STUDIES ON ANTIBIOTICS BN-227 AND BN-227-F, NEW ANTIBIOTICS

I. TAXONOMY, ISOLATION AND CHARACTERIZATION

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The two new antibiotics, BN-227 and BN-227-F, were isolated from the fermentation broth of *Pseudomonas* sp. BN-227. BN-227 has a molecular formula $C_7H_9NO_3$, and melts at 115°C. BN-227-F has a molecular formula $C_{21}H_{24}N_8O_9Fe$, and melts at 156°C. BN-227-F is a chelate compound consisting of three similar ligands (antibiotic BN-227) and ferric ion. The two antibiotics have antimicrobial activity against Gram-positive and Gram-negative bacteria.

In our screening studies of antibiotics produced by bacteria isolated from soils, two new antibiotics, BN-227 and BN-227-F, were isolated from the culture filtrate of *Pseudomonas* sp. BN-227. In this paper, the taxonomy of the strain, and the isolation and characterization of the antibiotics are described.

Taxonomic Studies

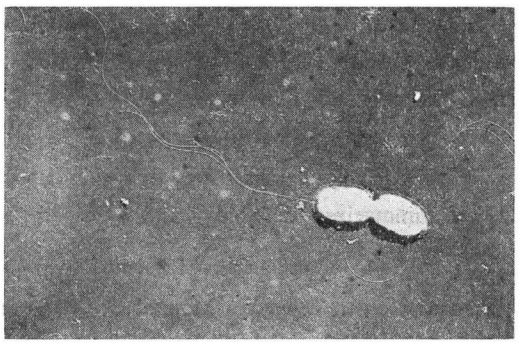
1. Morphological Characteristics

Morphological observation of the strain BN-227 was carried out by both optical and electron microscopy with cells cultured mainly on nutrient agar for 18~24 hours at 28°C. The following results were obtained.

The culture consists of short rods with round ends, $0.6\sim0.8\times1.0\sim2.0\ \mu$, occurring singly or sometimes in pairs. The strain is motile, possessing polar flagella. It is Gram-negative and has no endospore.

The typical electron microscopic photograph of strain BN-227 is shown in Fig. 1.

Fig. 1. Electron microscopic photograph of *Pseudomonas* sp. BN-227.



2. Cultural Characteristics

Cultural observation of the strain was carried out using cultures grown on various media for 2~7 days.

The following results were obtained.

(1) Nutrient agar slants (for 2 days at 28°C): Abundant growth, raised in center, smooth surface, glistening opaque and creamy white color. No soluble pigment.

(2) Nutrient broth (for 2 days at 28°C): Moderate growth, turbid.

Table 1. Physiological characteristics of *Pseudomonas* sp. BN-227.

Nitrite formation from nitrate	+
Denitrification	−
Methyl red test	+
VOGES-PROSKAUER test	−
Indole formation	−
Hydrogen sulfide formation	−
Hydrolysis of starch	−
Utilization of citrate	+
Utilization of inorganic nitrogen sources	+
Producing soluble pigment	−
Oxidase test	±
Arginine dehydrolase	−
Temperature range for growth	10～37°C
Optimum temperature for growth	26～30°C
pH range for growth	5～10
Optimum pH for growth	6～8
Aerobic	+
Acid and gas formation from glucose*	−
Growth factor required	−
Egg yolk reaction	+
Accumulation of poly-β-hydroxybutyrate	+
Pathogenicity (mouse i.p.)	−

+: positive, −: negative

* by HUGH and LEIFSON's method (HUGH, R. & E. LEIFSON: J. Bacteriol. 62: 377, 1951)

(3) Gelatin stab (for 7 days at 20°C): Moderate growth, liquefaction.

(4) Litmus milk (for 7 days at 28°C): Moderate growth. Positive peptonization without coagulation, an alkaline reaction to litmus.

3. Physiological Characteristics

Physiological characteristics of strain BN-227 are summarized in Tables 1 and 2.

Table 2. Utilization of carbon sources by *Pseudomonas* sp. BN-227.

Carbon source	Utilization
D-Glucose	+
D-Xylose	+
D-Ribose	+
Rhamnose	−
Sucrose	+
D-Arabinose	+
D-Sorbitol	+
Inositol	+
Erythritol	−

+: growth, −: no growth

Table 3. Comparisons of *Pseudomonas* sp. BN-227 with *Pseudomonas cepacia*.

	Ps. cepacia	BN-227
Number of flagella	>1	>1
Pigments	+	−
Arginine dehydrolase	−	−
Denitrification	−	−
Hydrolysis of starch	−	−
Hydrolysis of gelatin	d	+
Utilization of carbon sources		
D-Glucose	+	+
D-Xylose	d	+
D-Ribose	+	+
Saccharate	+	+
Levulinate	+	−
Citraconate	+	+
Mesaconate	−	−
Mesotartarate	+	−

+: positive, −: negative, d: positive for more than 10% but less than 90% of all strains.

4. Comparison of *Pseudomonas* sp. BN-227 with Closely Related Bacteria

According to BERGEY's Manual of Determinative Bacteriology[1], strain BN-227 was considered to belong to genus *Pseudomonas* with those characteristics described above. Furthermore, the present organism belongs to Section II of *Pseudomonas* species, which accumulate poly-β-hydroxybutyrate as intercellular carbon reserve, utilize arginine and betaine as sole carbon sources and never require growth factors. Among six species of Section II described in BERGEY's manual, strain BN-227 is similar to *Pseudomonas cepacia* BURKHOLDER.

The comparison of strain BN-227 with *Pseudomonas cepacia* is summarized in Table 3. As shown in Table 3, strain BN-227 is different from *Pseudomonas cepacia* in some of cultural and physiological characteristics.

These characteristics of strain BN-227, however, were not enough to designate the strain to be a new species. Therefore, strain BN-227 was tentatively named *Pseudomonas* sp. BN-227. The type strain has been deposited in the collection of the Fermentation Research Institute, Agents Industrial Science and Technology, Chiba, Japan and assigned the designation FERM-P No. 4357.

Fermentation

A well-grown agar slant of producing organism was used to inoculate seed medium containing 2.0% glycerin, 1.5% wheat germ, 1.5% soybean meal, 0.3% NH_4Cl, 0.05% K_2HPO_4 and 0.2% $CaCO_3$. The seed culture was incubated at 28°C for 20 hours on a reciprocal shaker.

The seed culture (1~2%) was inoculated into a 30-liter jar fermentor contained the same medium.

The fermentation was conducted at 28°C, the agitation was at 200 rpm, and aeration was at 20 liters/min.

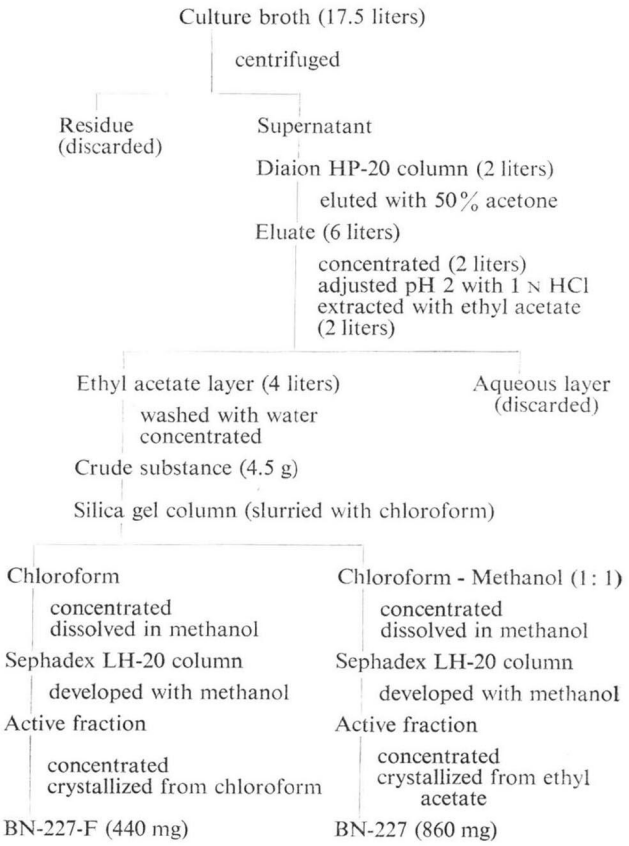
Antibiotic concentration reached a maximum after 40~42 hours. The antibiotic activity in the fermentation broth was determined by a paper disc-agar diffusion assay using *Staphylococcus aureus* FDA 209P.

In the jar fermentation, strain BN-227 produced titres of 1,500~2,000 mcg/ml as antibiotic BN-227.

Isolation and Purification

The isolation and purification of antibiotics are summarized in Fig. 2.

Fig. 2. Isolation and purification of antibiotics BN-227 and BN-227-F.



Physical and Chemical Properties

Physical and chemical properties of antibiotics BN-227 and BN-227-F are summarized in Table 4.

The IR spectrum of antibiotic BN-227 is illustrated in Fig. 3, which shows characteristic bands at 3470, 1650, 1540, 1460, 1280, 1160, 1100, 820, 760 cm^{-1}.

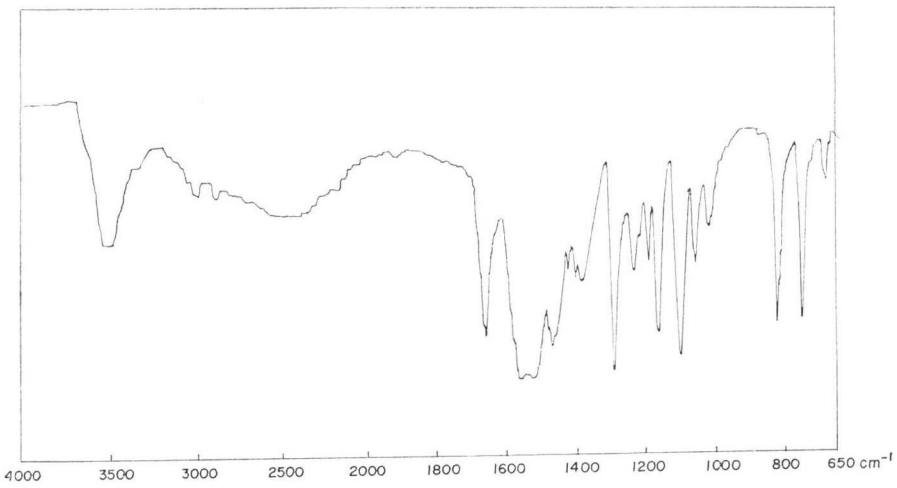
The NMR spectrum is shown in Fig. 4, which revealed a methyl signal at 2.43 ppm, a methoxy signal at 3.73 ppm, two olefinic protons at 6.53 and 7.20 ppm and hydroxy signal at 9.70 ppm.

The IR spectrum of antibiotic BN-227-F is illustrated in Fig. 5, which shows characteristic bands

Table 4. Physical and chemical properties of antibiotics BN-227 and BN-227-F.

	BN-227	BN-227-F
Appearance	colorless prism	reddish needle
Melting point	115°C	156°C
UV max.	333 nm ($E^{1\%}_{1cm}$ 570)	318 nm ($E^{1\%}_{1cm}$ 275)
Optical activity	$[\alpha]^{20}_{D}$ 0 (*c* 1, MeOH)	could not detect
Molecular weight (Mass spectrometry)	155	518
Molecular formula	$C_7H_9NO_3$	$C_{21}H_{24}N_3O_9Fe$
Found	C 53.79, H 5.84, N 8.73	C 48.19, H 4.63, N 8.15, Fe 9.78
Calcd.	C 54.18, H 5.86, N 9.02	C 48.66, H 4.68, N 8.10, Fe 10.78
Solubility		
Soluble	methanol, ethanol	chloroform, methanol
Slightly soluble	chloroform, benzene, acetone, ethyl acetate	acetone, benzene, ethyl acetate
Insoluble	water, petroleum ether	water, petroleum ether
Color reaction		
Positive	ferric chloride, potassium permanganate	potassium permanganate
Negative	ninhydrin, MOLISCH, SAKAGUCHI, biuret	ferric chloride, ninhydrin, MOLISCH, SAKAGUCHI, biuret

Fig. 3. IR spectrum of antibiotic BN-227 (KBr pellet).



at 3450, 1640, 1520, 1390, 1280, 1200, 1160, 1110, 1060, 880, 760, 705 cm^{-1}.

The NMR spectrum of antibiotic BN-227-F can not be measured because it contains iron.

Table 5 indicates comparative Rf values on thin-layer chromatogram. In every case a single spot was detected with ferric chloride reagent and by bioautography on *Staphylococcus aureus* FDA 209P.

On the basis of physical and chemical data, and the simplicity of IR spectrum, it was predicted that the antibiotic BN-227-F is a chelate compound consisting of three similar ligands (antibiotic BN-227) and ferric ion. Its chemical structure, which will be described in a separate paper, was presumed to be as follows.

Fig. 4. NMR spectrum of antibiotic BN-227 (60 MHz in $CDCl_3$).

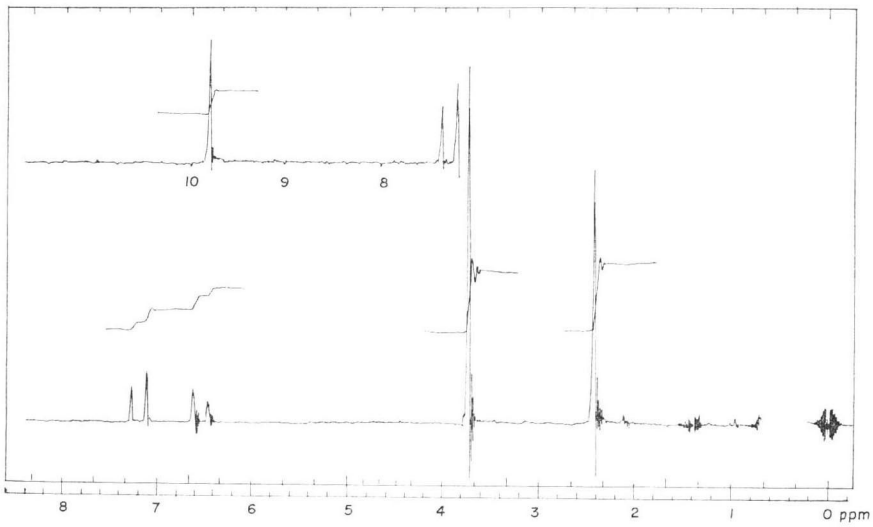


Fig. 5. IR spectrum of antibiotic BN-227-F (KBr pellet).

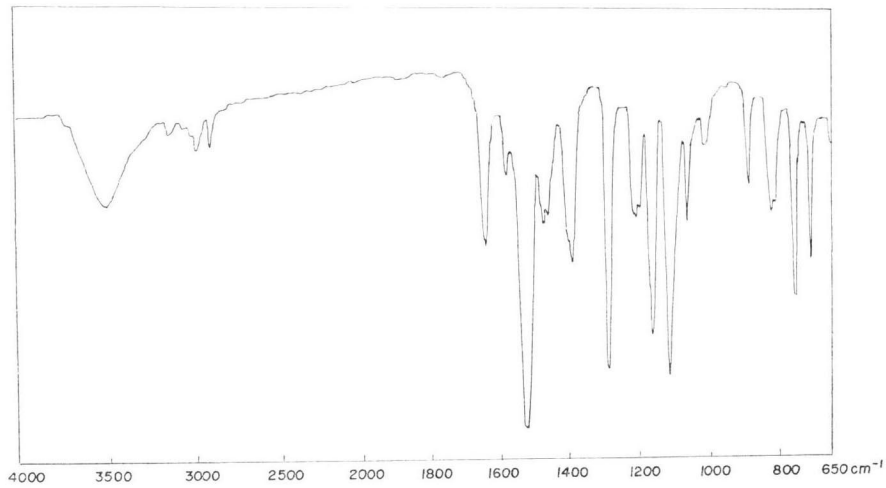
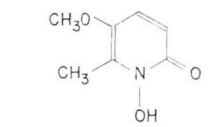


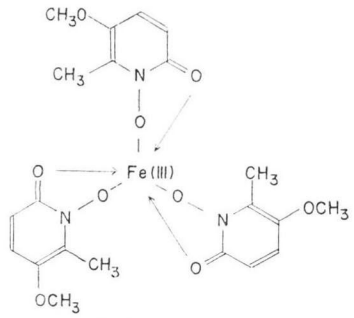
Table 5. Comparison of Rf values of antibiotics BN-227 and BN-227-F on thin-layer chromatogram.

Solvent systems	Rf*	
	BN-227	BN-227-F
$CHCl_3$-CH_3OH (4 : 1)	0.69	0.89
C_6H_6-C_2H_5OH (4 : 1)	0.54	0.71
C_6H_6-$(CH_3)_2CO$ (3 : 2)	0.22	0.70
C_6H_6-$CH_3COOC_2H_5$ (1 : 9)	0.07	0.10

* Silica gel $60F_{254}$ (Merck).



Antibiotic BN-227



Antibiotic BN-227-F

Biological Properties

Table 6 summarizes the antimicrobial spectra of antibiotics BN-227 and BN-227-F. Antibiotics BN-227 and BN-227-F are active against Gram-positive and Gram-negative bacteria.

Table 6. Antimicrobial spectra of antibiotics BN-227 and BN-227-F.

Test microorganism	MIC (mcg/ml)*	
	BN-227	BN-227-F
Staphylococcus aureus Smith S-424	50	50
Staphylococcus aureus N-0003	50	50
Staphylococcus aureus No.26	50	50
Staphylococcus aureus N-0032	25	25
Staphylococcus epidermidis ATCC 14990	50	25
Streptococcus faecalis ATCC 8043	1.56	1.56
Bacillus subtilis ATCC 6633	50	100
Bacillus anthracis No. 119	12.5	25
Escherichia coli K-12 IAM-1264	25	>100
Escherichia coli No. 29	25	>100
Salmonella typhi 0-901-W	12.5	>100
Salmonella typhimurium LT-2	50	>100
Klebsiella pneumoniae	50	>100
Proteus vulgaris OX19	25	>100
Proteus vulgaris J-0001	50	>100
Proteus mirabilis J-0013	50	>100
Proteus rettgeri J-0026	50	>100
Serratia marcescens No. 1	>100	>100
Pseudomonas aeruginosa IAM-1007	>100	>100
Pseudomonas aeruginosa M-0025	>100	>100

* Agar dilution method.

The acute toxicities of antibiotics BN-227 and BN-227-F were examined by intraperitoneal injection into mice. Administration of 100 mg/kg of each substance to mice caused no death, indicating low toxicity.

Discussion

The physical, chemical and biological properties of an antibiotic BN-227 were compared with those of known antibiotics, but no antibiotic could be found to be identical with antibiotic BN-227. RAPOPORT *et al.*[2] reported a compound having the molecular formula $C_7H_9NO_3$. But the antibiotic BN-227 was differentiated from this compound by ultraviolet absorption spectra and melting point. AMES *et al.*[3] reported an N-hydroxypyridone derivative having the molecular formula $C_7H_9NO_3$, the melting point of which is same as that of antibiotic BN-227. However, the chemical structure of antibiotic BN-227, which will be described in a companion paper[4], is different from that of the compound.

The antibiotic BN-227-F has a molecular formula $C_{21}H_{24}N_3O_9Fe$. Several antibiotics containing iron in its molecule have been reported to date, such as albomycin[5], grisein[6], fluopsin F[7], danomycin[8] and ferrimycin[9]. When compared with related antibiotics, antibiotic BN-227-F appears to be different from those antibiotics both by its iron content and IR spectrum.

From these comparison, no antibiotic could be found to be identical with antibiotics BN-227 and BN-227-F. Therefore BN-227 and BN-227-F were considered as new antibiotics.

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