B-1008, A NEW ANTIBIOTIC OF BACTERIAL ORIGIN CONTAINING A SPERMIDINE MOIETY

PRODUCTION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL PROPERTIES

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A new antibiotic, B-1008 has been isolated from the cultured broth of *Pseudomonas fluorescens* No. 101 B-13L. B-1008 is a water-soluble basic substance containing the spermidine moiety and possessing antibacterial activity against a wide range of bacterial species.

During our screening for water-soluble antibiotics containing a free amino group, a new antibiotic designated B-1008 has been isolated from a fermentation broth of *Pseudomonas fluorescens* No. 101 B-13L. The present paper deals with the production, isolation, and the physico-chemical and biological properties of B-1008.

Experimental

All melting points (mp) are uncorrected. IR spectra were obtained in KBr disc using a Hitachi infrared spectrophotometer Model 260–10. ^{1}H-NMR spectra were determined in D_2O either with a Hitachi Perkin-Elmer R-20A or a Jeol FX-100 spectrometer with 3-(trimethylsilyl)propionic acid-d_4 sodium salt as an internal standard. ^{13}C-NMR spectra were obtained in D_2O on a Jeol FX-100 spectrometer. UV spectra were taken with a Hitachi UV spectrophotometer Model 124.

Thin-layer chromatography (TLC) was carried out on silica gel (Kieselgel 60F_{254}, Merck) or cellulose (Merck) plates using the following systems.

System No.	Solvent system
MN-11	Methanol - 28% ammonia water (1: 1)
MN-12	Methanol - 28% ammonia water (1: 2)
PPAW	*n*-Propanol - pyridine - acetic acid - water (30: 20: 6: 24)
CMN-132	Chloroform - methanol - 28% ammonia water (1: 3: 2)
CM17N-211U	Chloroform - methanol - 17% ammonia water (2: 2: 1, upper phase)

Organism:

The producing organism was isolated from a soil sample collected in Asa, Yamaguchi Prefecture, Japan and identified as *Pseudomonas fluorescens* No. 101 B-13L.

Assay:

Samples obtained during fermentation and purification were assayed by an agar well method using *Pseudomonas aeruginosa* IFO 3445 as test organism and pure B-1008 as standard.

Antibiotic production:

The producing organism was grown at 28°C on a nutrient agar slant. A well-grown agar slant of the bacteria was used to inoculate the seed medium containing 1% glycerol, 0.5% sodium glutamate and 0.25% yeast extract, the pH being adjusted to 7.2 before sterilization. The seed culture (5 ml) was incubated at 28°C for 24 hours on a reciprocal shaker, and the culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of fermentation medium composed 1% glycerol, 1% sodium glutamate

and 0.25% yeast extract (pH 7.0 prior to sterilization). Antibiotic production reached a maximum after 3~4 days shaking at 28°C.

Fermentation studies were also carried out in a 50-liter jar-fermentor. The seed culture (600 ml) was transferred to the fermentation medium containing 1% glycerol, 1% sodium glutamate and 0.25% yeast extract (pH 7.0 prior to sterilization). Cultivation was carried out at 28°C for 72~96 hours with agitation at 200 rpm and aeration of 8 liters/min. At harvest, the broth pH reached 8.4~8.8.

Isolation:

The fermentation broth (27 liters) was clarified by centrifugation and the supernatant passed through an Amberlite CG-50 (NH_4^+ form) column (7.5×40 cm). After washing with water (12 liters), the column was eluted with 0.2 N ammonia water (10 liters). Appropriate fractions were concentrated *in vacuo* and an aqueous solution of the concentrate was applied to a column of Diaion HP-30 (3.2×25 cm), which was then developed with water. The active eluate was concentrated and charged to a column (3.2×35 cm) of CM-Sephadex C-25 (NH_4^+ form). The column was eluted with 0.06 N ammonia water. Active fractions were concentrated *in vacuo* and lyophilized to give pure B-1008 (780 mg).

Preparation of the helianthate:

B-1008 hydrochloride (0.484 g) was dissolved in water. To this solution was added methyl orange (1.00 g) dissolved in water at 55°C. The reaction mixture was cooled in a refrigerator for 20 hours and the crystals then separated by filtration. Recrystallization from water gave reddish-orange crystals of the helianthate, 0.231 g. Mp 187~190°C (dec.).

Anal. Calcd. for $C_{16}H_{27}N_4O_7 \cdot 2(C_{14}H_{15}N_3O_3S)$: C, 52.9; H, 5.77; N, 14.03
Found: C, 52.8; H, 5.78; N, 13.99

Acid hydrolysis:

After a solution of B-1008 in 6 N hydrochloric acid was refluxed for 13 hours, the reaction mixture was evaporated *in vacuo* to remove excess hydrochloric acid. The residue was purified by Sephadex G-10 and silica gel column chromatography to give crystalline product, which was recrystallized from methanol. Mp 253~254°C. IR (KBr): 1590, 1490, 1442, 1404, 1152, 992, 876 cm^{-1}. TLC: Rf 0.18 (MN-12, silica gel) and 0.33 (PPAW, cellulose).

Anal. Calcd. for $C_7H_{19}N_3 \cdot 3HCl$: C, 33.01; H, 8.73; N, 16.50
Found: C, 32.89; H, 8.57; N, 16.19

This product was identical with authentic spermidine in all respects.

Results

The producing organism was cultured for 3~4 days at 28°C and the peak antibiotic potency of 100~330 mcg/ml was obtained. A schematic representation of the isolation process is shown in Fig. 1. B-1008 was a white amorphous solid ($[\alpha]_D$+82°, decomposed over a wide range above *ca.* 140°C), readily soluble in water and insoluble in ethanol, acetone and other organic solvents. The substance gave positive reactions with ninhydrin, TOLLENS, MILLON and ferric chloride reagents, but was negative to SAKAGUCHI and anthrone reactions. B-1008 formed a complex with mercuric chloride. The UV absorption spectrum exhibited a characteristic peak at 276 nm (H_2O), which did not change in 1 N sodium hydroxide and 1 N hydrochloric acid solutions. The IR spectrum showed characteristic absorption at 1570 cm^{-1} (Fig. 2). On TLC, B-1008 showed the

Fig. 1. Isolation of B-1008.

Culture broth
↓ centrifuged
Supernatant
↓
Amberlite CG-50 column chromatography
↓ eluted with 0.5 N ammonia water
Diaion HP-30 column chromatography
↓ eluted with water
CM-Sephadex column chromatography
↓ eluted with 0.06 N ammonia water
Active fraction
↓ lyophilized
Pure B-1008

Fig. 2. IR spectrum of B-1008.

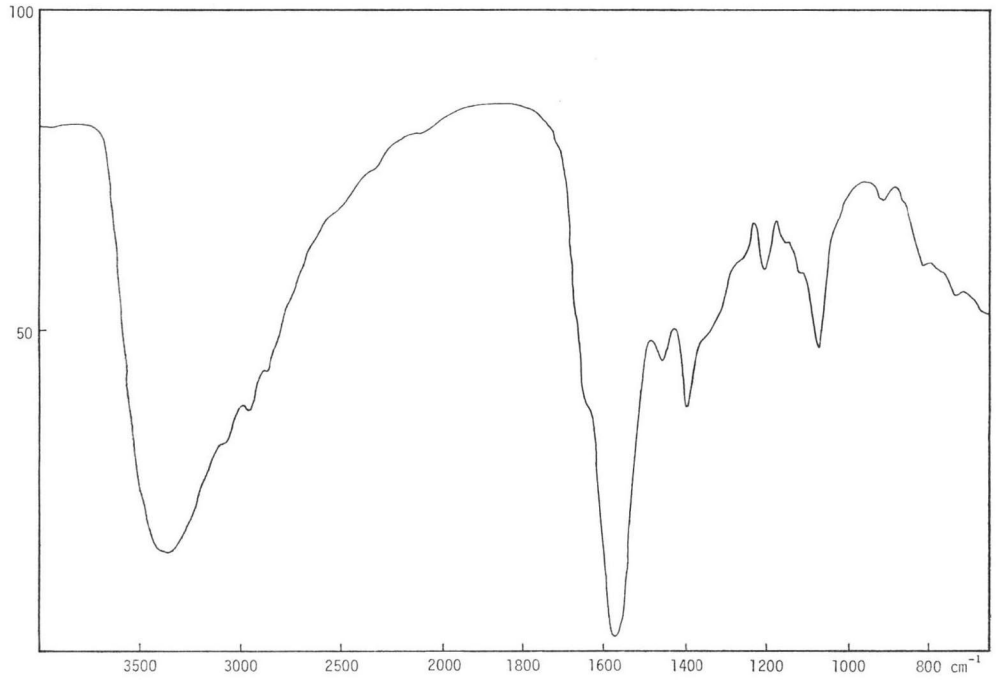
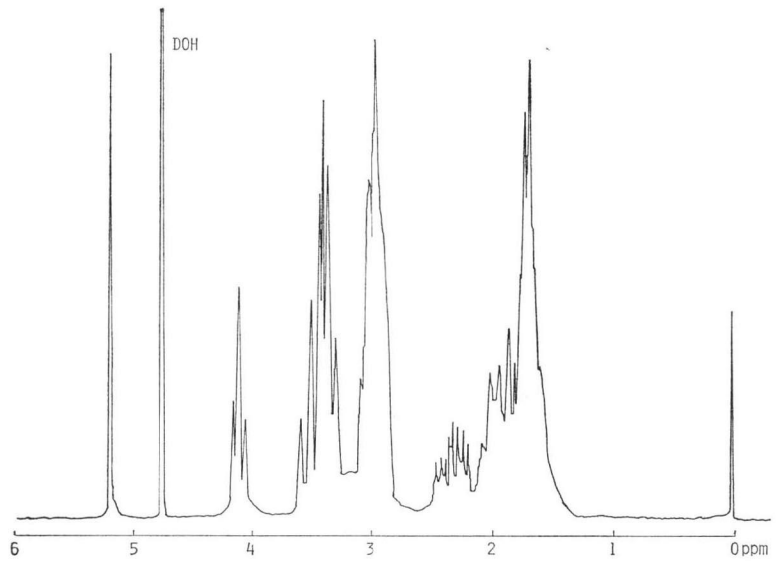


Fig. 3. ^{1}H-NMR spectrum of B-1008 (100 MHz, D_2O).



Rf values given in Table 1. The ^{1}H-NMR spectrum of B-1008 is shown in Fig. 3. ^{13}C-NMR experiments of B-1008 indicated clearly the presence of sixteen carbons as summarized in Table 2. Elemental analysis of the helianthate salt agreed with the formula, $C_{16}H_{27}N_4O_7 \cdot 2(C_{14}H_{15}N_3O_3S)$.

Acid hydrolysis of B-1008 gave a ninhydrin-positive product, that was purified by column chromato-

Table 1. TLC of B-1008.

Solvent system	Rf value
MN-11	0.39
PPAW	0.05
CMN-132	0.30
CM17N-211U	0.46

TLC was carried out on silica gel plates.

Table 2. ^{13}C-NMR spectrum data of B-1008.

Carbon No.	δ (ppm)	Multiplicity
1	201.3	s
2	181.2	s
3	177.3	s
4	97.9	d
5	77.7	s
6	70.9	d
7	67.3	d
8	55.7	d
9	47.9	t
10	46.0	t
11	42.5	t
12	39.8	t
13	31.9	t
14	26.0	t
15	25.2	t
16	24.0	t

Table 3. Antibacterial spectrum of B-1008.

Test organism	MIC (mcg/ml)
Staphylococcus aureus IFO 12732	12.5
Bacillus subtilis ATCC 6633	100
Escherichia coli IFO 12734	50
Salmonella enteritidis IFO 3313	25
Pseudomonas aeruginosa IFO 3445	100
Pseudomonas aeruginosa IFO 12689	100
Proteus mirabilis IFO 3849	50
Proteus vulgaris IFO 3169	50
Serratia marcescens IFO 3736	50

Table 4. Characterization of spermidine-containing antibiotics.

Spermidine-containing antibiotic	Molecular formula	Aromatic component
B-1008	$C_{16}H_{27}N_4O_7$	None
LL-BM 123β	$C_{36}H_{61}N_{13}O_{12}$	Hydroxycinnamic acid
LL-BM 123γ_1	$C_{37}H_{59}N_{13}O_{13}$	Hydroxycinnamic acid
LL-BM 123γ_2	$C_{37}H_{59}N_{13}O_{13}$	Hydroxycinnamic acid
Edeine A_1	$C_{33}H_{53}N_{10}O_{10}$	3-Amino-3-(4-hydroxyphenyl)-propionic acid
Edeine D	$C_{33}H_{53}N_{10}O_9$	3-Amino-3-phenylpropionic acid
Bleomycin A_5	$C_{57}H_{85}N_{18}O_{21}S_2$	3-Amino-3-(4-amino-6-carboxy-5-methylpyrimidino-2-yl)-propionic acid
Laterosporamine	$C_{17}H_{35}N_7O_4$	None

graphy. Elemental analysis of the purified product agreed with the formula, $C_7H_{19}N_3 \cdot 3HCl$ and the IR spectrum showed the characteristic absorption of aliphatic amines. The NMR spectrum also revealed the presence of only methylene protons. The product was identified as spermidine by comparing IR spectrum, NMR spectrum, Rf values on TLC and elemental analysis of the product with those of an authentic sample.

The minimum inhibitory concentration (MIC) of B-1008 was determined by the serial two-fold agar dilution method (Nutrient Agar—Eiken). The results are shown in Table 3. The median lethal dose (LD_{50}) of B-1008 for mice was 9.5 mg/kg (for female, i.v.) and 9.1 mg/kg (for male, i.v.).

Discussion

B-1008 can be distinguished from other antibiotics containing spermidine such as LL-BM-123[1], edeines[2], bleomycin A_5[3] and laterosporamine[4] by comparing either its IR spectrum or its elemental

analysis with those of the known antibiotics (Table 4). Accordingly, B-1008 is considered to be a new antibiotic. The chemical structure is now under investigation.

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