ISOLATION OF OCTAPEPTIN D

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

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

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

(Received for publication November 30, 1979)

A new peptide antibiotic complex, named octapeptin D, was isolated from culture broth of a microorganism belonging to the genus *Bacillus*. The trihydrochloride of the antibiotic was obtained as a colorless powder, soluble in water and methanol. The empirical formula, $C_{47}H_{88}N_{12}O_{11}\cdot 3HCl\cdot H_2O$, was indicated by elemental analysis. Amino acid analysis on the acid hydrolyzate demonstrated the presence of 2,4-diaminobutyric acid (4 moles), serine (1 mole) and leucine (3 moles). Gas chromatographic analysis with the methylated product of the ethereal extract of the acid hydrolyzate revealed the presence of β-hydroxy isodecanoic acid, β-hydroxy decanoic acid, β-hydroxy isoundecanoic acid and β-hydroxyanteisoundecanoic acid. Octapeptin D is active against Gram-negative and Gram-positive bacteria *in vitro* and *in vivo*.

In the course of our screening program for new antibiotics from the genus *Bacillus*[1], a strain numbered JP-301 was found to produce two peptide antibiotics active against Gram-positive and Gram-negative bacteria. One of the two was named octapeptin D, since it was recognized as a new member of the octapeptin group antibiotics[2]. In this paper, fermentation, isolation as well as the physico-chemical and biological properties of octapeptin D are described.

Taxonomic Characteristics of the Producing Microorganism

The bacterium is a moderately sized rod with rounded ends, $0.6\times 1.5\sim 3.0\ \mu$ in main, and produces an elliptical dormant endospore in a central to terminal position. The sporangium is definitely swollen, and the wall of the spore is thick and easily stainable. Gram staining shows clear positive at all stages. The bacterium grows well at $25\sim 35^{\circ}$C and requires oxygen for growth. Colonies on Gly-IM agar* are circular, raised, dull shiny, with a butyrous structure, and of grayish white color. On agar slants of Gly-IM, moderate, filiform growth with dull shiny surface and opaque density is observed. In broth (Gly-IM), uniform, scanty growth occurs. The bacterium produces acid but no gas from glucose. Starch hydrolysis is negative, casein hydrolysis is positive and the VOGES PROSKAUER reaction is negative. The above results suggest that this bacterium is a *Bacillus* species closely related to *Bacillus brevis* and *Bacillus circulans*[3]. Since further taxonomic study has not yet been done, the bacterium has been tentatively designated as *Bacillus* species JP-301.

Fermentation and Isolation

Spores of the strain JP-301 were inoculated into 100 ml of a medium composed of glucose 1.0%, peptone 0.5%, meat extract 0.5%, NaCl 0.1%, KH_2PO_4 0.05%, $MgSO_4\cdot 7H_2O$ 0.05%, $MnSO_4$ 0.001%

* Gly-IM agar: Glycerol 0.5, peptone 0.25, beef extract 0.25, yeast extract 0.25, Bacto soytone (Difco) 0.25, NaCl 0.3, agar 1.25% (w/v), pH 6.8.

and $Fe_2(SO_4)_3$ 0.001% (pH 7.0) in a SAKAGUCHI flask and shake-cultured for 2 days at 27°C. About 3 ml of the culture were then transferred to 100 ml of a medium composed of glucose 1.0%, soy bean meal 2.0%, corn steep liquor 0.25%, $(NH_4)_2SO_4$ 0.5%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $Fe_2(SO_4)_3$ 0.01% and $CaCO_3$ 0.4% (pH 7.0) in a SAKAGUCHI flask, which was shake-cultured for 4 days at 27°C.

The culture broth (6 liters) was mixed with an equal volume of a mixture of *n*-butanol and methanol (1: 1) and filtered at pH 5.0. The filtrate was evaporated to a nearly aqueous solution, from which the antibiotics were adsorbed on an Amberlite IRC-50 (Na) column. The column was washed with water and eluted with 0.5 N HCl. That portion of the eluate exhibiting activity by paper disk agar diffusion method on an assay plate seeded with *Escherichia coli* JC-2 was extracted with *n*-butanol at pH 8.0. The *n*-butanol extract was washed with diluted sodium bicarbonate solution and water. The antibiotic principle was then extracted with acidified water (pH 2.0 by HCl) from the *n*-butanol solution and re-extracted with *n*-butanol at pH 8.0. After washing with water, the *n*-butanol extract was concentrated to dryness under reduced pressure to give 1.34 g of a crude powder.

The crude powder was preparatively chromatographed on silica gel plates (Merck, Silica Gel GF_{254}, 500 μ) with chloroform - ethanol - 14% ammoniacal water (4: 7: 2). Two separated zones of antibiotics (I: Rf *ca.* 0.25 and II: Rf *ca.* 0.13) were detected by bioautography on an assay plate seeded with *E. coli* and ninhydrin coloration. Zone I was extracted with slightly acidified 50% aqueous methanol (pH 2.0, by HCl). The extract was evaporated to nearly aqueous solution, from which the antibiotic was transferred to *n*-butanol at pH 8.0. The *n*-butanol solution was washed with water, and after slightly acidified concentrated to dryness, giving 380 mg of a powder. It was then subjected to Sephadex LH-20 column (2.5 × 100 cm), developed with 40% aqueous methanol. The active eluate fractions (to *E. coli*) were collected, evaporated and finally lyophilized. The resulting powder was then dissolved into a small amount of methanol and slightly acidified with HCl. By addition of acetone to the solution, octapeptin D trihydrochloride was precipitated as a colorless powder (280 mg).

Characterization of Octapeptin D

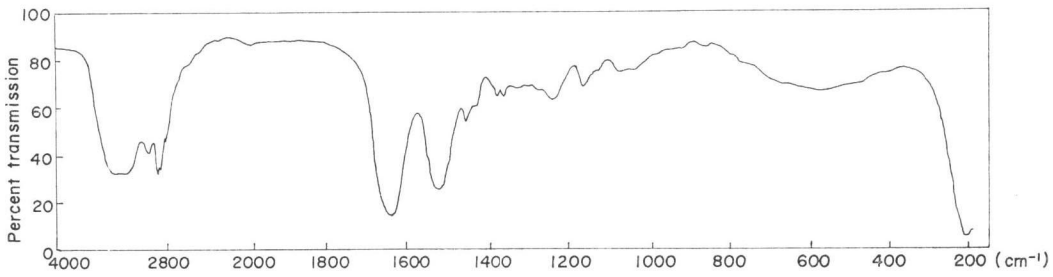
Octapeptin D trihydrochloride obtained as a colorless amorphous powder melts with decomposition at about 216~225°C. Elemental analysis indicated an empirical formula of $C_{47}H_{88}N_{12}O_{11} \cdot 3HCl \cdot H_2O$.

Anal. Found: C, 50.38; H, 8.42; N, 14.25; Cl, 9.53.
Calcd.: C, 50.19; H, 8.33, N, 14.94; Cl, 9.61.

The hydrochloride is soluble in water and methanol, and insoluble in acetone, ethyl acetate and chloroform. It gives positive reaction to ninhydrin reagent, but negative to SAKAGUCHI, PAULY, EHRLICH, BENEDICT and ferric chloride reagents. It shows a specific rotation: $[\alpha]_D^{22} -41.7 \pm 1.5°$ (*c* 0.549, 0.5 N HCl). In the ultraviolet absorption spectrum measured in methanol, only end absorption was observed. The strong absorption bands at 1650 cm^{-1} and 1540 cm^{-1} in the infrared absorption spectrum (Fig. 1) indicated that this substance is a peptide.

Octapeptin D trihydrochloride was hydrolyzed with constant boiling hydrochloric acid at 110°C for either 20 hours or 40 hours. The results of analysis with an automatic amino acid analyzer, Hitachi KLA-5 (Table 1), indicated that the constituent amino acids on a molar basis are 2,4-diaminobutyric acid (4 moles), serine (1 mole) and leucine (3 moles). The hydrochloride was also hydrolyzed with

Fig. 1. Infrared absorption spectrum of octapeptin D trihydrochloride (KBr).



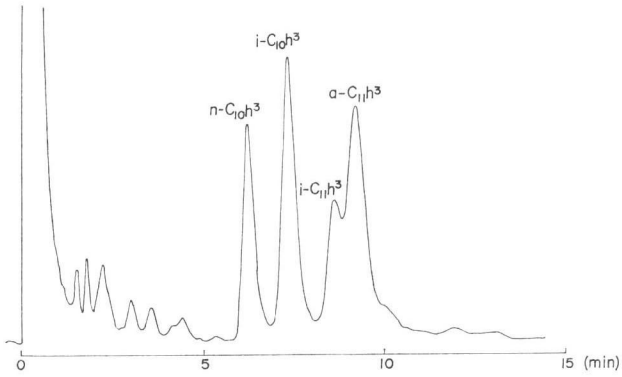
constant boiling hydrochloric acid at 110°C for 1 hour and the hydrolyzate was extracted with ethyl ether. Then the ethereal extract was methylated with diazomethane and analyzed with gas chromatography. Methyl esters of four β-hydroxy fatty acids: β-hydroxy isodecanoic acid, β-hydroxy decanoic acid, β-hydroxy isoundecanoic acid and β-hydroxy anteisoundecanoic acid, were identified by comparison with the respective specimens prepared in our previous studies[4,5] (Fig. 2).

Table 1. Amino acid analysis of octapeptin D trihydrochloride.

	Found (μ moles/mg)		
	Dab*	Ser	Leu
20 hours hydrolyzate	3.51	0.84	2.63
40 hours hydrolyzates	3.14	0.69	2.40
(moles/mole)	(4)	(1)	(3)

* Dab: 2,4-diaminobutyric acid.

Fig. 2. Gas chromatogram of fatty acid methyl esters from octapeptin D trihydrochloride.
Column: 15% DEGS, 3.2 mm × 1.6 m
Carrier gas: N_2, 40 ml/min
Temperature: 150°C



Octapeptin D is active against Gram-negative and Gram-positive bacteria *in vitro* as shown in the antimicrobial spectrum obtained by the usual agar dilution method (Table 2). It exhibits a higher order of activity against Gram-negative bacteria than against Gram-positive bacteria. When octapeptin D was administered by two subcutaneous doses, given 1 and 5 hours postinfection, to ICR mice infected with *E. coli* EC-14, a therapeutic effect: ED_{50} 5.0 mg/kg × 2, was observed.

Acute toxicity to the mice by intraperitoneal route was: LD_{50} 10 ~ 50 mg/kg.

Consideration

From the above properties and the constituent amino acids and fatty acids, it is clear that octapeptin D is a new member of the octapeptin group of antibiotics[2]. Octapeptin A, B complex (EM 49)[6,7] and octapeptin C[8,9], are produced by microorganisms belonging to *Bacillus circulans*, whereas octa-

peptin D is produced by a strain of *Bacillus* related to both *B. circulans* and *B. brevis*. From the constituents described above, octapeptin D is conjectured to be a complex of four acyl octapeptides differing in the identity of the fatty acyl residue. Separation of these four acylpeptide components and determination of their structures are presented in the accompanying publication[10].

Table 2. Antimicrobial spectrum of octapeptin D.

Organism	MIC (mcg/ml)
Escherichia coli NIHJ JC-2	6.25
Escherichia coli EC-14	12.5
Klebsiella pneumoniae	6.25
Pseudomonas aeruginosa	12.5
Salmonella typhimurium	6.25
Shigella sonnei	3.13
Staphylococcus aureus 209P JC-1	50
Staph. aureus Smith	50
Staph. epidermidis TB-548	25
Bacillus subtilis PCI 219	6.25

Obtained by the usual agar dilution method.

References

1) SHOJI, J.; T. KATO, S. TERABE & R. KONAKA: Resolution of peptide antibiotics, cerexins and tridecaptins, by high performance liquid chromatography. (Studies on antibiotics from the genus *Bacilus*. XXVI). J. Antibiotics 32: 313~319, 1979
2) MEYERS, E.; W. L. PARKER, W. E. BROWN, J. SHOJI & Y. WAKISAKA: A nomenclature proposal for the octapeptin antibiotics. J. Antibiotics 29: 1241~1242, 1976
3) BUCHANAN, R. E. & N. N. GIBBONS *ed*: BERGEY's Manual of Determinative Bacteriology 8th, The Williams & Wilkins Company, Baltimore, U.S.A., 1974
4) SHOJI, J. & T. KATO: The structure of cerexin B. (Studies on antibiotics from the genus *Bacillus*. XVII). J. Antibiotics 29: 1275~1280, 1976
5) SHOJI, J.; T. KATO, K. MATSUMOTO, Y. TAKAHASHI & M. MAYAMA: Production and isolation of cerexins C and D. (Studies on antibiotics from the genus *Bacillus*. XVIII). J. Antibiotics 29: 1281~1285, 1976
6) MEYERS, E.; W. E. BROWN, P. A. PRINCIPE, M. L. RATHNUM & W. L. PARKER: EM 49, a new peptide antibiotic. I. Fermentation, isolation and preliminary characterization. J. Antibiotics 26: 444~448, 1973
7) PARKER, W. L. & M. L. RATHNUM: EM 49, a new peptide antibiotic. IV. The structure of EM 49. J. Antibiotics 28: 379~389, 1975
8) SHOJI, J.; H. HINOO, Y. WAKISAKA, K. KOIZUMI, M. MAYAMA, S. MATSUURA & K. MATSUMOTO: Isolation of a new antibiotic 333-25, related to antibiotic EM 49. (Studies on antibiotics from the genus *Bacillus*. XI). J. Antibiotics 29: 516~520, 1976
9) KATO, T. & J. SHOJI: The amino acid sequence of octapeptin C_1 (333-25). (Studies on antibiotics from the genus *Bacillus*. XIX). J. Antibiotics 29: 1339~1340, 1976
10) KATO, T. & J. SHOJI: The structure of octapeptin D. (Studies on antibiotics from the genus *Bacillus*. XXVIII). J. Antibiotics 33: 186~191, 1980