BN-183B, A NEW ANTITUMOR ANTIBIOTIC PRODUCED BY *PSEUDOMONAS*

TAXONOMY, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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BN-183B is a new antitumor antibiotic with chlorine in its molecule found in the culture broth of *Pseudomonas* sp. BN-183. The compound was weakly basic and isolated as a hydrochloride in a pure state. The molecular formula of its free base was determined as $C_{14}H_{20}N_2O_6Cl_2$. The antibiotic showed not only strong antimicrobial activity against both Gram-positive and Gram-negative bacteria but also marked activity toward experimental tumors such as lymphoid leukemia L-1210 and lymphocytic leukemia P-388 in mice. No mutagenicity of BN-183B was noted.

In the course of our screening program for new antibiotics from bacteria, we isolated a strain designated BN-183 which produced an antibiotic active against both Gram-positive and Gram-negative bacteria.

The novel antibiotic which was named BN-183B was found to be a weakly basic compound containing chlorine in its molecule. Besides excellent antimicrobial activities, the substance exhibited marked activity against experimental tumors of lymphoid leukemia L-1210 and lymphocytic leukemia P-388 in mice.

In this paper, we report taxonomic studies on strain BN-183, fermentation, isolation, physicochemical and biological properties of BN-183B.

Taxonomic Studies of Strain BN-183

The producing microorganism, strain BN-183, was isolated from a soil sample collected at Tokai district of Ibaragi prefecture in Japan.

Strain BN-183 is a non-sporulating Gram-negative rod of $0.6\sim0.8$ by $1.0\sim2.0\ \mu$ in size and is motile with polar flagella. Poly-β-hydroxybutyrate granules are observed by a phase contrast microscope. No sheath, stalk or slime is produced. The electromicrograph of strain BN-183 is shown in Fig. 1.

Physiological and biological properties of strain BN-183 are summarized in Tables 1 and 2. According to BERGEY's Manual of Determinative Bacteriology[1] and the observation reported by STANIER *et al.*[2], the following conclusions have been made.

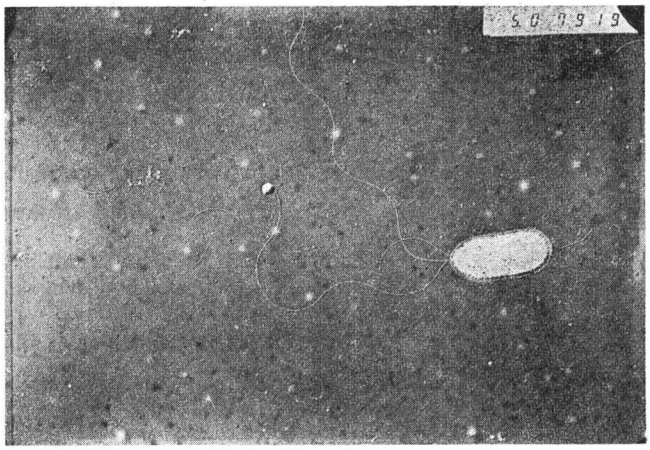
Strain BN-183 appears to belong to the genus *Pseudomonas* in view of the morphological, physiological and biological characteristics described above.

Strain BN-183 is considered to belong to Section II of genus *Pseudomonas* because it requires no growth factors, accumulates poly-β-hydroxybutyrate and uses arginine or betain as sole carbon source.

The results of Tables 1 and 2 show that strain BN-183 closely resembles the species *Pseudomonas cepacia* and *Pseudomonas marginata*, but differs from these two with respect to the utilization of several carbon sources.

Thus strain BN-183 has been conventionally named *Pseudomonas* sp. BN-183 and deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology of Japan, under the accession number of FERM-P No. 3332.

Fig. 1. Electron microscopic photograph of strain BN-183.



Fermentation and Isolation of BN-183B

As the result of preliminary medium test for fermentation of BN-183B, a combination of glycerin and dextrin as carbon source and soybean meal as nitrogen source produced a tolerable level of potency. Addition of sodium chloride or potassium chloride was indispensable for furnishing broths of high potency. The

Table 1. Physiological properties of strain BN-183.

Test	Response
Growth in anaerobic conditions	−
Denitrification	−
Production of diffusible pigments	−
Hydrolysis of starch	−
Accumulation of poly-β-hydroxybutyrate	+
Levan formation from sucrose	−
Decomposition of arginine (MOELLER's method)	−
Egg yolk reaction	−
Oxdase test	±
Growth at 41°C	−
Requirement of growth factors	−
Liquefaction of gelatin	+

The scores of +, − and ± represent positive, negative and faintly positive, respectively.

Table 2. Utilization of various carbon sources of strain BN-183.

Carbon sources	BN-183	*P. cepacia**	*P. marginata**
Glucose	+	+	+
D-Xylose	+	±	+
D-Fucose	+	+	+
D-Arabinose	+	+	+
D-Ribose	+	+	+
Cellobiose	+	+	+
Saccharate	+	+	+
L-Rhamnose	−	±	−
Levulinate	+	+	−
Citraconate	+	+	+
Sebacate	+	+	+
Mesaconate	−	−	+
D(−)Tartarate	−	−	+
meso-Tartarate	+	+	+
o-Hydroxybenzoate	−	+	−
m-Hydroxybenzoate	−	+	−
Erythritol	−	−	−
Adonitol	−	+	+
2,3-Butyleneglycol	−	+	−
Betain	+	+	+
Threonine	+	+	+
Histidine	+	+	+
Arginine	+	+	+
Tryptamine	−	+	−
Ethanolamine	+	+	+
Nicotinate	−	−	+
Acetamide	−	+	−
Anthranilate	−	+	±

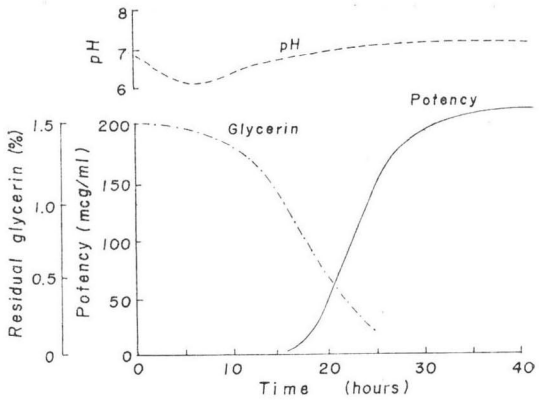
The scores of +, − and ± mean the same in Table 1.

* Data from R. W. BALLARD *et al.*[4]

maximum activity against *Bacillus subtilis* ATCC 6633 was obtained around fortyeight hours in cultivation of *Pseudomonas* sp. BN-183. The typical example of time course of fermentation is illustrated in Fig. 2.

Fig. 2. Time course of BN-183B production in 300-liter fermentor.

Concentration of glycerin was determined by the method of IWAI *et al.*[3]



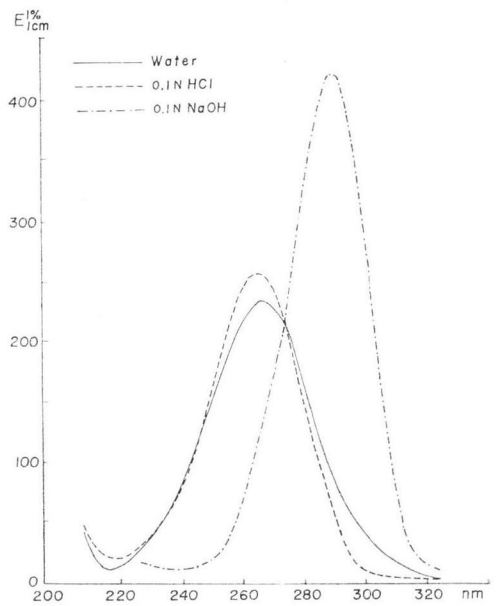
Isolation of BN-183B in a pure state was a rather difficult task owing to its weak basic nature which behaved ambiguously on the tests of resins and absorbents. BN-183B was absorbed on Diaion HP-20, and eluted with aqueous methanol. After removal of methanol, the active fraction was absorbed on CM-Sephadex C-25 column. The active fractions eluted with sodium chloride solution were desalted by the procedures with Diaion HP-20 mentioned above. The resulting solution was concentrated under reduced pressure to furnish a partially purified powder. Further purification of BN-183B was performed by an extraction with methanol and the successive column chromatographies with active charcoal and DEAE-Sepharose CL-6B by developing both with water. The purified dry material was dissolved in methanol and concentrated under reduced pressure until precipitation of BN-183B resulted. The condensate was kept at 5°C overnight and the precipitates were collected by filtration. The colorless powder of BN-183B hydrochloride was obtained in a pure state.

Physico-chemical Properties of BN-183B

BN-183B, isolated as the hydrochloride salt, is an amorphous and colorless powder. BN-183B hydrochloride is soluble in water, methanol and sparingly soluble in ethanol, but very insoluble in acetone, benzene and hexane. The hydrochloride did not show a melting point and decomposed at 214°C. The specific rotation of BN-183B hydrochloride was $[\alpha]_D^{22} -9.2°$ (*c* 1, H_2O).

The molecular formula of BN-183B free base was determined as $C_{14}H_{20}N_2O_6Cl_2$ on the basis of the mass spectrum and elemental analysis of mono-N-acetyl BN-183B which was obtained as crystalline by treatment of BN-183B with acetic anhydride in methanol. The molecular ion peak of mono-N-acetyl BN-183B was observed at *m/e* 424. Elemental analysis of BN-183B hydrochloride; Found: C 39.84; H 5.25; N 6.34; Cl 22.90. Anal. calcd. for $C_{14}H_{20}N_2O_6Cl_2 \cdot HCl$: C 40.05; H 5.01; N 6.67; Cl 25.39.

Fig. 3. Ultraviolet spectra of BN-183B hydrochloride.



Elemental analysis of mono-N-acetyl BN-183B; Found: C 44.99; H 5.10; N 6.84; Cl 16.27. Anal. calcd. for $C_{16}H_{22}N_2O_7Cl_2$: C 45.18; H 5.18; N 6.59; Cl 16.71.

The BN-183B hydrochloride showed a UV maximal absorption at 264 nm ($E^{1\%}_{1cm}$ 258) in water, at 264 nm ($E^{1\%}_{1cm}$ 253) in 0.1 N hydrochloric acid and at 288 nm ($E^{1\%}_{1cm}$ 413) in 0.1 N sodium hydroxide as illustrated in Fig. 3. The IR and PMR spectra are illustrated in Figs. 4 and 5, respectively.

The chromatographic mobility of BN-183B hydrochloride by using silica gel thin-layer plate ($60F_{254}$, E. Merck) is shown in Table 3. The antibiotic was positive in ninhydrin and ferric chloride reaction, and negative in biuret reaction.

Fig. 4. Infrared spectrum of BN-183B hydrochloride in KBr.

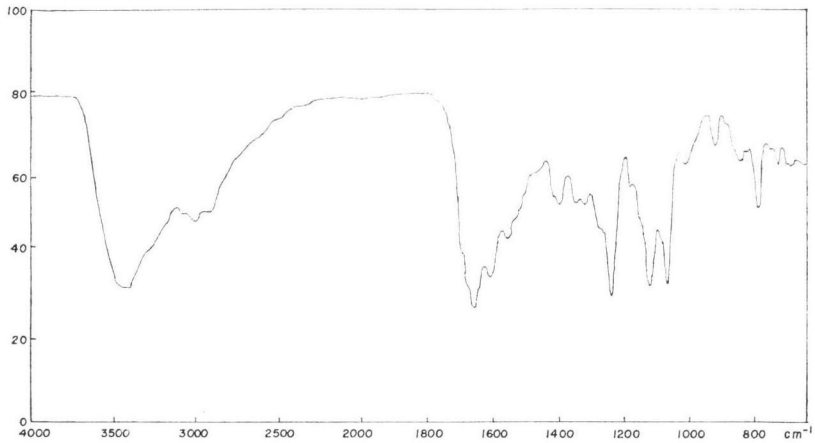
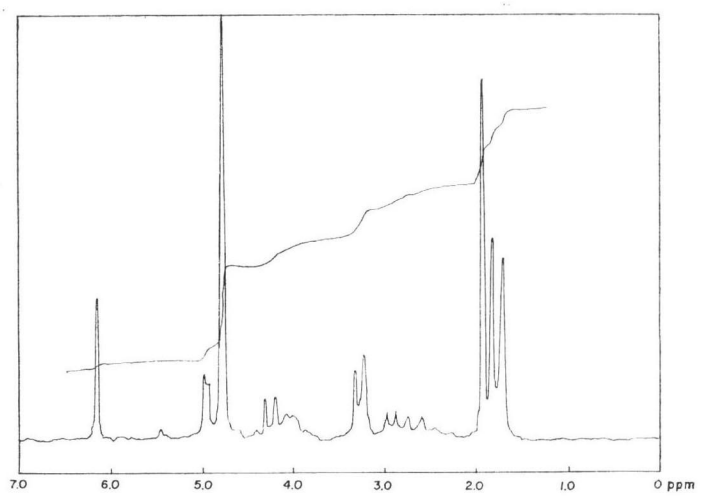


Fig. 5. 60 MHz PMR spectrum of BN-183B hydrochloride in D_2O.



Antimicrobial Activity of BN-183B

The minimum inhibitory concentrations (MIC) of BN-183B hydrochloride against nineteen strains of eight genera of bacteria were determined by a two fold serial dilution method in agar, according to the standard methods recommended by the Japan Society of Chemotherapy[5].

Table 3. Chromatographic mobility of BN-183B hydrochloride.

Solvent system	Rf value
BuOH - AcOH - H_2O (2: 1: 1)	0.66
AcOEt - AcOH - H_2O (60: 17: 17)	0.34
BuOH - Pyridine - AcOH - H_2O (6: 4: 1: 3)	0.65

Detection was made by ninhydrin reaction and bioautography with agar plate of *Bacillus subtilis* ATCC 6633.

As shown in Table 4, BN-183B hydrochloride was found to possess fairly strong antimicrobial activities toward both Gram-positive and Gram-negative bacteria. In another test of paper-disc agar-diffusion method using a concentration range of 100 mcg/ml to 1,000 mcg/ml, this compound showed clear inhibitory zones against not only *Pseudomonas aeruginosa* 8151, *Pseudomonas cepacia* 8241 (both clinical strain) and *Mycobacterium smegmatis* ATCC 607 but also *Candida albicans* IAM 4888. Thus BN-183B appears to be one of the prominent antibiotics produced by bacteria with broad antimicrobial spectrum.

LD_{50} values of BN-183B hydrochloride were determined by varied administration routes, *i.e.* oral (po), intravenous (iv), intramuscular (im), intraperitoneal (ip) and subcutaneous (sc) routes, and the results were tabulated in Table 5. BN-183B appears to be a compound possessing comparatively high acute toxicity.

Table 4. MIC values of BN-183B.

Test organisms	MIC (mcg/ml)
Staphylococcus aureus Rosenbach FDA 209P JC-1	0.39
Staphylococcus aureus Smith S-424	0.39
Staphylococcus aureus No. 26*	0.78
Staphylococcus aureus N-0032*	0.78
Staphylococcus epidermidis ATCC 14990	0.39
Staphylococcus epidermidis N-0028*	0.10
Streptococcus faecalis ATCC 8043	0.10
Bacillus anthracis No. 119	12.5
Bacillus subtilis PCI 219	0.10
Escherichia coli Cast. & Chalm. NIHJ JC-2	3.13
Escherichia coli K-12 IAM 1264	3.13
Escherichia coli W677 (A-20684)*	1.56
Escherichia coli JR 66/W 677 (A-20683)*	1.56
Salmonella typhi 0-901-W	6.25
Shigella dysenteriae Shigae	1.56
Klebsiella pneumoniae PCI 602	25
Klebsiella pneumoniae 22#3038 (A-20680)	12.5
Klebsiella pneumoniae CN 69 (PCase: H)	12.5
Proteus vulgaris OX 19	12.5

* Clinical isolates.

Table 5. LD_{50} values of BN-183B hydrochloride by varied administration routes.

Route	po	iv	im	ip	sc
LD_{50} (mg/kg)	50.0	4.1	4.1	4.3	6.0

Ten JCL-ICR mice weighing 20±0.5 g were used for each administration route.

Antitumor Activity of BN-183B

The effect of BN-183B hydrochloride on lymphoid leukemia L-1210 and lymphocytic leukemia P-388 was investigated, and the results were summarized in Table 6.

Seventy one percent and 92% increase in life span against L-1210 and P-388 were observed respectively at an optimal effective dose, 4 mg/kg, in the administration schedule of day 1 ~ 3 (intraperitoneal injection for 3 consecutive days) as compared to their respective untreated control. However, no animal survived for more than twenty days through the experiment. At a higher dose, 6 mg/kg, no effectiveness was found against both tumors. This appears to be due to the acute toxicity of the substance.

Mutagenicity of BN-183B

The mutagenicity of BN-183B hydrochloride was examined by *in vitro* microbial test[6] known as the AMES' test. The strains *Escherichia coli* (B/r) WP2[6] and *Salmonella typhimurium*[7,8] were all kindly supplied from Dr. T. KADA, National Institute of Genetics, Mishima.

As shown in Table 7, BN-183B hydrochloride did not demonstrate any mutagenic activity toward all strains tested, whereas two carcinogenic agents as positive control, 4-nitro-quinoline-N-oxide (4-NQO) and N-methyl-N-nitro-N'-nitrosoguanidine (NTG) were both clearly positive in the revertant forming test.

Considering the biological properties described above, BN-183B appears to be characterized by its antitumor activities lacking mutagenicity rather than its antimicrobial activities. Thus, this substance is probably the first antitumor antibiotic found of bacterial origin.

Table 6. Effect of BN-183B by intraperitoneal injection on L-1210 and P-388.

Tumor	Dose* (mg/kg)	Survival days (T/C)**	ILS*** (%)
L-1210, 10^6 cell (ip) (BDF_1)	6	3.2/7.6	−57.9
	4	13.0/7.6	71.0
	2	12.2/7.6	60.5
	1	11.0/7.6	44.7
	0.5	10.2/7.6	34.2
	0.25	9.4/7.6	23.7
P-388, 10^6 cell (ip) (CDF_1)	6	7.0/10.3	−32.0
	4	19.8/10.3	92.2
	2	17.2/10.3	67.0
	1	15.6/10.3	51.5
	0.5	14.4/10.3	39.8
	0.25	14.0/10.3	35.9
	0.125	11.0/10.3	6.8

Five males of BDF_1 hybrid and CDF_1 hybrid mice weighing 21±1 g were used in each administrated dose. One million cell of L-1210 or P-388 were intraperitoneally transplanted into mice.

* Intraperitoneal injection of BN-183B hydrochloride was begun at 24 hours after the transplantation and performed once a day for 3 days.

** T/C=treated/control

*** Increase in life span.

Table 7. Results of AMES' test of BN-183B hydrochloride.

Test compounds	Concentration (mcg/ml)	Strains							
		E. coli		*S. typhimurium*					
		hcr+	hcr−	TA1535	TA1536	TA1537	TA1538	TA98	TA100
BN-183B hydrochloride	10,000	−	−	−	−	−	−	−	−
	1,000	−	−	−	−	−	−	−	−
	100	−	−	−	−	−	−	−	−
4NQO	100	+	+	+	−	−	++	++	+++
NTG	1,000	++	−	+++	−	−	−	++	+++

A paper disc of 8 mm diameter containing 20 μl of each sample was placed on the center of each agar plate and incubated at 37°C for 2 days. The revertant colonies formed around the paper disc were counted and scored as +++ : >100, ++ : >50, + : >20 and − : <20.

Experimental

(1) Fermentation

Antimicrobial activity of BN-183B was investigated by a paper-disc agar-diffusion method using *Bacillus subtilis* ATCC 6633 as an assay organism. A 500-ml Erlenmeyer flask containing 100 ml of a seed medium composed of 1% glycerine, 0.5% glucose, 1% Polypeptone, 0.5% meat extract and 0.2% $CaCO_3$ (pH 7.0, before strerilization) was inoculated with a loopful of strain BN-183 slant culture

and incubated at 28°C for 2 days on a rotary shaker (200 rpm). The first seed culture thus obtained was transferred into a 30-liter jar fermentor containing 20 liters of a medium consisted of 1.5% glycerin, 1.5% dextrin, 2% soybean meal, 0.5% KCl and 0.2% $CaCO_3$ (pH 6.8), and operated at 28°C for 2 days using an agitation rate of 180 rpm and an air flow rate of 0.5 vol./vol./min. During the cultivation Silicon KM 68-2F (Shin-etsu Kagaku Co., Tokyo) as antifoamer was automatically added. The production of BN-183B was conducted in a 300-liter stainless steel fermentation vessel containing 200 liters of the above medium. One liter of the second seed culture mentioned above was transferred into the vessel and the fermentation was carried out at 28°C for two days with an agitation rate of 120 rpm and an air flow rate of 0.5 vol./vol./min.

(2) Isolation

The culture broth (150 liters) having a titer of 200 mcg/ml of BN-183B was successively centrifuged. To the clarified supernatant (pH 6.8), Diaion HP-20 resin (15 liters) was added and the mixture was vigorously stirred for an hour. The separated adsorbent was packed in a column and washed with 30 liters of 50% methanol. Active fractions (90 liters), eluted with 80% methanol, were pooled, evaporated and diluted with water to make the volume 50 liters. The aqueous solution (pH 7.0) was passed through a column of CM-Sephadex C-25 (4.5 liters), and washed with deionized water (12 liters). The column was developed with 0.1 M sodium chloride solution. The resulting active fractions (10 liters) was passed through a column of Diaion HP-20 resin (5 liters) and washed with deionized water (7.5 liters) and followed by an elution with 50% methanol. The active fractions (10 liters) collected were evaporated to dryness to give brownish powder (4.1 g, 750 mcg/mg). The material was then dissolved in methanol and filtered to remove the insoluble matter. The resulting clarified solution was applied onto a charcoal column (100 ml) and the elution with methanol was performed. The resulting active fraction (500 ml) was evaporated to dryness to furnish a yellowish powder (3.3 g, 920 mcg/mg). After dissolving with a small volume of water (12 ml), the purified material was subjected to a column of DEAE-Sepharose CL-6B (370 ml) and developed with water. The active fractions (170 ml) were collected and evaporated to dryness to give pale yellowish powder (3.0 g) which showed a single spot on TLC (Table 3). The purified powder was dissolved in methanol (150 ml) and concentrated until a precipitate began to come out. The mixture was kept in a refrigerator (5°C) overnight. The resulting precipitates deposited were collected by filtration and dried under reduced pressure. The purified powder of BN-183B hydrochloride (2.5 g) was obtained at the overall yield of 8.3 percent.

Addendum

After this manuscript was submitted, a paper on bactobolin was reported in J. Antibiotics 32: 1069~1071, 1979 by S. KONDO, Y. HORIUCHI, M. HAMADA, T. TAKEUCHI & H. UMEZAWA. Our antibiotic seems to be identical with bactobolin. The similarity of both antibiotics will be reported elsewhere.

Acknowledgements

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