ISOLATION OF A NEW PEPTIDE ANTIBIOTIC COMPLEX 61-26

STUDIES ON ANTIBIOTICS FROM THE GENEUS *BACILLUS*. V

JUN'ICHI SHOJI, RYUZI SAKAZAKI, YOSHIHARU WAKISAKA,
KENZO KOIZUMI and MIKAO MAYAMA

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

(Received for publication July 29, 1974)

A new antibiotic named 61-26 active against gram-positive bacteria and some fungi was isolated from a *Bacillus* strain. The antibiotic is a weakly basic peptide slightly soluble in aqueous alcohols. An approximate empirical formula of $C_{50}H_{93}N_{11}O_{17}$ and constituent amino acids of aspartic acid (1 mole), serine (2 moles), alanine (2 moles), and sum of valine and isoleucine (2 moles) are indicated.

In the course of our screening program for new antibiotics from the genus *Bacillus*,[1] a *Bacillus* strain 61-26, which was isolated from a soil sample collected in New Guinea, was found to produce a new antibiotic. The antibiotic named 61-26 was extracted with *n*-butanol from the culture broth of the strain and purified by TLC on silica gel with *n*-butanol-acetic acid-water (4 : 1 : 2).

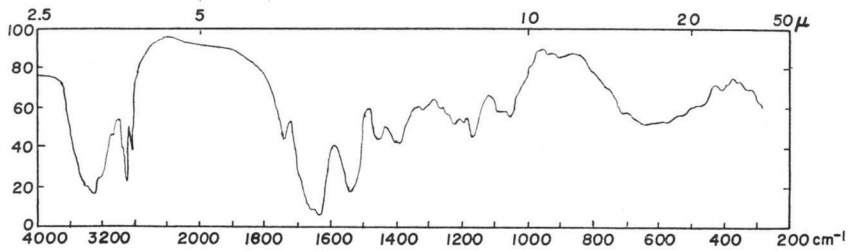
A weakly basic nature of the antibiotic was indicated by paper electrophoresis. The free base was obtained as a colorless amorphous powder, m.p. 170~180°C (dec.), and the hydrochloride as a colorless amorphous powder, m.p. 193~230°C (dec.). Elemental analyses indicated an approximate empirical formula of $C_{50}H_{93}N_{11}O_{17}$ for the free base.

The hydrochloride is soluble in pyridine and dimethylsulfoxide, slightly soluble in methanol, ethanol and aqueous butanol, but insoluble in other organic solvents and water. It is positive to DRAGENDORFF reagent, but negative to ninhydrin reaction.

The hydrochloride shows optical activity: $[\alpha]_D^{24°}+51.0\pm1.9°$ (*c* 0.494, dimethylsulfoxide). Only an end absorption was observed in the ultraviolet absorption spectrum measured in methanol. The infrared absorption spectrum (Fig. 1) indicated this antibiotic to be a peptide, possibly containing a lactone or ester linkage.

Amino acid analysis with the acid hydrolyzate of this antibiotic indicated the presence of aspartic acid (1 mole), serine (2 moles), alanine (2 moles), valine (*ca.* 1.4 moles) and isoleucine (*ca.* 0.6 moles), and a molecular weight of approximately 1,200. The sum of

Fig. 1. Infrared absorption spectrum of antibiotic 61-26 (KBr).



valine and isoleucine was shown to be 2 moles in several analyses, suggesting that the antibiotic preparations were not homogeneous in the parts of valine and isoleucine residues, but a complex of homologous peptides.[2)]

No fatty acid was detected by means of GLC with the ethereal extract of the hydrolyzate. The remaining portion of the antibiotic other than peptide part is yet obscure.

The antibiotic 61-26 is active against gram-positive bacteria and some yeasts and fungi (Table 1). Toxicity to mice was observed at a dose of 50 mg/kg intraperitoneally. Some effect against sheath blight of rice plant was observed in a pot test.

Table 1. Antimicrobial spectrum of antibiotic 61-26

Test organism	MIC (mcg/ml)	Test organism	MIC (mcg/ml)
Bacillus subtilis PCI 219	6.25	*Pseudomonas aeruginosa* Ps-24	>50
Bacillus anthracis	3.13	*Mycobacterium tuberculosis* $H_{37}Rv$	>50
Staphylococcus aureus FDA 209P JC-1	3.13	*Candida albicans* M-9	12.5
Staphylococcus aureus Smith	3.13	*Trichophyton rubrum*	>50
Diplococcus pneumoniae type I	12.5	*Trichophyton mentagrophytes*	>50
Streptococcus pyogenes C-203	6.25	*Trichophyton purpureum*	>50
Escherichia coli NIHJ JC-2	>50	*Epidermophyton floccosum*	12.5
Klebsiella pneumoniae	>50	*Microsporum gypseum*	25
Salmonella typhimurium	>50		

Obtained by the usual agar dilution method

Some sixty members of peptide antibiotics isolated from the genus *Bacillus* have been reported up to the present time. On comparison of the antibiotic complex 61-26 with the above members, it is evident that none is identical or similar in species of constituent amino acids. Thus, the antibiotic is concluded to be new.

Experimental

Fermentation

Spores of the strain 61-26 were inoculated into 120 ml of a medium consisting of peptone 1.0%, meat extract 0.5% and sodium chloride 0.3%, pH 7.0, in a 500-ml shake flask, and shake-cultured for 18 hours at 27°C. About 4 ml of the culture was then transferred to a flask containing 130 ml of a medium containing glucose 1.0%, glycerine 0.25%, soybean meal 1.0%, peptone 0.25%, and sodium chloride 0.3%, pH 7.0. Fermentation was carried out for 6 days at 27°C on a reciprocal shaker.

Isolation and Purification

About 5 liters of the culture broth were adjusted to pH 4.0 with hydrochloric acid, mixed with an equal volume of a mixture of *n*-butanol and methanol (1 : 1), and filtered. The filtrate was evaporated under reduced pressure and extracted with *n*-butanol at pH 8.0 three times. The *n*-butanol extracts were combined and washed with 2% sodium bicarbonate, water, dilute hydrochloric acid and water successively. It was then concentrated to a small volume, to which ethyl acetate was added to precipitate a crude powder (1.2 g).

The crude material was applied to silica gel plates (Silica gel GF, thickness 750 μ, 20 $\times$100 cm) and developed with *n*-butanol-acetic acid-water (4 : 1 : 2). The zone of the antibiotic was visualized by iodine and extracted with slightly acidified 50% aqueous methanol containing 5% chloroform. The extract was neutralized to pH 5.0 with dilute ammonium

hydroxide, concentrated to a nearly aqueous solution, from which the antibiotic was transferred to *n*-butanol at pH 7.0. The *n*-butanol solution was washed with water, concentrated to a small volume, from which the antibiotic free base was precipitated as a colorless amorphous powder (200 mg) by addition of acetone.

Anal. Found: C, 52.58; H, 8.27; N, 13.71; MW, 1150 (Osmometry in pyridine)
Calcd. for $C_{50}H_{93}N_{11}O_{17}\cdot H_2O$: C, 52.75; H, 8.41; N, 13.54; MW, 1138.35.
No sulfur was found.

The free base was dissolved into methanol, concentrated and slightly acidified with hydrochloric acid. Hydrochloride of the antibiotic was obtained as a colorless amorphous powder by precipitating and washing with acetone.

Anal. Found: C, 52.19; H, 7.98; N, 14.05; Cl, 3.35
Calcd. for $C_{50}H_{93}N_{11}O_{17}\cdot HCl$: C, 51.91; H, 8.19; N, 13.32; Cl, 3.07

Amino Acid Analysis

The antibiotic 61-26 was hydrolyzed with constant-boiling hydrochloric acid at 110°C for 48 hours. By a Hitachi Automatic amino acid analyzer, Model KLA-5, the following amino acids (μmoles per mg of the antibiotic) were found: aspartic acid (0.84), serine (1.43), alanine (1.57), valine (1.07), isoleucine (0.51) and ammonia (1.01).

Acknowledgement

The authors are grateful to Dr. S. MATSUURA of this Laboratory for his *in vivo* assay studies and Dr. K. WATANABE for his pot test with the antibiotic 61-26.

References

1) SHOJI, J.; H. HINOO, Y. WAKISAKA, K. KOIZUMI & M. MAYAMA: Isolation of a new peptide antibiotic TL-119. Studies on antibiotics from the genus *Bacillus*. IV. J. Antibiotics 28: 126~128, 1975
2) SHOJI, J. & R. SAKAZAKI: A new peptide antibiotic complex S-520. II. Further characterization and degradative studies. J. Antibiotics 23: 432~436, 1970