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

IN VITRO EVALUATION

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Nocardicin A, a new monocyclic β-lactam antibiotic, exerts a comparatively potent antimicrobial activity against gram-negative organisms, especially *Pseudomonas aeruginosa*, the indole-positive and indole-negative *Proteus* groups (except *Pr. morganii*), *Serratia marcescens* and the *Neisseria* groups. The *in vitro* antimicrobial activity of nocardicin A against clinical isolates of *Ps. aeruginosa* was about twice that of carbenicillin. The mean MICs of nocardicin A for *Pr. mirabilis*, *Pr. rettgeri* and *Pr. inconstans* ranged from 3.13 to 12.5 μg/ml and were 25~50 μg/ml for *Pr. vulgaris*. Nocardicin A in concentrations of 12.5~50 μg/ml inhibited 30 strains (48%) of *S. marcescens* usually resistant to β-lactam antibiotics. However, nocardicin A had no significant *in vitro* activity against *Staphylococci* and *Escherichia coli*. No cross-resistance was seen between nocardicin A and other β-lactam antibiotics. This antibiotic was stable to β-lactamase. The *in vitro* activity of nocardicin A against *Ps. aeruginosa* and *Pr. mirabilis* was greatly influenced by the assay media used. Nocardicin A was bactericidal and appeared to act synergistically with serum bactericidal factors against *Ps. aeruginosa* and with polymorphonuclear leukocytes against *Ps. aeruginosa*, *E. coli* and *Pr. vulgaris*. The bactericidal activity of nocardicin A against the above 3 organisms, therefore, increased markedly in the presence of fresh serum and polymorphonuclear leukocytes.

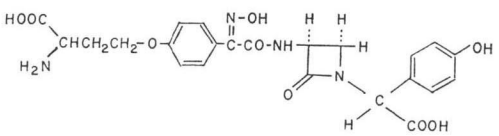
Nocardicin A is a newly developed β-lactam antibiotic which was isolated from culture filtrates of *Nocardia uniformis* subsp. *tsuyamanensis* ATCC-21806[1]. This antibiotic differs from penicillins and cephalosporins in antimicrobial activity and biological properties and structurally has the monocyclic β-lactam ring depicted in Fig. 1.[2] This paper deals with the *in vitro* antimicrobial activity of nocardicin A.

Materials and Methods

1. Test antibiotics

The test antibiotics were nocardicin A (Fujisawa Research Laboratories), carbenicillin (CBPC, Beecham Research Laboratories), cefazolin (CEZ, Fujisawa Pharmaceutical Co., Ltd.), ampicillin (ABPC, Beecham Research Laboratories) and gentamicin (GM, Schering Co.).

Fig. 1. Chemical structure of nocardicin A.



$C_{23}H_{24}N_4O_9$: M.W. 500.47

2. Test strains

Standard strains stored in our Research Laboratories and clinical isolates from patients in several hospitals in Japan were used.

3. Test media

Commercially available culture media such

as heart infusion agar (HI, Difco), antibiotic medium No. 3 (A-No. 3, Difco), antibiotic medium No. 5 (A-No. 5, Difco) and Nutrient agar (NA, Difco) were used.

4. *In vitro* antimicrobial activity

The MICs of the test antibiotics were determined by agar dilution with as the replicating device. Unless otherwise specified, each strain was cultured at 37°C for 20 hours in trypticase soy broth (TSB, BBL) and was diluted with 0.9% saline to contain 10^6 cells/ml for inoculation. HI agar with 10% defibrinated rabbit's blood was used for *Streptococcus* and *Corynebacterium*, and GC agar (Eiken Chemical Co.) was used for the *Neisseria* groups. The MICs were determined after incubation at 37°C for 20 hours and expressed in terms of μg/ml.

5. Bactericidal activity

(1) Bactericidal activity of nocardicin A against growth phase: *Ps. aeruginosa* NCTC-10490 was cultured in TSB at 37°C for 20 hours and suspended in A-No. 3 broth containing nocardicin A or carbenicillin at concentrations equal to twice the MIC. A final cell concentration of about $1 \sim 3 \times 10^6$/ml was obtained. The cultures were incubated at 37°C with shaking, and the viable cells were counted at regular intervals.

(2) Bactericidal activity of nocardicin A against stationary phase: *Ps. aeruginosa* NCTC-10490 was cultured in TSB at 37°C for 20 hours and further incubated with shaking at 37°C for 4 hours to obtain the organism in the stationary phase. These cultures were centrifuged and resuspended in fresh A-No. 3 broth to obtain their original volume. After incubating at 37°C with shaking, the viable cells were counted at regular intervals.

6. Bioassay method for nocardicin A

The concentrations of nocardicin A were determined by the cylinder plate method or paper-disc method on A-No. 3 broth containing 1% agar seeded with a 0.2% inoculum of overnight broth cultures of *Alcaligenes faecalis* 773-9.

7. Stability of nocardicin A to β-lactamases

(1) Preparation of β-lactamase: The cells were grown at 37°C in HI broth to which benzylpenicillin was added as an inducer. After overnight incubation, the cells were harvested by centrifugation, washed once with saline and suspended in 10 mM phosphate buffer (pH 7.0). The cell suspensions were sonicated at maximum power for 20 minutes. After cellular debris was removed by centrifugation, ammonium sulfate was added to the supernatant to 90% saturation. The resulting precipitate was collected by centrifugation, dissolved in the phosphate buffer and then applied to gel filtration of Sephadex G100. The enzyme fractions were pooled and stored at -20°C.

(2) Assay of β-lactamase activity: β-Lactamase activity was determined with a Hitachi 124 spectrophotometer equipped with a thermostatted cell holder. The enzyme was mixed in a 1-cm crystal cuvette with 120 μg of substrate and 30 micromoles of phosphate buffer (pH 7.0) to make a final volume of 3.0 ml and incubated at 37°C. The hydrolysis of nocardicin A, penicillins and cephalosporins was carried out at 220 nm, 240 nm and 260 nm, respectively.

8. Serum-protein binding

The extent of binding of nocardicin A and carbenicillin to serum protein of various animals was measured by ultrafiltration of serum containing 100 μg/ml or 25 μg/ml of the antibiotics through a cellulose acetate (Visking) dialysis membrane.

9. Bactericidal activity of nocardicin A in the presence of fresh serum

To determine the bactericidal activity between the antibiotics and fresh serum, various concentrations of nocardicin A, carbenicillin and gentamicin were added to test media (A-No 3 broth) containing 20% rabbit's fresh serum. The cultures were then inoculated with 10^6 cells/ml of *Ps. aeruginosa* and incubated with shaking at 37°C for 5 hours, and the viable cells were counted.

10. Effect of nocardicin A on phagocytosis and killing by polymorphonuclear leukocytes (PMN)

(1) Preparation of PMN suspension: As reported in our previous paper[3], healthy rabbits were given intraperitoneally 200 ml of 0.1% glycogen solution. PMN samples were collected from the

peritoneal cavity 4~16 hours later and were adjusted to obtain a final concentration of 8×10^6 PMN/ml in HANKS' balanced salt solution.

(2) Determination of bactericidal activity of nocardicin A in the presence of PMN: *Ps. aeruginosa* 7095, *E. coli* 331 and *Pr. vulgaris* 627 cultured overnight in TSB were washed once with HANKS' balanced salt solution (HANKS' BSS) and suspended in the solution to obtain 1×10^8 cells/ml; 4.8 ml of the PMN suspension, 0.1 ml of nocardicin A solution, and 0.1 ml of the bacterial suspensions were placed in silicon-coated tubes. Media without the drug or PMN were used as the control. The mixtures were incubated at 37°C with shaking for 4 hours and centrifuged at 4,000r.p.m. for 20 minutes to precipitate the PMNs and the test organisms. Sterile distilled water was added to the precipitate to release the viable organism from the PMNs. The remaining viable cells were counted in the usual manner.

Results

1. Antimicrobial Spectrum

Nocardicin A provides an antimicrobial activity against gram-negative organisms such as *Ps. aeruginosa*, the *Proteus* group (except *Pr. morganii*) and *Neisseria* groups, but is ineffective against pathogenic gram-positive organisms except *C. diphtheriae*. The activity of this substance against *E. coli*, *Klebsiella* and *Salmonella* spp. was weaker than that of carbenicillin (Table 1).

2. Influence of Various Cultural Conditions on the Antimicrobial Activity of Nocardicin A

(1) Test Media

The antimicrobial activity of nocardicin A against *Ps. aeruginosa* and *Pr. mirabilis* was greatly influenced by the test media used, as shown in Table 2. The MICs of nocardicin A against *Ps. aeruginosa* No. 5 and *Pr. mirabilis* No. 3 in HI agar were 100 μg/ml and 200 μg/ml, respectively. However, the MICs of nocardicin A against *Ps. aeruginosa* No. 5 in A-No.3 agar and *Pr. mirabilis* No. 3 in A-No.

Table 1. Antimicrobial spectrum of nocardicin A against aerobic and facultatively anaerobic bacteria

Organism	MIC (μg/ml)		Organism	MIC (μg/ml)	
	Nocardicin A	Carbenicillin		Nocardicin A	Carbenicillin
S. aureus 209P JCl	800	0.78	*Sal. paratyphi* A 1015	200	0.78
S. epidermidis 1602-1	800	1.56	*Sal. typhimurium* 1406	25	0.78
**S. pyogenes* S-23	200	0.2	*S. marcescens* 1421-4	800	12.5
**S. faecalis* 6733	>800	25	*E. aerogenes* 1402-10	200	6.25
**S. pneumoniae* III	100	0.78	*E. cloacae* 1401-4	800	12.5
**C. diphtheriae* PW8	12.5	0.05	*C. freundii* 1381-3	200	6.25
B. subtilis ATCC 6633	50	0.2	*A. lwoffii* 1641-4	3.13	0.78
M. luteus PCI 1001	25	0.2	*A. faecalis* 1311-1	800	3.13
E. coli NIHJ JC2	100	12.5	*Pr. mirabilis* 1432-75	1.56	0.78
E. coli 1341-18 (R^+)	100	>800	*Pr. vulgaris* IAM 1025	1.56	0.39
K. pneumoniae NCTC 418	200	50	*Pr. rettgeri* 1434-3	3.13	>800
			Pr. inconstans 1436-21	12.5	0.78
Sh. flexneri Ia EW8	100	6.25	*Pr. morganii* 1433-2	200	3.13
Sh. sonnei I EW33	12.5	0.78	*Ps. aeruginosa* 1101-75	12.5	50
Sal. enteritidis 1891	100	0.78	***N. gonorrhoeae*	1.56	N.D.
Sal. typhi 0-901	100	1.56	***N. meningitidis*	1.56	N.D.

MIC: HI agar, 10^6cells/ml, 37°C, 20 hours

* Supplemented with 10% rabbit blood ** GC-agar supplemented with 10% rabbit blood

Table 2. Influence of various factors on antimicrobial activity of nocardicin A and carbenicillin

Factor		Antibiotic	MIC (μg/ml)	
			Ps. aeruginosa No. 5	*Pr. mirabilis* No. 3
Medium	HI agar	Nocardicin A	100 (>520.5)*	200 (106.3)*
	Nutrient agar		50	6.25
	Antibiotic medium No. 3		3.13 (53.7)	50
	Antibiotic medium No. 5		25	3.13 (5.7)
	HI agar	Carbenicillin	12.5	1.56
	Nutrient agar		12.5	1.56
	Antibiotic medium No. 3		6.25	1.56
	Antibiotic medium No. 5		12.5	3.13
Inoculum size	10^8	Nocardicin A	100	12.5
	10^6		6.25	3.13
	10^4		1.56	3.13
	10^8	Carbenicillin	>200	25
	10^6		6.25	3.13
	10^4		0.78	0.78
Medium pH	6.0	Nocardicin A	12.5	6.25
	7.0		6.25	6.25
	8.0		3.13	6.25
	6.0	Carbenicillin	12.5	0.78
	7.0		12.5	1.56
	8.0		12.5	1.56

MIC: Antibiotic medium No. 3 (*Ps. aeruginosa* No. 5), Antibiotic medium No. 5 (*Pr. mirabilis* No. 3), 10^6 cells/ml, stamp method, 37°C, 20 hours.

* Mean MIC of nocardicin A against clinical isolates (*Ps. aeruginosa*, 50 strains; *Pr. mirabilis*, 47 strains)

5 agar were both 3.13 μg/ml. Therefore, A-No.3 medium (*Ps. aeruginosa*) and A-No. 5 medium (*Pr. mirabilis*) were found to be the most suitable of several conventional culture media. Though not shown in this table, the antimicrobial activity of nocardicin A against other species of *Proteus* and other organisms was not influenced by the culture medium. The relation between the kind of media and MICs was studied using a large number of strains of *Ps. aeruginosa* and *Pr. mirabilis*. The median MICs of nocardicin A against these organisms showed similar tendencies as shown in *Ps. aeruginosa* No. 5 and *Pr. mirabilis* No. 3 (Table 2). On the other hand, CBPC activity was not influenced by the test media.

(2) Inoculum size and pH of the test medium

As shown in Table 2, the antimicrobial activity of nocardicin A was readily influenced by inoculum size. Against *Ps. aeruginosa* No. 5 and *Pr. mirabilis* No. 3, nocardicin A was respectively 64-fold and 4-fold less active at 10^8 cells/ml inocula than at 10^4 cells/ml. The antimicrobial activity of nocardicin A was generally higher when small inoculum sizes were used. This was also true for carbenicillin.

The MICs of nocardicin A against *Ps. aeruginosa* No. 5 were 12.5 μg/ml at pH 6 and 3.13 μg/ml at pH 5. However, no such wide variation was observed in the case of *Pr. mirabilis* No. 3. The MICs of carbenicillin against these organisms were not influenced by the pH of the medium.

3. Susceptibility of Clinical Isolates to Nocardicin A

Distribution of MICs of nocardicin A and carbenicillin against clinical isolates of *Ps. aeruginosa*,

Table 3. Distribution of susceptibility of clinical isolates to nocardicin A and carbenicillin

Organism	Antibiotic	MIC (μg/ml)											
		≦0.39	0.78	1.56	3.13	6.25	12.5	25	50	100	200	400	≧800
Ps. aeruginosa (50)*	Nocardicin A						2	11	13	12	5	3	4
	Carbenicillin							4	6	23	8	5	4
Pr. mirabilis (63)	Nocardicin A		3	2	10	37	11						
	Carbenicillin	3	36	17		1	1	1				2	2
Pr. vulgaris (50)	Nocardicin A				1	4	5	12	15	9	3	1	
	Carbenicillin	1	4	7	10	14	4	4	4	1		1	
Pr. rettgeri (37)	Nocardicin A				3	20	10	3				1	
	Carbenicillin	12	4	5	6		2	2	1	1		2	2
Pr. inconstans (21)	Nocardicin A				15	5	1						
	Carbenicillin	2	11	6	2								
E. coli (63)	Nocardicin A					1			18	24	13	4	3
	Carbenicillin				7	26	3	1					26
S. marcescens (63)	Nocardicin A						3	12	15	8	4	4	3
	Carbenicillin						3	3	6		2		50
A. calcoaceticus (63)	Nocardicin A				1		2	5	9	5	12	4	2
	Carbenicillin		1	2	2	7	8	11	6	1	2		

MIC: Antibiotic medium No. 3 (*Ps. aeruginosa* and *E. coli*), Antibiotic medium No. 5 (*Proteus* spp., *S. marcescens* and *A. calcoaceticus*), 10^6 cells/ml, stamp method, 37°C, 20 hours

* Numbers in parenthesis indicate number of isolates used.

Pr. mirabilis, Pr. vulgaris, Pr. rettgeri, Pr. inconstans, E. coli, S. marcescens and *A. calcoaceticus* is shown in Table 3. Of the 50 strains of *Ps. aeruginosa*, 26 were inhibited by 12.5~50 μg/ml of nocardicin A, whereas 7 were highly resistant. In contrast, carbenicillin inhibited only 10 strains at 12.5~50 μg/ml. Nocardicin A inhibited 52 of the 63 strains of *Pr. mirabilis* at 0.78~6.25 μg/ml, whereas carbenicillin inhibited 60 of the same 63 strains at 0.39~1.56 μg/ml. Nocardicin A inhibited 37 of the 50 strains of *Pr. vulgaris* at 50 μg/ml or less, however carbenicillin inhibited 48 of the same strains at the same concentrations. All 37 strains of *Pr. rettgeri* were inhibited by 3.13~12.5 μg/ml of nocardicin A. Carbenicillin was more active than nocardicin A against most of the strains, whereas 4 strains were highly resistant. Against the 21 strains of *Pr. inconstans*, nocardicin A was less active than carbenicillin, but nocardicin A in concentrations of 3.13~12.5 μg/ml inhibited all the strains. Nocardicin A also inhibited at 50 μg/ml or less 30 of the 63 strains of *S. marcescens* which are not susceptible to most β-lactam antibiotics. The activity of nocardicin A against the 63 strains of *E. coli* was generally weak and almost all of the strains were inhibited at 50~200 μg/ml. Against the 63 strains of *A. calcoaceticus*, nocardicin A was less active than carbenicillin.

4. Activity of Nocardicin A against Gram-negative Organisms Resistant or Non-susceptible to Other β-Lactam Antibiotics

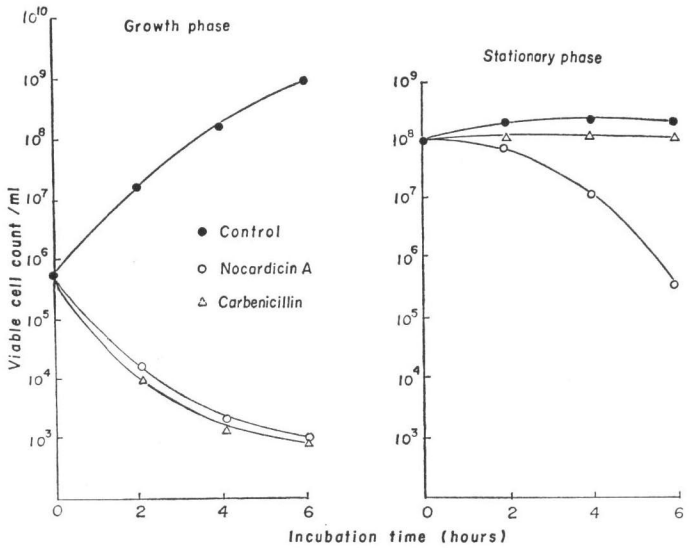
Ps. aeruginosa and *Proteus* species which are resistant or not susceptible to other β-lactam antibiotics such as carbenicillin, ampicillin and cefazolin were generally susceptible to nocardicin A (Table 4). These results show that no cross-resistance was seen between nocardicin A and the other

Table 4. Antimicrobial activity of nocardicin A against gram-negative bacilli resistant or non-susceptible to other β-lactam antibiotics

Organism		MIC (μg/ml)			
		Nocardicin A	Carbenicillin	Ampicillin	Cefazolin
Ps. aeruginosa	67	50	>800	>800	>800
	139	100	>800	>800	>800
Pr. mirabillis	89	6.25	>800	>800	12.5
	105	6.25	>800	>800	6.25
Pr. rettgeri	3	3.13	>800	>800	>800
	28	25	>800	>800	>800
Pr. inconstans	3	3.13	6.25	50	200
	7	6.25	100	50	200

MIC: Antibiotic medium No. 3 (*Ps. aeruginosa*), Antibiotic medium No. 5 (*Proteus* species), 10^8 cells/ml, stamp method, 37°C, 20 hours.

Fig. 2. Bactericidal activity of nocardicin A and carbenicillin against *Ps. aeruginosa* NCTC-10490.



β-lactam antibiotics tested.

5. Bactericidal Activity

As clearly shown in Fig. 2, viable cells of *Ps. aeruginosa* NCTC-10490 in the logarithmic growth phase decreased markedly in the presence of nocardicin A at double the MIC. This tendency was similar to that observed with carbenicillin, although carbenicillin was not bactericidal against the organism in the stationary phase. On the other hand, nocardicin A showed apparent bactericidal activity, though weaker than that against the organism in the growth phase.

6. Stability of Nocardicin A to β-Lactamases

Degradation of nocardicin A by β-lactamase-producing gram-negative bacilli was compared with that of other β-lactam antibiotics. As shown in Table 5, nocardicin A was very stable to all types of β-lactamases produced by the organisms tested except *Pr. vulgaris* 9.

Table 5. Hydrolysis of nocardicin A and other β-lactam antibiotics by β-lactamases from gram-negative bacteria

Enzyme type*		Organism	Relative activity							Rate of hydrolysis (μg/min/ml)
			Nocardicin A	CER**	CET	CEZ	PCG	ABPC	CBPC	
Chromosomal enzyme	PCase*	*K. pneumoniae* 92	0	15	4	4	100	146	25	13.6
		Pr. mirabilis 23	0	9	41	6	100	41	0	0.2
	CSase*	*E. coli* 36	0.06	100	357	56	5	0.8	0	8.5
		Pr. vulgaris 9	27	100	249	527	35	50	5	4.1
		Pr. morganii 6	0	100	214	74	72	2	0	10.8
		Ps. aeruginosa 59	0	100	271	341	32	8	0	1.2
		C. freundii 50	0	100	105	98	10	0	0	3.9
R-plasmid	PCase	*E. coli* 40	0.07	15	6	5	100	140	10	21.8
		Pr. rettgeri 3	0	51	78	33	100	215	29	66.7
		Pr. morganii 53	0	43	51	36	100	48	14	16.7
		Ps. aeruginosa 47	0	5	0.7	2	100	92	74	85.2

β-lactamase activity: Initial rate of inactivation at concentration of 40 μg/ml at 37°C by U.V. method (Nocardicin A, 220 nm; cephalosporins, 260 nm; penicillins, 240 nm)

* PCase: Penicillinase. CSase: Cephalosporinase.

** CER: Cephaloridine. CET: Cephalothin. CEZ: Cefazolin. PCG: Penicillin G. ABPC: Ampicillin. CBPC: Carbenicillin.

Table 6. Extent of serum protein binding of nocardicin A and carbenicillin

Antibiotic	Concentration	% Bound			
		Human	Dog	Rabbit	Rat
Nocardicin A	100 μg/ml	22.3	13.1	29.8	34.4
	25 μg/ml	23.6	17.9	31.4	37.3
Carbenicillin	100 μg/ml	44.6	31.3	59.4	29.6
	25 μg/ml	42.7	26.0	61.2	27.6

Ultra-filtration method

7. Binding of Nocardicin A to Serum Protein

As shown in Table 6, the extents of binding of nocardicin A to serum protein of human, dog and rabbit were about twice lower than those of carbenicillin. But, the extent of binding of nocardicin A to serum protein of rat was slightly higher than that of carbenicillin.

8. Effect of Fresh Serum and Polymorphonuclear Leukocytes (PMN) on Bactericidal Action of Nocardicin A

(1) Fresh Serum

The bactericidal activities of nocardicin A, carbenicillin and gentamicin against *Ps. aeruginosa* 7095 were compared in media with and without 20% fresh rabbit serum (Fig. 3). The bactericidal activity of carbenicillin was not influenced by fresh serum, however, the activity of gentamicin clearly decreased in the presence of fresh serum. Conversely, the activity of nocardicin A was rather enhanced in the presence of fresh serum, a tendency seen at all concentrations tested. However, this enhanced bactericidal activity in the presence of fresh serum was observed only against *Ps. aeruginosa* and not against the other organisms.

Fig. 3. Influence of fresh rabbit serum on bactericidal activity of nocardicin A, carbenicillin and gentamicin against *Ps. aeruginosa* 7095.
serum concentration: 20% fresh rabbit serum.

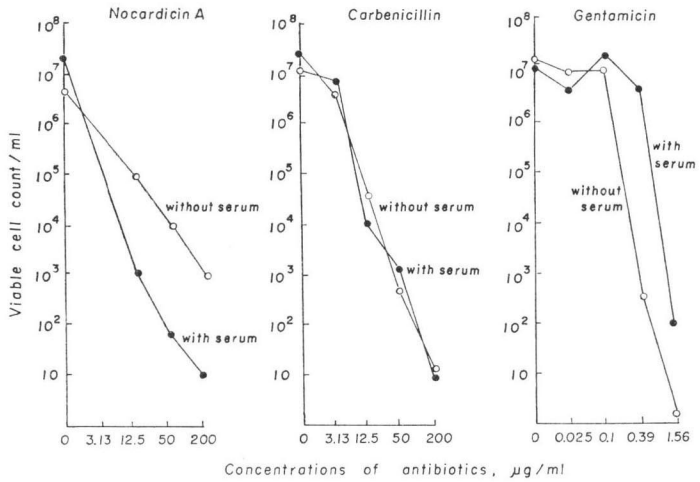
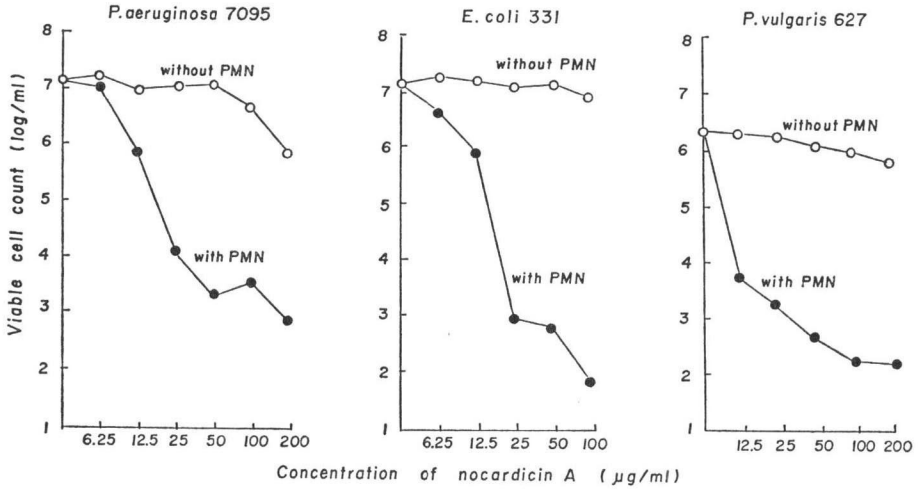


Fig. 4. Effect of nocardicin A on phagocytosis and killing of *Ps. aeruginosa, E. coli* and *Pr. vulgaris* by rabbit polymorphonuclear leukocytes.



(2) Polymorphonuclear Leukocytes (PMN)

The question whether the antimicrobial activity of nocardicin A against *Ps. aeruginosa, E. coli* and *Pr. vulgaris* would also be influenced in the presence of rabbit PMN was investigated. As shown in Fig. 4, no marked decrease of viable cells was observed in the above 3 kinds of gram-negative bacilli when nocardicin A and PMN were given alone. However, with nocardicin A and PMN in combination, the viable cells of the test organisms decreased markedly relative to the concentration of nocardicin A given, and declined to $10^2 \sim 10^3$ cells/ml. It is of particular interest that the weak *in vitro* antimicrobial activity of nocardicin A against *E. coli* was intensified in the presence of PMN. However, against *S. aureus* the bactericidal activity of nocardicin A was not enhanced by PMN.

Discussion

Bleomycin,[4] pachystermines[5], phleomycin[4], wildfire toxin[6] and (s)-alanyl-3-[α-(s)-chloro-3-(s)-hydroxy-2-oxo-3-azetidinyl methyl]-(s)-alanine[7] have been reported as natural substances having a monocyclic β-lactam ring. These substances, however, are weaker in antimicrobial activity than nocardicin A. The antimicrobial activity of nocardicin A is characterized by the absence of cross-resistance with other β-lactam antibiotics such as cephalosporins and penicillins. Hence, nocardicin A shows a potent activity against organisms resistant to these antibiotics. This may be explained by the fact that nocardicin A is stable to β-lactamases. However, the fluctuation of *in vitro* sensitivity of *Ps. aeruginosa* and *Pr. mirabilis* to nocardicin A is attributed to salt and other factors in the test media[8]. Since the MICs of nocardicin A changed according to experimental conditions, especially test media and inoculum size, the effectiveness of nocardicin A needs to be evaluated by studies on its therapeutic efficacy against experimental infections in mice. Generally the *in vitro* antimicrobial activity of most antibiotics decreases in the presence of body components, especially serum. On the contrary, the antimicrobial activity of nocardicin A against *Ps. aeruginosa* increased in the presence of fresh serum. In addition, nocardicin A appeared to act synergistically with polymorphonuclear leukocytes. For a typical example, the *in vitro* antimicrobial activity of nocardicin A against *E. coli* was very weak but its therapeutic effect was strong in preliminary studies in mice infected with *E. coli*. This phenomenon is considered to be due to the *in vitro* synergistic action between nocardicin A and PMN.

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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.

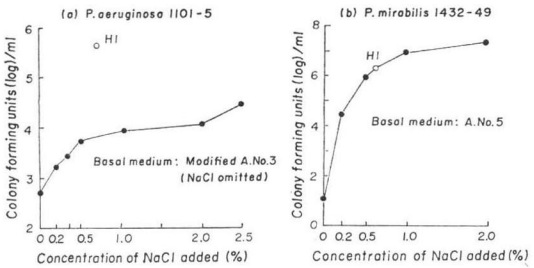


Fig. 2. Influence of sodium chloride on the *in vitro* activity of nocardicin A (II).

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.

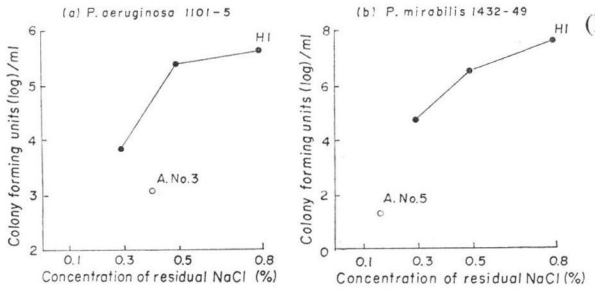
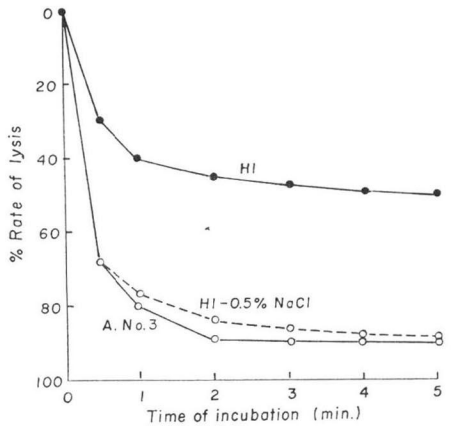


Fig. 3. Influence of culture media on the sensitivity of *P. aeruginosa* 1101-5 to EDTA and lysozyme.

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.

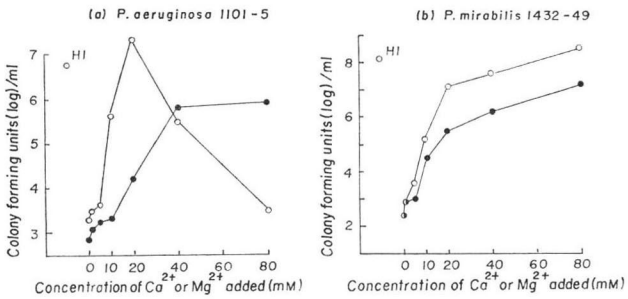
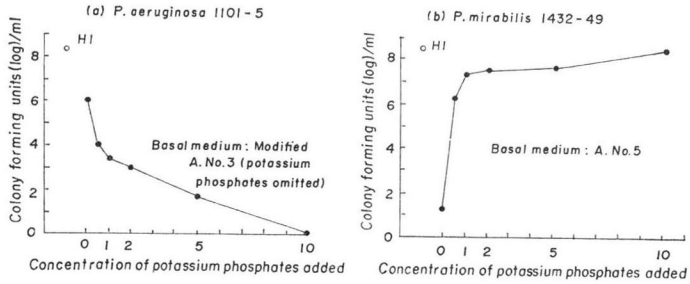


Fig. 5. Influence of potassium phosphates on the *in vitro* activity of nocardicin A.

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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