ISOLATION OF BREVISTIN, A NEW PEPTIDE ANTIBIOTIC

(STUDIES ON ANTIBIOTICS FROM THE GENUS *BACILLUS*. IX[1])

JUN'ICHI SHOJI, RYUZI SAKAZAKI, YOSHIHARU WAKISAKA, KENZO KOIZUMI, MIKAO MAYAMA, SHINZO MATSUURA and KOICHI MATSUMOTO

Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553, Japan

(Received for publication December 15, 1975)

A new peptide antibiotic, brevistin, was isolated from the culture broth of *Bacillus brevis* 342-14. Brevistin is an acylpeptide with an approximate empirical formula of $C_{63}H_{91}N_{15}O_{18}$ containing aspartic acid, threonine, glycine, valine, isoleucine, phenylalanine, tryptophan and 2,4-diaminobutyric acid. The antibiotic is soluble in acid and alkaline water. Its hydrochloride is soluble in methanol. Brevistin is active against Ggam-positive bacteria *in vitro* and *in vivo*, and is of low toxicity to mice.

In the course of our screening program for new antibiotics from the genus *Bacillus*,[1] a strain numbered 342-14 and identified as *B. brevis* was found to produce three antibiotic components active against gram-positive bacteria. Components I, II and III (tentatively named according to their decreasing order of Rf values in TLC) were isolated. Component I exhibited an excellent chemotherapeutic effect in *in vivo* studies, and was named brevistin. Brevistin is an acylpeptide, the structure of which is described in a succeeding paper.[2]

This paper reports on the characterization of the producing strain as well as the isolation and preliminary characterization of brevistin.

Taxonomic Characterization of the Producing Strain

Strain 342-14 was isolated from a soil sample collected in Thailand and has been deposited in the Fermentation Research Institute, Chiba, Japan, and in the American Type Culture Collection, U.S.A. under the accession numbers, FERM-P 2363 and ATCC 21991, respectively. The taxonomic characteristics of the strain are described below.

A. Morphology

I. Vegetative cells (28°C, 1~4 days): Gram-positive rods on IM-agar medium* are 0.6 ~0.7 by 2.5~6.0 μ with rounded ends. They occur singly or in masses and are actively motile with peritrichous flagella. Irregular forms are not observed.

II. Spore and Sporangium (28°C, 3~7 days): Spores on IM-agar medium are mostly 0.8 by 1.2 μ, easily stainable, elliptical, terminal to subterminal. The sporangium is definitely swollen.

B. Cultural Characteristics

I. Nutrient agar colony (28°C, 1~7 days): Circular, convex, entire, smooth colonies of semitranslucent density and butyrous structure. The surface of young colonies is shiny but becomes dull with age.

* IM-Agar: Soluble starch 0.2 %, glycerol 0.2 %, Polypeptone 0.25 %, beef extract 0.25 %, yeast extract 0.25 %, NaCl 0.3 %, agar 1.0~1.2 % (w/v), pH 6.8.

II. Nutrient agar slant (28°C, 1~7 days): Growth moderate, filiform, whitish gray, surface shiny (at 1 day) changing to dull after 2 days. No production of pigment.

III. Glucose nutrient broth (28°C, 1~7 days): Moderate turbidity with sediment. Ring formation (pellicle) may be observed.

C. Physiological Characters

I. Relation to oxygen (28°C, 1~4 days): Aerobic (GPYB-agar stab*). No acid or gas is produced from glucose.

II. Optimum temperature (IM-agar, 1~2 days): About 28°C.

III. pH for growth (IM-broth, 28°C, 1~2 days): Grows at pH 4.9~8.5 or more alkaline. Optimum is between 6.0 and 8.5.

IV. Citrate utilization (28°C, 1~8 days): No growth on KOSER's synthetic medium or CHRISTENSEN's nutrient medium.

V. Starch hydrolysis (28°C, 1~7 days): Negative.

VI. Gelatin stab (28°C, 1~18 days): Slowly liquefied.

VII. Litmus milk (28°C, 1~8 days): Peptonized slowly without color change.

VIII. Nitrate reduction to nitrite (28°C, 1~5 days): Negative.

IX. Acetylmethylcarbinol production (28°C, 1~7 days): Negative.

X. Indole production (28°C, 1~7 days): Negative.

XI. H_2S formation (28°C, 1~7 days, Difco peptone iron agar): Negative.

XII. Cytochrome oxidase (28°C, 1 day, nutrient agar): Positive.

XIII. Urease activity: Negative.

XIV. Catalase (28°C, 1 day, nutrient agar): Positive.

XV. Tyrosinase activity (28°C, 1~7 days): Negative.

XVI. Carbohydrate utilization (28°C, 1~7 days): No acid and gas formation is observed from L-arabinose, D-xylose, D-glucose, D-mannose, D-galactose, D-fructose, sucrose, maltose, lactose, trehalose, starch, glycogen, inulin, glycerol, inositol, adnitol, D-mannitol, D-sorbitol, salicin and α-methylglucoside.

XVII. Growth in NaCl (glucose nutrient broth, 28°C, 1~5 days): Grows poorly in 5 % and 7.5 % NaCl. No growth at 10 % NaCl.

D. Speciation

The above observations indicate that this bacterium should be classified as *Bacillus brevis*[3,4], *B. freudenreichii*[3] or *B. aneurinolyticus*.[3] *Bacillus freudenreichii* differs from 342-14 by (a) growth on 5 % NaCl and (b) urease activity. *B. aneurinolyticus* also differs from this by (a) starch hydrolysis and (b) casein hydrolysis. The description of *B. brevis* is very similar to that of 342-14. Therefore, we concluded that 342-14 is a strain of *Bacillus brevis*.

Procedures for the taxonomic study were in accordance to those described in the Manual of Microbiological Methods[5] and Taxonomy and Identification of Microorganisms[6] except where indicated otherwise.

Fermentation and Isolation

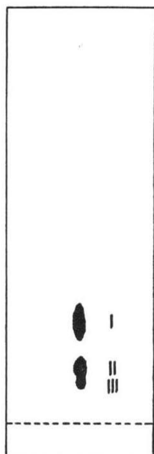
Spores of the strain 342-14 were inoculated into 100 ml of a medium consisting of glucose 1.0 %, glycerol 0.5 %, peptone 1.0 %, meat extract 0.5 % and sodium chloride 0.3 % (pH 7.0) in a 500-ml shaking flask (SAKAGUCHI flask), and cultured with shaking at 28°C for 2 days. About 5 ml of the above culture was seeded into 130 ml of a medium consisting of glucose 1.0 %, glycerol 0.25 %, peptone 0.25 %, soy bean meal 1.0 %, and sodium chloride 0.3 %

* GPYB-Stab: Glucose 1.0 %, Polypeptone 0.5 %, yeast extract 0.2 %, beef extract 0.3 %, BCP (bromocresol purple) 0.015 %, agar 0.4 % (w/v), pH 6.6.

(pH 7.0) in a 500-ml shaking flask (SAKAGUCHI flask). Cultivation was performed at 28°C for four days on a reciprocal shaker.

To about 5 liters of the culture broth obtained as described above, 2.5 liters of *n*-butanol and 2.5 liters of methanol were added, and the mixture was filtered. The filtrate was concentrated under reduced pressure until it separated into two phase, and was then extracted twice with *n*-butanol at pH 8.0. The *n*-butanol extract was found to contain three antibiotic components, I (brevistin), II and III when examined by TLC as illustrated in Fig. 1.

Fig. 1. Chromatogram of three antibiotics produced by strain 342-14.
Silica gel GF plate
Chloroform-ethanol-14% ammonia (4:7:2)
Bioautographed on a *S. aureus* assay plate
(1) 342-14-I (Brevistin)
(2) 342-14-II
(3) 342-14-III



When the *n*-butanol solution containing the three components was washed with water adjusted to pH 2.0 with hydrochloric acid, brevistin remained mainly in the *n*-butanol phase, whereas components II and III were transferred into the aqueous phase. The butanol phase was concentrated under reduced pressure to a syrup. By addition of ethyl acetate to this concentrate, crude brevistin (300 mg) precipitated.

The crude brevistin was purified by preparative TLC carried out on silica gel GF plates with chloroform - ethanol - 14 % aqueous ammonia (4:7:2). The separated zone of brevistin was detected by a UV-lamp and eluted with 50 % aqueous methanol, slightly acidified with hydrochloric acid (pH 2.0). After concentration, the eluate was again chromatographed on silica gel GF plates with chloroform - ethanol - 1% aqueous acetic acid (4:7:2). The separated zone of the antibiotic was detected and eluted as above. After concentration of the eluate, the antibiotic was extracted with *n*-butanol. The *n*-butanol phase was washed first with water adjusted to pH 7.0 with sodium bicarbonate and then with a small amount of distilled water. By concentration of the butanol phase under reduced pressure the free form of brevistin was obtained as a colorless amorphous powder (100 mg).

The free form of brevistin was dissolved in methanol, slightly acidified with hydrochloric acid. After concentration, the hydrochloric acid salt of brevistin was precipitated by addition of ethyl acetate.

Physico-chemical Properties

Brevistin shows a single spot on TLC using a silica gel GF plate with the following solvents: chloroform - ethanol - 14 % aq. ammonia (4:7:2), Rf *ca.* 0.20; chloroform - ethanol - 10 % acetic acid (4:7:2), Rf *ca.* 0.14; and *n*-butanol - acetic acid - water (3:1:1), Rf *ca.* 0.40. The spots were visualized with bioautography, ninhydrin or sulfuric acid.

The free form of brevistin is a colorless amorphous powder, m.p. 190~195°C (dec.).

Analysis Found: C, 54.63; H, 7.12; N, 14.81. $C_{63}H_{91}N_{15}O_{18} \cdot 2H_2O$
Requires: C, 54.73; H, 6.78; N, 15.20; M.W., 1382.49.

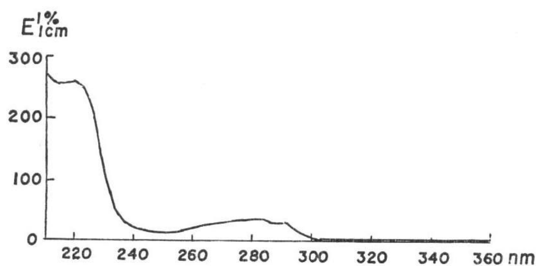
The hydrochloric acid salt of brevistin is a colorless amorphous powder, m.p. 195~200°C.

Analysis Found: C, 50.75; H, 6.82; N, 13.89; Cl, 5.05. $C_{63}H_{91}N_{15}O_{18} \cdot 2HCl \cdot 4H_2O$
Requires: C, 50.73; H, 6.83; N, 14.09; Cl, 4.75; M.W., 1491. 44.

Brevistin is insoluble in water at neutral pH, but soluble in acid and alkali. The hydrochloric acid salt is soluble in methanol, slightly soluble in ethanol, but insoluble in acetone, ethyl acetate, chloroform and ethyl ether. The antibiotic is positive in the ninhydrin and EHRLICH tests, but shows negative SAKAGUCHI, PAULY and BENEDICT reactions.

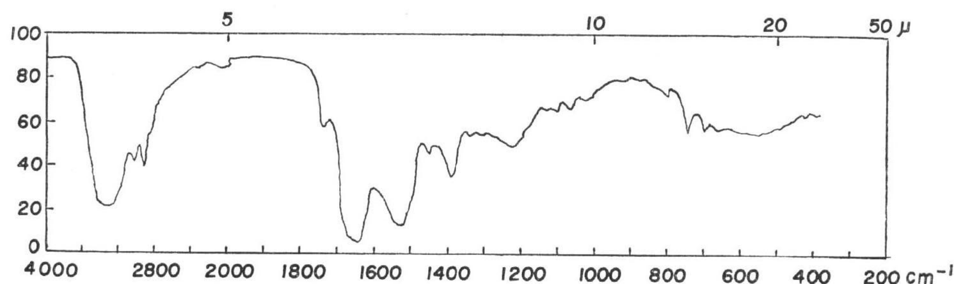
The optical rotation of the hydrochloric acid salt is: $[\alpha]_D^{22.0} +6.1 \pm 0.5°$ (*c* 1.036, MeOH). In the ultraviolet absorption spectrum measured with the hydrochloric acid salt in methanol, the following maxima are observed: 274 nm ($E_{1cm}^{1\%}$ 36), 283 nm ($E_{1cm}^{1\%}$ 39), and 290.5 nm ($E_{1cm}^{1\%}$ 34) (Fig. 2). The infrared absorption spectrum of the hydrochloric acid salt measured on potassium bromide tablet is shown in Fig. 3.

Fig. 2. Ultraviolet absorption spectrum of brevistin (in methanol).



When brevistin was hydrolyzed with hydrochloric acid and the hydrolyzate analyzed with an automatic amino acid analyzer, aspartic acid, threonine, glycine, valine, isoleucine, phenylalanine, tryptophan and 2, 4-diaminobutyric acid were found. GLC of the methylated product from the ether extract of the hydrolyzate indicated the presence of a fatty acid. Thus, brevistin is thought to be

Fig. 3. Infrared absorption spectrum of brevistin (KBr).



an acylpeptide antibiotic. Details of the degradation experiments used to elucidate the structure of brevistin will be published in the next report.[2]

By comparison with the amino acid composition of known peptide antibiotics isolated from bacilli, it is concluded that brevistin is a new peptide antibiotic.

Table 1. Antimicrobial spectrum of brevistin.

Test microorganism	MIC (mcg/ml)
Bacillus subtilis PCI 219	6.25
Bacillus anthracis	6.25
Staphylococcus aureus FDA 209P JC-1	3.13
Staphylococcus aureus 80257	6.25
Staphylococcus aureus Smith	6.25
Streptococcus pyogenes C-203	3.13
Streptococcus pneumoniae type 1	6.25
Escherichia coli NIHJ JC-2	>50
Escherichia coli 80750	>50
Klebsiella pneumoniae	>50
Salmonella typhimurium	>50
Pseudomonas aeruginosa PS-24	>50

Obtained by the usual agar dilution method.

Biological Properties

Brevistin is active against Gram-positive bacteria *in vitro*, but not against Gram-negative bacteria. The antimicrobial spectrum obtained by the agar dilution method is shown in Table 1.

Brevistin is also active against Gram-positive bacteria *in vivo*. The ED_{50} values obtained against *Streptococcus pyogenes*, *Staphylococcus aureus* and *Streptococcus pneumoniae* are listed in Table 2. Moreover, the acute toxicity of this antibiotic is relatively low.

The LD_{50} values observed in mice are as follows: 400~500 mg/kg (ip), >500 mg/kg (sc) and >500 mg/kg (po).

Table 2. Therapeutic effect of brevistin administered subcutaneously to ICR mice infected with *Streptococcus pyogenes*, *Staphylococcus aureus* or *Streptococcus pneumoniae*.

	S. pyogenes	*S. aureus*	*S. pneumoniae*
ED_{50}*(mg/kg×2)	1.23	0.72	3.54

*The ED_{50} is expressed as mg/kg in two subcutaneous doses, given 1 and 5 hours postinfection.

References

1) SHOJI, J.; H. HINOO, Y. WAKISAKA, K. KOIZUMI, M. MAYAMA, S. MATSUURA & K. MATSUMOTO: Isolation of three new antibiotics, thiobacillins I, II and III, related to micrococcin P. (Studies on antibiotics from the genus *Bacillus*. VIII). J. Antibiotics 29: 366~374, 1976
2) SHOJI, J. & T. KATO: The structure of brevistin. (Studies on antibiotics from the genus *Bacillus*. X). J. Antibiotics 29: 380~389, 1976
3) BUCHANAN, R.E. & N.E. GIBBONS (edited): "BERGEY's Manual of Determinative Bacteriology, Eighth Edition, The Williams and Wilkins Co., Baltimore, 1975
4) GIBBS, B.M. & D.A. SHAPTON (edited): "Identification Methods for Microbiologists. Part II", Academic Press, London & New York, 1968
5) Society of American Bacteriologists: "Manual of Microbiological Methods", McGrow-Hill, New York, 1956
6) HASEGAWA, T. (edited): "Taxonomy and Identification of Microorganisms." University of Tokyo Press, Tokyo, 1975