A. No3-P.P.*	
	10^8	10^6	10^8	10^6
P. aeruginosa				
1101-05	50	12.5	100	25
1101-63	400	25	800	200
1101-64	400	50	800	200
P. mirabilis				
1432-80	100	12.5	25	6.25
1432-84	50	12.5	12.5	3.13
1432-85	50	12.5	12.5	3.13

* P.P.: K_2HPO_4 (3.68 g/liter)+KH_2PO_4 (1.32 g/liter)

(Fig. 3). The results suggested that the structure of the outer layer of *P. aeruginosa* cells may have been modified by sodium chloride making them more tolerant to the antibiotic. These findings were further supported by the fact that mutants with altered penetrability to drugs were sensitive to nocardicin A even in HI medium (Table 3).

2. Influence of Other Ions

The *in vitro* activity of nocardicin A against *P. aeruginosa* and *P. mirabilis* was affected by divalent cations such as calcium and magnesium. The addition of the divalent cations to A.No3 or A.No5 resulted in a decrease of nocardicin A activity to the level as low as in HI medium (Fig. 4). However, each medium contains no more than 1 mM of the cations although the inhibitory activities of these cations appear over than 10 mM (Table 1). Hydrogen ion concentrations also influence on the *in vitro* activity of nocardicin A. The

Fig. 4. Influence of calcium and magnesium ions on the *in vitro* activity of nocardicin A.

Influence of magnesium (-●-●-) and calcium (-○-○-) on the nocardicin A activity was examined by colony formation method. The basal medium used for *P. aeruginosa* and *P. mirabilis* were A. No3 and A. No5, respectively. Selective concentrations of nocardicin A for *P. aeruginosa* and *P. mirabilis* were 100 and 50 μg/ml, respectively.

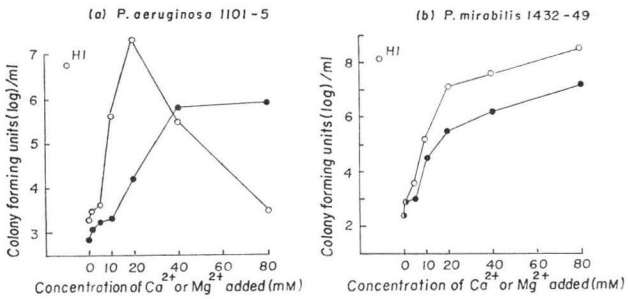
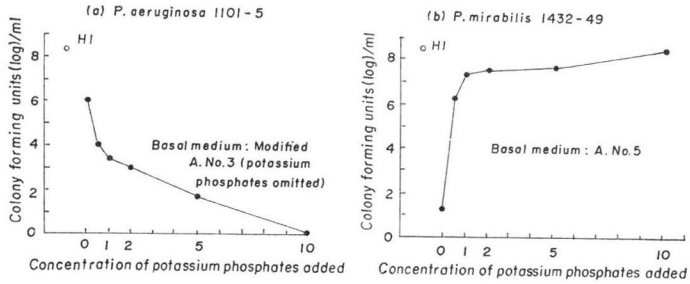


Fig. 5. Influence of potassium phosphates on the *in vitro* activity of nocardicin A.

Selective concentrations of nocardicin A for *P. aeruginosa* and *P. mirabilis* were 12.5 and 50 μg/ml, respectively. The potassium phosphates content in A. No3 (0.35%) was expressed as 1 p.p.



drug inhibited the growth of *P. aeruginosa* and *P. mirabilis* more distinctly in alkaline pH than in acidic pH. Potassium phosphates antagonized the *in vitro* activity against *P. mirabilis*, whereas high concentration of potassium phosphates enhanced the activity against *P. aeruginosa* (Fig. 5 and Table 4). These results explain the discrepancy that the *in vitro* activity of nocardicin A against *P. mirabilis* in A. No5 was superior to that in A.No3, since A.No3 (but not A.No5) contains potassium phosphates.

3. Amino Acids and Sugars as Minor Inhibitors

The *in vitro* activity of nocardicin A in M9 minimal medium was about 2~4 times greater than that in rich natural media. The *in vitro* activity of nocardicin A against various bacteria was reduced by addition of casein hydrolysate and yeast extract to M9 minimal medium. It was confirmed that five amino acids in the casein hydrolysate but none of the vitamins in the yeast extract reduced the *in vitro* activity of nocardicin A. They were glycine, homocystine, methionine, threonine, valine. The addition of each amino acid (1 mg/ml) to A.No3 or A.No5 also resulted in the decrease of the *in vitro* activity of nocardicin A to some extent (Table 5). However, they were considered to be minor inhibitors since the difference of each amino acid content between HI medium and A.No3 or A.No5 never exceeded 100 μg/ml. The addition of more than 1% glucose also tended to decrease in the *in vitro* activity of nocardicin A (Table 6).

Table 5. Influence of five kinds of amino acids on MICs of nocardicin A

MIC: μg/ml, 37°C, 20 hours.

Organism		HI		A. No3		A. No5		A. No3+ 5A.A		A. No5+ 5A.A	
		10^8	10^6	10^8	10^6	10^8	10^6	10^8	10^6	10^8	10^6
P. aeruginosa	1101–44	>800	800	50	12.5			200	25		
	1101–55	800	800	100	12.5			200	50		
P. mirabilis	1432–49	800	200			25	6.25			100	25
	1432–56	>800	800			25	6.25			100	25

5A.A: glycine, homocystine, methionine, threonine, valine each 1 mg/ml

Table 6. Influence of carbon source on MICs of nocardicin A against *P. aeruginosa*

MIC: μg/ml, 10^6/ml, 37°C, 20 hours.

Antibiotic	Strain	HI	A. No3	M9+ 0.2% glucose	M9+ 1% glucose
Nocardicin A	1101–36	800	6.25	25	100
	1101–51	>800	25	12.5	>800
	1101–56	800	25	50	>800
Carbenicillin	1101–36	25	12.5	25	50
	1101–51	50	50	50	100
	1101–56	50	50	100	100

Discussion

The present study elucidated the factors influencing on the *in vitro* antimicrobial activity of nocardicin A. These factors are summarized in Table 7. It is, however, still unsettled how these factors inhibit the antimicrobial activity of nocardicin A, although data suggest that sodium chloride may modify the structure of bacterial outer layer to decrease the penetrability of nocardicin A. Further investigation is necessary to determine if these factors are related to *in vivo* activity. Preliminary

Table 7. Effective antagonists and synergists on the *in vitro* activity of nocardicin A

	P. aeruginosa	*P. mirabilis*
Antagonists	Major component 1) Sodium chloride Minor components 1) Amino acids (Gly.Hom.Met.Thr.Val.) 2) Divalent cations (Ca^{++}, Mg^{++}) 3) Carbon source (Succinate, malate)	Major component 1) Sodium chloride Minor components 1) Amino acids (Gly.Hom.Met.Thr.Val.) 2) Divalent cations (Ca^{++}, Mg^{++}) 3) Carbon source (Succinate, malate) 4) Potassium phosphates
Synergists	1) Potassium phosphates 2) Citrate	

studies indicated that the activity of nocardicin A in serum is scarcely affected by the addition of sodium chloride. Significant therapeutic effect of nocardicin A in mice experimentally infected with some enteric bacilli and *Pseudomonas* may be elucidated both by the bacterial activity determined *in vitro* with suitable assay media such as A.No3 or A.No5, and by modification of surface structure of the bacteria by nocardicin A so as to be easily ingested and killed by the leukocytes. The latter phenomenon will be reported elsewhere.

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