NOCARDICIN A, A NEW MONOCYCLIC β-LACTAM ANTIBIOTIC

I. DISCOVERY, ISOLATION AND CHARACTERIZATION

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Nocardicin A is a new monocyclic β-lactam antibiotic obtained from the fermentation broth of a strain of actinomycetes. The producing organism, strain WS 1571, was identified as *Nocardia uniformis* subsp. *tsuyamanensis* ATCC 21806. The antibiotic, obtained as colorless crystals, exhibits moderate *in vitro* antibacterial activity against a broad-spectrum of Gram-negative bacteria including *Proteus* and *Pseudomonas*. It has low toxicity in laboratory animals.

In the course of an investigation directed toward the discovery of new and novel β-lactam antibiotics, an actinomycete, designated strain WS 1571, was isolated from a soil sample collected in Tsuyama city, Okayama Prefecture, Japan. Shake flask cultures possessed stronger inhibitory activity on a mutant strain of *Escherichia coli* which is specifically supersensitive to β-lactam antibiotics than on the parent strain (Table 1).

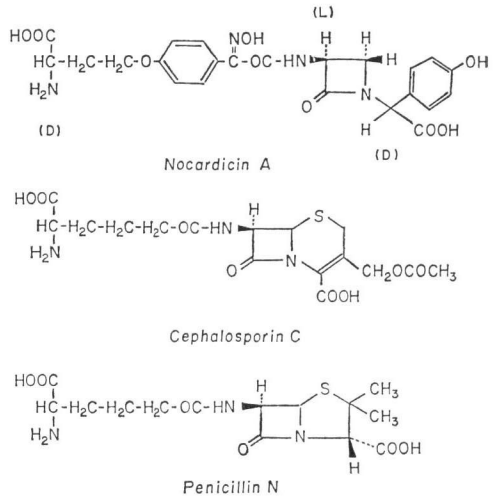
Its selective inhibitory activity on β-lactam antibiotic supersensitive cells, together with its sensitivity to some β-lactamases, made us presume that it was a β-lactam antibiotic. An isolation program was therefore undertaken to obtain

Table 1. Sensitivity of a mutant of *Escherichia coli* which is supersensitive to β-lactam antibiotics.

Antibiotic	MIC (mcg/ml)*	
	E. coli (parent strain)	Es-11 (mutant strain)
Penicillin G	100	0.8
Cephalosporin C	400	0.4
Cephamycin C	100	1.6
Nocardicin A	400	0.4
Fosfomycin	1.6	1.6
Cycloserine	25	25
Kanamycin	6.3	6.3
Actinomycin C	>100	>100

* MIC was determined by serial agar dilution method with heart infusion medium.

Fig. 1. Chemical structure of nocardicin A and other β-lactam antibiotics.



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pure antibiotic for further evaluation. The antibiotic was obtained as colorless crystals. At first it was called by the code number of FR-1923,[1] but afterward it has been designated nocardicin A.

The elucidation of its chemical structure by chemists in our laboratories (manuscript in preparation) has shown it is a monocyclic β-lactam antibiotic with novel acyl side chain as shown in Fig. 1.

The isolation and properties of the antibiotic have been reported briefly in the preliminary report.[1] This paper describes the characteristics of strain WS 1571, fermentation, isolation procedures and biological and chemical properties of nocardicin A.

There are several minor components with β-lactam structure produced in the fermentation broth of this strain along with nocardicin A. They are now under investigation. The result will be published elsewhere.

Taxonomic Studies on Strain WS 1571

Strain WS 1571 is an actinomycete isolated from a soil sample collected in Tsuyama city, Okayama Prefecture, Japan.

The methods and media of taxonomic studies recommended by the International Streptomyces Project (ISP)[2] were used primarily, along with several supplementary tests. Stock slant cultures were maintained on BENNETT's agar. These slants, as well as subsequent cultures used in this study, were incubated at 30°C.

Microscopic observations were made on cultures that were grown from 10 to 14 days on CZAPEK's, BENNETT's and inorganic salts-starch media. Sporophore morphology was observed on undisturbed plate cultures. Carbon replica technique was used to obtain electron micrographs.

Strain WS 1571 produced an aerial mycelium which is composed of a network of sympodially branched aerial hyphae that eventually segment into spores. Short, clavate side branches are formed that usually produce several spores each (Fig. 2). Spores are oblong to short cylindrical, averaging 0.5~1.0 by 0.5~1.5 μ in size, with smooth spore surface when observed by electron microscopy (Fig. 3). Neither fragmentation of hyphae nor formation of spores occurred in the substrate mycelium.

Colony characteristics were observed from slant cultures on nine media after 10 and 21 days of incubation. The formulae for glucose-asparagine, nutrient and CZAPEK's agars are those of WAKSMAN.[3] Temperature requirement was determined on BENNETT's agar slants using temperature gradient incubator (Toyo Kagaku Sangyo Co., Ltd.). Gelatin liquefaction was examined at 21 days on a medium co-

Fig. 2. Aerial mycelium of strain WS 1571; undisturbed agar plate.

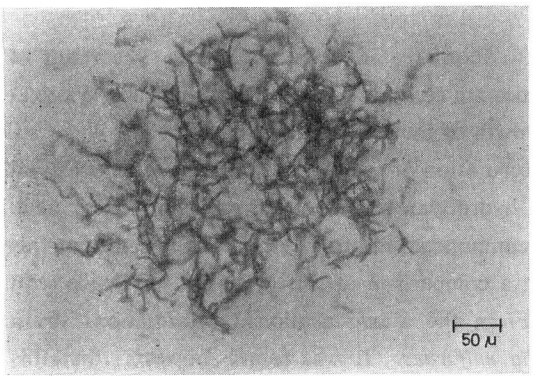


Fig. 3. Electron micrograph of the spores of strain WS 1571; carbon replica method.

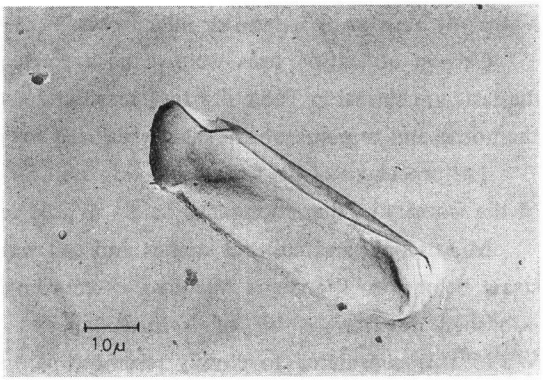


Table 2. Cultural characteristics of strain WS 1571

Medium	Characteristics
CZAPEK's agar	Growth abundant, reverse orange-yellow; no aerial mycelium; no soluble pigment
Glucose-asparagine agar	Growth abundant; reverse yellow to pale cream; no aerial mycelium; no soluble pigment
Glycerin-asparagine agar	Growth fair; reverse orange-yellow; aerial mycelium abundant, white; no soluble pigment
Inorganic salts-starch agar	Growth fair, reverse orange-yellow; aerial mycelium abundant, white; no soluble pigment
Tyrosine agar	Growth abundant, reverse yellow; aerial mycelium abundant, white; no soluble pigment
Nutrient agar	Scant growth
Yeast-malt extract agar	Growth fair, yellowish brown wrinkled lether like; no aerial mycelium; no soluble pigment
Oat meal agar	Growth abundant, reverse brown to pale yellow; no aerial mycelium no soluble pigment
Peptone-yeast iron agar	Growth abundant, reverse yellow; no soluble pigment

Table 3. Physiological properties of strain WS 1571

Property observed	Characteristics
Action on milk	No coagulation; peptonization faint
Gelatin liquefaction	Faint
Hydrolysis of starch	Strongly hydrolyzed
Melanin production (Peptone-yeast iron agar, tyrosine agar and tryptone-yeast extract broth)	None
Temperature requirements	Growth and sporulation good from 26°C to 30°C; no growth at 40°C or above
Cell wall pattern	IV (meso-diaminopimelic acid)

Table 4. Utilization of various carbon compounds by strain WS 1571

Carbon compound	Growth
L-Arabinose	±
D-Xylose	±
D-Glucose	+
D-Fructose	−
L-Rhamnose	±
Sucrose	+
Raffinose	−
D-Mannitol	±
Inositol	−

Symbols: +; positive utilization, ±; doubtful utilization, −; no utilization.

posed of 20% gelatin, 2% glucose and 0.5% peptone. The medium was refrigerated after incubation to detect liquefaction. Strength of starch hydrolysis was observed by the starch-iodine reaction after incubation on inorganic salts-starch agar plate for 14 days.

The cultural characteristics and summarized physiological properties of strain WS 1571 are presented in Tables 2 and 3, respectively. Unless otherwise stated, all cultures were incubated at 30°C for 2 weeks before observation. On most media, orange vegetative growth develops moderately and the aerial mass is thin and white. No soluble pigment formed on the media tested including tyrosine agar, nutrient agar, yeast-malt extract agar and other proteinous media such as gelatin or milk. Accordingly, strain WS 1571 is considered to be non-chromogenic. Starch hydrolysis is good. The hydrolytic activity on gelatin or milk is weak.

Carbon utilization tests were made according to PRIDHAM-GOTTLIEB method.[4] The result of the tests are shown in Table 4. D-Glucose and sucrose are easily utilized and L-arabinose,D-xylose,L-rhamnose and D-mannitol are slightly utilized for growth of the organism.

The procedure of BECKER *et al.*[5] was used for preparation of cells and chromatographic detection of the isomers of diaminopimelic acid. Whole cell hydrolysates contain meso-diaminopimelic acid.

Microscopic and cultural studies and cell wall components of strain WS 1571 indicate that this strain belongs to the genus *Nocardia*. Accordingly, a comparison of this organism was made with published descriptions[3,7,8] of *Nocardia* species. From the above-mentioned informations strain WS 1571 is considered to closely resemble *Nocardia uniformis*. It was found, however, that this

species was differentiated from strain WS 1571 in hydrolytic activities on gelatin and starch. As a result of the above comparisons, strain WS 1571 is considered a subspecies of *Nocardia uniformis*, and the name *Nocardia uniformis* subsp. *tsuyamanensis* is proposed. This strain has been deposited in the American Type Culture Collection, Rockville, Md., as ATCC 21806.

Production of Nocardicin A

For production of the antibiotic, 30-liter fermentors with 20 liters of the medium shown in Table 5 were inoculated with 2~5% of the mature seed broth. Seed flasks (500 ml) containing 100 ml of the seed medium were inoculated with spores from the slant culture and incubated at 30°C on a shaker with 3-inch throw at 180 rev/min for 2~3 days to obtain good growth. Fermentations were carried out at 30°C for 4 days under aeration of 20 liters/min and agitation of 300 rev/min.

Table 5. Media used for production of nocardicin A

Seed medium		Fermentation medium	
Sucrose	2%	Glycerin	3%
Cotton seed meal	2%	Cotton seed meal	2%
Dried yeast	1%	Dried yeast	2%
		KH_2PO_4	2.18%
		$Na_2HPO_4 \cdot 12H_2O$	1.43%
		$MgCl_2 \cdot 6H_2O$	0.5%

Progress of the fermentation was monitored by diffusion plate assays performed on supernatant fluid from centrifuged broth samples (4,000 rev/min for 10 minutes). *Pseudomonas aeruginosa* NCTC 10490 was used as a test organism for the bioassay.

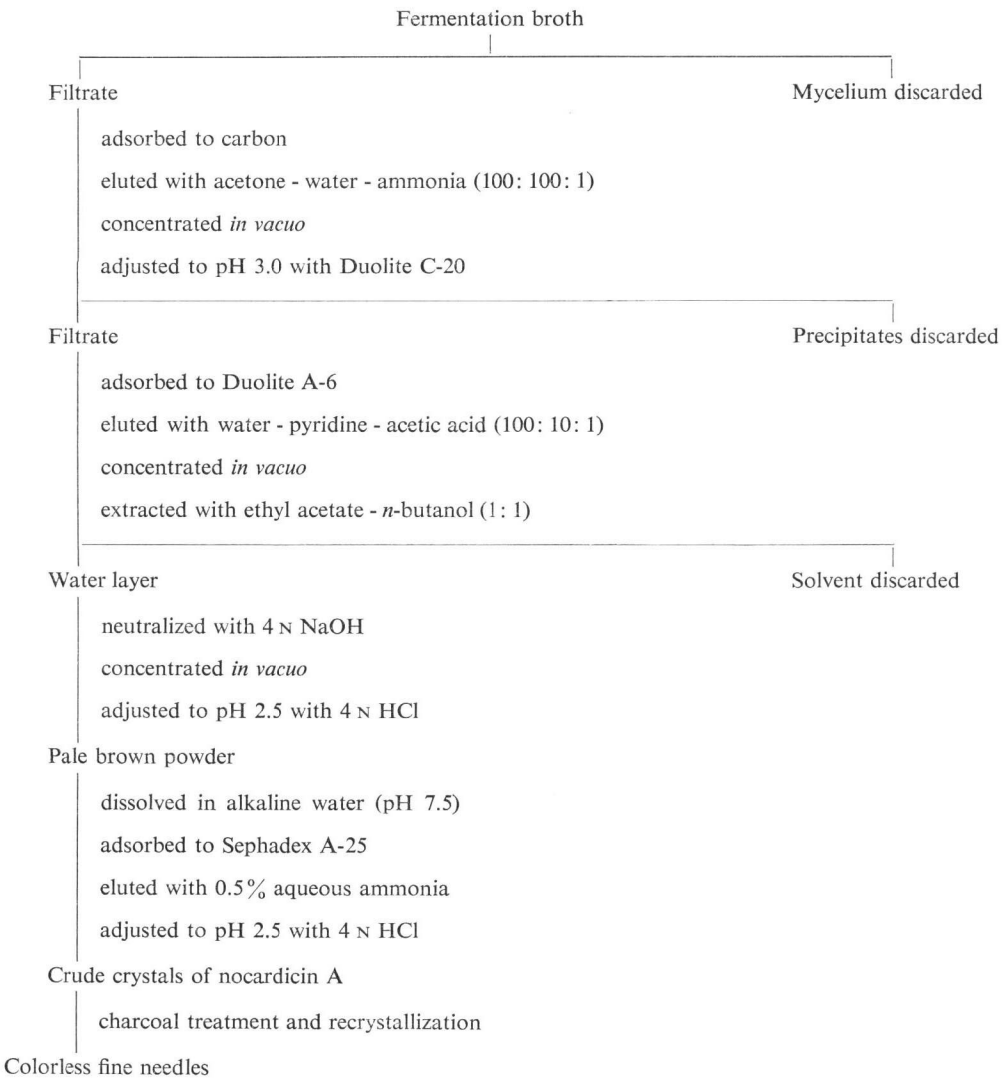
Isolation Procedure

The isolation method used for nocardicin A is outlined in Fig. 4. Most of the antibiotic activity was found in the broth filtrate. After the fermentation was completed the culture broth was filtered with the aid of filter aid (Radiolite). The active principle was adsorbed to activated carbon and extracted from the carbon with acetone - water - 25% aqueous ammonia (100: 100: 1 by volume). The extract was concentrated under reduced pressure and adjusted to pH 3.0 with cation-exchange resin (Duolite C-20, H^+ form). Precipitate which appeared after acidification was removed by filtration. The filtrate was passed through a column of an anion-exchange resin (Duolite A-6, acetate form). The column was washed with water and the absorbate was eluted with water - pyridine - acetic acid (100: 10: 1). The active eluate was concentrated under reduced pressure and organic solvent-extractable impurities were removed. The active solution was adjusted to pH 2.5 to give a pale brown precipitate. The collected precipitate was suspended in water and the suspension was adjusted to pH 7.5 with 4 N aqueous sodium hydroxide. The solution was passed through a column of an anion-exchange resin (DEAE-Sephadex A-25, Cl^- form). The column was washed with water and the absorbate was eluted with 0.5% aqueous ammonia solution. The eluate was adjusted to pH 2.5 with 4 N hydrochloric acid to give a crystalline powder. After treatment with charcoal, the crude crystals were recrystallized from acidic water to yield colorless fine needles. From 20 liters of fermentation broth (500 mcg/ml), 3.6 g of nocardicin A was obtained.

Physicochemical Properties

Nocardicin A is a colorless crystalline material, soluble in alkaline solutions such as aqueous ammonia, aqueous pyridine and aqueous sodium hydroxide and dimethyl sulfoxide, sparingly soluble

Fig. 4. Isolation procedure for nocardicin A.



in methanol and insoluble in chloroform, ethyl acetate and ethyl ether. It gradually changes to brown at 187°C and decomposes at 214~216°C. The optical rotation of the sodium salt of nocardicin A is $[\alpha]_D^{25}$ −135 (*c* 1, water). The elementary analysis gave the following data; C, 54.31; H, 4.90; N, 10.71; O, 30.08. Attempts were made to determine the molecular weight by mass spectroscopy, the vapor pressure depression method and the RAST method but satisfactory results were not obtained.

Fig. 5 shows the ultraviolet absorption spectrum of nocardicin A in 1/15 M phosphate buffer solution (pH 8.0) and in 0.1 N aqueous sodium hydroxide solution. It shows shoulder at 220 nm and a maximum at 272 nm ($E_{1cm}^{1\%}$ 310) in phosphate buffer and maxima at 244 nm ($E_{1cm}^{1\%}$ 460) and 283 nm ($E_{1cm}^{1\%}$ 270) in alkaline solution.

Its infrared absorption spectrum measured in nujol gave peaks at the following frequency as shown in Fig. 6; 3450, 3250, 3200, 2700~2500, 1725, 1655, 1605, 1590, 1510, 1395, 1260, 1240, 1220, 1175, 1045, 930, 840, 810, 720 cm^{-1}. NMR spectrum of the antibiotic is presented in Fig. 7.

Color reactions of the sodium salt of nocardicin A are as follows: positive in ninhydrin, DRAGENDORFF and ferric chloride tests and negative in EHRLICH, MOLISCH, FEHLING and TOLLENS tests.

The Rf values of the sodium salt of no-

Table 6. Rf values of nocardicin A on cellulose plates

Solvent	Rf
Wet butanol	0.02
70% Propanol	0.21
Butanol - acetic acid - water (4: 1: 2)	0.34

Fig. 5. UV spectra of nocardicin A.

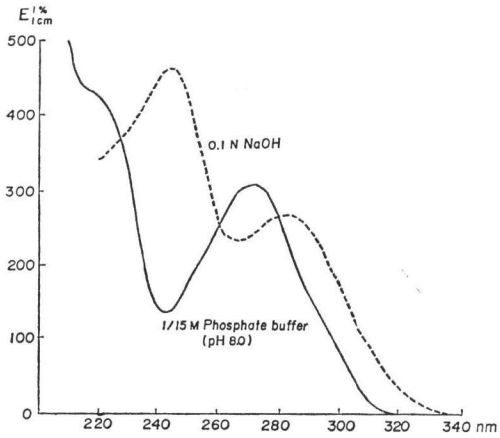


Fig. 6. IR spectrum of nocardicin A (nujol).

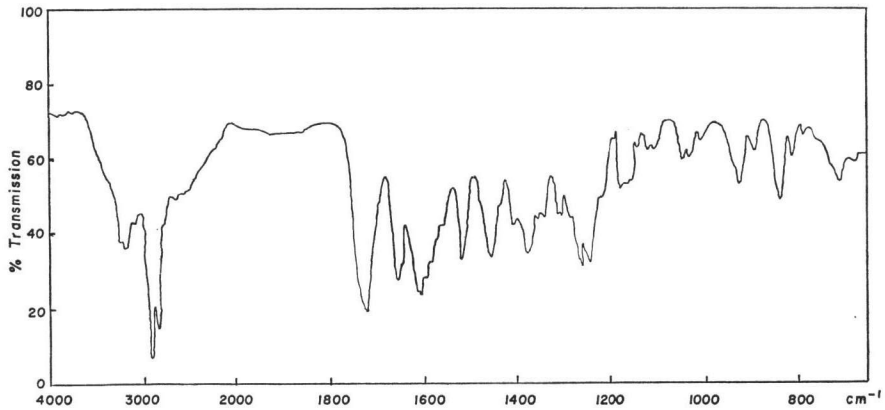
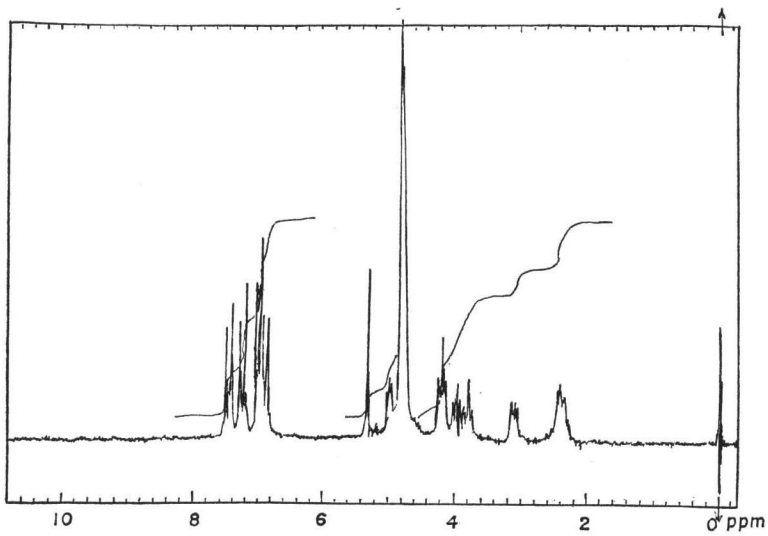


Fig. 7. NMR spectrum of nocardicin A.



cardicin A in thin-layer chromatography are shown in Table 6.

Biological Characteristics

The antibacterial spectrum of nocardicin A is shown in Table 7. This test was conducted by the serial agar dilution streak method. One loopful of an overnight culture of each test strain in Trypticase-soy broth (about 10^8 viable cells/ml) was streaked on heart infusion agar containing graded concentrations of the drugs and the minimal inhibitory concentration (MIC) was expressed in terms of mcg/ml after incubation at 37°C for 20 hours except for *Mycobacterium* and fungi, in which MIC was determined after 3 days of incubation. For the determination of MICs against *Diplococcus* and *Streptococcus*, alteration was made by adding 10% rabbit serum to the Trypticase-soy broth and 10% defibrinated rabbit blood to the HI -agar medium. For fungi malt extract medium was used and spore suspension (10^6/ml) was streaked.

Nocardicin A shows selective antibacterial activity, showing moderate activity against a broad spectrum of Gram-negative bacteria including *Proteus* and *Pseudomonas*. It has no inhibitory effect on *Staphylococcus*, *Mycobacterium*, fungi and yeast. The antibiotic shows no cross resistance with streptomycin, kanamycin, chloramphenicol, tetracycline, α-aminobenzylpenicillin and cephaloridine.

The acute toxicity is shown in Table 8. The toxicity is very low, LD_{50} in laboratory animals

Table 7. Antimicrobial spectrum of nocardicin A

Test microorganisms	MIC (mcg/ml)	
	Nocardicin A	Cephazolin
Staphylococcus aureus FDA 209P	>800	0.39
Bacillus subtilis ATCC 6633	50	0.39
Sarcina lutea PCI-1001	6.25	0.78
Diplococcus pneumoniae III	100	0.2
Streptococcus hemolyticus S-23	200	0.2
Escherichia coli NIHJ JC-2	100	1.56
Klebsiella aerogenes NCTC-418	200	1.56
Proteus vulgaris IAM-1025	3.13	>100
Salmonella typhi O-901	50	1.56
Shigella sonnei I EW-33	3.13	0.78
Pseudomonas aeruginosa IAM-1095	400	>100
Mycobacterium phlei	>1,600	12.5
Penicillium chrysogenum Q-176	>1,600	>1,600
Aspergillus niger	>1,600	>1,600
Candida albicans	>1,600	>1,600

Table 8. Acute toxicity of nocardicin A

Species	Sex	LD_{50} (mg/kg)			
		i.v.	i.p.	s.c.	p.o.
Mouce	Male	2,100	2,500	2,900	>8,000
	Female	2,400	2,500	3,100	>8,000
Rat	Male	>2,000	2,600	3,100	>8,000
	Female	>2,000	2,800	5,100	>8,000

being larger than 2 g/kg in any route tested.

Discussion

Penicillin and cephalosporin are among the most important chemotherapeutic agents produced by microorganisms. They are characterized chemically by the presence of a β-lactam ring in their molecules and biologically by their selective inhibitory effect on bacterial cell wall synthesis.

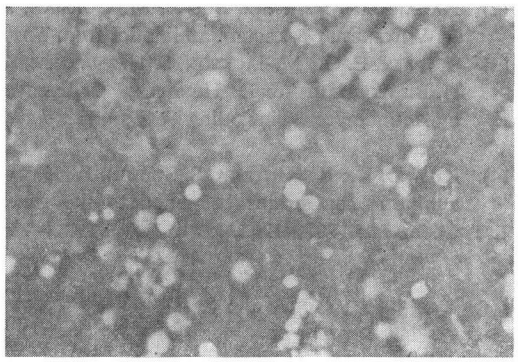
Compounds with β-lactam structures are not so often encountered in nature. Only a few have been found in fermentation broths of microorganisms[9,10,11] other than penicillins and cephalosporins. So far the penicillins and cephalosporins are the only two types of "β-lactam antibiotics" that have been found.

In order to obtain new β-lactam antibiotics, we have developed a series of mutants of *Escherichia coli* selected *in vitro* for specific high level of susceptibility to penicillins and cephalosporins. As a consequence of this selection process, the cultures aquired specific hypersensitivity to β-lactam antibiotics. The supersensitivity is restricted to β-lactam antibiotics, as the sensitivity of the mutants to most of other antibiotics obtainable through commercial routes is not much different from that of the parent strain. By the use of one of these mutants as test organism nocardicin A has been found.

As shown in Fig. 1, it has monocyclic β-lactam with a novel acyl side chain. The partial structure of nocardicin A resembles that of penicillin and cephalosporin. Cells of susceptible strain of *Pseudomonas* treated with lethal concentration (25 mcg/ml) of nocardicin A in the presence of 20% sucrose were transformed into protoplasts or spheroplasts as shown in Fig. 8. Thus it seem likely that the antibiotic inhibits bacterial cell wall synthesis. The effect of nocardicin A on enzymes of cell wall synthesis is now under investigation.

Fig. 8. Morphology of cells treated with nocardicin A

One tenth ml of logarithmic culture of *Pseudomonas aeruginosa* NCTC 10490 was put on the Difco Antibiotic Medium 3 plate with 30% sucrose. Soft agar (0.7%) containing 25 mcg/ml of nocardicin A and 20% sucrose was overlayed. After overnight incubation, cell morphology was examined under microscope (×240).



Since the time of discovery of penicillin G and cephalosporin C, only a limited number of fungi had been known to produce β-lactam antibiotics. In 1971, several strains of streptomyces were found to produce cephalosporin type β-lactam antibiotics.[12,13] Recently researchers of Takeda Pharmaceutical Co., Japan, detected a large number of fungi producing β-lactam antibiotics of penicillin and cephalosporin types by the use of a β-lactam supersensitive mutant of *Pseudomonas* as test organism.[14]

This is the first report that a microorganism belonging to *Nocardia* produces a β-lactam antibiotic.

The discovery of nocardicin A produced by a nocardia strain shows that the search for new and novel chemotherapeutic agents in microbial products is still promising. At the same time, the rarity of certain types of synthetic abilities is shown by the fact that only one strain of actinomycetes has been found to produce nocardicin A among tens of thousands of strains that have been tested by our sensitive method.

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