ISOLATION OF TRIDECAPTINS A, B AND C

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

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Three new antibiotics, tridecaptins A, B and C, were isolated from culture broths of strains of *Bacillus polymyxa* AR-110, B-2 and E-23, respectively. All are acyl tridecapeptides differing from each other in the fatty acid components and amino acid residues. They are weakly active against Gram-negative and Gram-positive bacteria *in vitro* and *in vivo*.

While screening for new antibiotics from the genus *Bacillus*, several new antibiotic substances belonging to a new class of antibiotics were found in culture broths of strains of *Bacillus polymyxa*. As all are acyl tridecapeptides, "tridecaptin" is proposed as the class name for these antibiotics. To distinguish individual members, a capital letter is assigned in order of discovery. Tridecaptins B and C are complexes requiring resolution. When the individual components are separated appropriate suffixes will be assigned.

In this report, the production, isolation and primary characterization of tridecaptins A, B and C are presented.

The Producing Organisms

Strain AR-110 produces tridecaptin A, strain B-2 tridecaptin B, and strain E-23 tridecaptin C. The three strains are closely related to each other, and are judged to belong to *Bacillus polymyxa* based on taxonomic studies. The taxonomic characteristics of these strains are as presented for strain E-23.

A. Morphology

(1) Vegetative cells (30°C, 1~3 days): Gram-positive rods on Gly-IM-agar* are 0.7~0.9× 2.0~5.5 μ with rounded ends. The cells occur singly or in a mass and are motile.

(2) Spores and sporangia (30°C, 1~7 days): Spores on Gly-IM agar are 1.0~1.2×1.5~2.0 μ, easily stainable, elliptical, central to subterminal. Sporangia are definitely swollen.

B. Cultural Characteristics

(1) Colony on Gly-IM-agar (30°C, 1~3 days): Circular, convex, entire, smooth, glistening, gummy, translucent to opaque.

(2) Gly-IM-agar slant (30°C, 1~7 days): Growth moderate, beaded to echinulate, whitish gray, surface shiny (at 1 day) changing to dull after 2 days. Gummy consistency and opaque density. Diffusible and non-diffusible pigments are not observed.

* Gly-IM-agar: Glycerol 0.5%, peptone 0.25%, beef extract 0.25%, yeast extract 0.25%, NaCl 0.3%, agar 1.25%, pH 6.8.

(3) Liquid culturing (NAM-medium*, 30°C, 1～3 days): Uniform, significant growth. No pellicle formation is observed.

C. Physiological Characters

(1) Relation to oxygen (30°C, 1～5 days): OF-test on S-medium agar** stab is facultative anaerobic. Acid and gas are produced from glucose.

(2) Temperature relation (MV-medium***, 1 day): Optimum is approximately 32～37°C. No growth was observed at 11°C or 45°C.

(3) Starch hydrolysis (30°C, 1～3 days): Positive.

(4) Gelatin stab (30°C, 1～7 days): Liquefied.

(5) Nitrate reduction to nitrite (30°C, 2～4 days): Positive.

(6) Litmus milk (37°C, 1～7 days): Peptonized slowly. Decoloration is observed.

(7) Acetylmethylcarbinol production (28°C, 2 days): Positive.

(8) H_2S formation (Difco peptone iron agar, 30°C, 1～7 days): Negative.

(9) Carbohydrate cleavage (30°C, 1～7 days): Acid formation is observed from L-arabinose, D-xylose, D-mannitol and D-glucose.

Essentially the same results were obtained with other two strains AR–110 and B–2. It was concluded that the three strains should be classified as *Bacillus polymyxa*[2,3].

Production and Isolation

Tridecaptin A

Spores of strain AR–110 were inoculated into 100 ml of a medium consisting of glucose 1.0%, peptone 0.5%, meat extract 0.5% and sodium chloride 0.1%, pH 7.0, in a SAKAGUCHI flask, and cultured for one day at 27°C on a reciprocal shaker. About 4 ml of the culture was transferred into 100 ml of a medium consisting of soluble starch 2.0%, Soytone (Difco) 3.0%, $MgSO_4 \cdot 7H_2O$ 0.2%, and $CaCO_3$ 1.0%, pH 7.0, in a SAKAGUCHI flask. Fermentation was carried out for one day at 27°C on a reciprocal shaker.

About 5 liters of the culture broth obtained by the above procedure was adjusted to pH 2.0 with HCl and after stirring with 2.5 liters each of butanol and methanol, it was filtered. The filtrate was evaporated under reduced pressure to a nearly aqueous solution and then extracted with *n*-butanol twice. The *n*-butanol extract was washed with dilute $NaHCO_3$ solution and then dilute HCl solution. Concentration to a small volume resulted in precipitation of a gel-like precipitate. The precipitate was collected by centrifugation and washed with ethanol to give a crude powder (4.0 g, content: approx. 70%).

About 500 mg of the crude powder was subjected to a column of Sephadex LH–20, which was developed with 50% aqueous methanol. The antibiotic was slightly retarded by the column and eluted shortly after the elution of excess chloride ion. Lyophilization of the active eluate gave hydrochloric acid salt of tridecaptin A (250 mg).

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* NAM-medium: Peptone 0.5%, beef extract 0.3%, yeast extract 0.2%, KNO_3 0.2%, pH 6.8.

** S-medium: Glucose 1.0%, peptone 0.5%, beef extract 0.3%, yeast extract 0.2%, NaCl 0.3%, bromcresol purple 0.008%, agar 0.4%, pH 6.8.

*** MV-medium: Soluble starch 2.0%, glycerol 0.5%, Difco soytone 1.5%, corn steep liquor 0.5%, NaCl 0.3%, agar 1.5%, pH 6.8.

Tridecaptin B

About 10 liters of the culture broth of the strain B–2 in the same medium as in tridecaptin A was filtered in the same manner as above. The filtrate was evaporated and then extracted with *n*-butanol at pH 2.0. The butanol extract was washed with 0.1 M KCl-HCl buffer, pH 2.0, and then dilute $NaHCO_3$ solution. Then the antibiotic was extracted with 0.1 N NaOH, and the aqueous extract was promptly adjusted to pH 8.0 from which the antibiotic was transferred to a small volume of butanol. When the butanol solution was repeatedly extracted with water adjusted to pH 3.0 with HCl, the antibiotic was transferred to the aqueous phase. The aqueous solution was extracted with butanol at pH 8.0, which was concentrated to dryness to afford a crude mixture of tridecaptin B (*ca.* 2.5 g) containing other antibiotic components of the tridecaptin group.

A 200-mg portion of the crude mixture was separated by droplet counter-current chromatography (DCC) with solvent system of *n*-butanol - acetic acid - water (3: 1: 5), using the lower phase as the moving solvent. Tridecaptin B moved faster and was separated from the other antibiotic components. Lyophilization of the tridecaptin B fraction gave a colorless powder (120 mg), which was then purified by preparative paper chromatography on Toyo Roshi No. 51 with *n*-butanol - pyridine - acetic acid - water (10: 6: 1: 4). The zone of the antibiotic was cut out and extracted with 50% aqueous methanol slightly acidified with HCl. Concentration and precipitation with acetone gave a colorless powder of hydrochloric acid salt of tridecaptin B (70 mg).

Tridecaptin C

In this case another medium was used that contained soluble starch 0.25%, glucose 1.0%, glycerine 0.25%, soy bean meal 1.0%, corn steep liquor 0.5%, yeast extract 0.1%, NaCl 0.1%, $CaCO_3$ 0.1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $MnSO_4$ 0.001% and $Fe_2(SO_4)_3$ 0.001%, pH 7.0.

About 6 liters of the culture broth of the strain E–23 was processed in essentially the same manner as for tridecaptin A and 90 mg of the hydrochloric acid salt of tridecaptin C was obtained.

Physico-chemical Properties

The preparations of tridecaptins A, B and C obtained as above showed a single spot on thin-layer chromatography and paper chromatography with Rf values listed in Table 1.

The hydrochloric acid salts of tridecaptins A, B and C are colorless amorphous powders. The decomposing points and the elemental analytical data are listed in Table 2. They are soluble in dilute alkaline and acid water, but not in neutralized water, partially soluble in aqueous alcohols, and insoluble in acetone, ethyl acetate, chloroform and ethyl ether. They are positive to ninhydrin and EHRLICH reactions, decolorlize $KMnO_4$ solution and are negative to SAKAGUCHI and PAULI reactions. Their $[\alpha]_D$ values and ultraviolet absorptions are shown in Table 3. The IR spectra, typical of peptides, are presented in Fig. 1.

Tridecaptins A, B and C were hydrolyzed

Table 1. Approximate Rf values of tridecaptins A, B and C.

	Solvent	A	B	C
TLC	I	0.10	0.05	0.10
	II	0.47	0.34	0.41
PC	III	0.65	0.60	0.60
	IV	0.34	0.20	0.25

TLC: Precoated silica gel plate (Merck).
PC: Toyo Roshi No. 51.
I: $CHCl_3$ - EtOH - 14% NH_4OH (4: 7: 2)
II: *i*-PrOH - 14% NH_4OH (2: 1).
III: BuOH - AcOH - H_2O (4: 1: 2).
IV: BuOH - pyridine - AcOH - H_2O (10: 6: 1: 4).

Table 2. Decomposing point and elemental analysis.

Hydrochloric acid salt of tridecaptin A	Colorless amorphous powder, d.p. 192∼197°C Anal. Found: C, 52.07; H, 7.03; N, 13.69; Cl, 6.73 Calcd. for $C_{73}H_{115}N_{17}O_{20}\cdot 3HCl\cdot H_2O$: C, 52.24; H, 7.21; N, 14.19; Cl, 6.34
Hydrochloric acid salt of tridecaptin B	Colorless amorphous powder, d.p. 191∼199°C Anal. Found: C, 48.06; H, 6.98; N, 14.09; Cl, 6.53 Calcd. for $C_{67}H_{111}N_{17}O_{20}\cdot 3HCl\cdot 5H_2O$: C, 48.06; H, 7.47; N, 14.23; Cl, 6.35
Hydrochloric acid salt of tridecaptin C	Colorless amorphous powder, d.p. 190∼197°C Anal. Found: C, 50.52; H, 7.15; N, 13.40; Cl, 5.84 Calcd. for $C_{74}H_{117}N_{17}O_{21}\cdot 3HCl\cdot 4H_2O$ C, 50.43; H, 7.32; N, 13.51; Cl, 6.03

Table 3. Optical rotation and ultraviolet absorption of tridecaptins A, B and C.

	A	B	C
$[\alpha]_D$ in MeOH - AcOH (1 : 1)	$-3.5\pm1.4°$	$-7.7\pm5.2°$	$-3.0\pm1.4°$
$\lambda_{max}^{H_2O}$: 274 nm	32($E_{1cm}^{1\%}$)	26($E_{1cm}^{1\%}$)	25($E_{1cm}^{1\%}$)
282 nm	34($E_{1cm}^{1\%}$)	26($E_{1cm}^{1\%}$)	27($E_{1cm}^{1\%}$)
289 nm	30($E_{1cm}^{1\%}$)	19($E_{1cm}^{1\%}$)	23($E_{1cm}^{1\%}$)

Fig. 1. Infrared absorption spectra of hydrochloric acid salts of tridecaptins A, B and C (KBr).

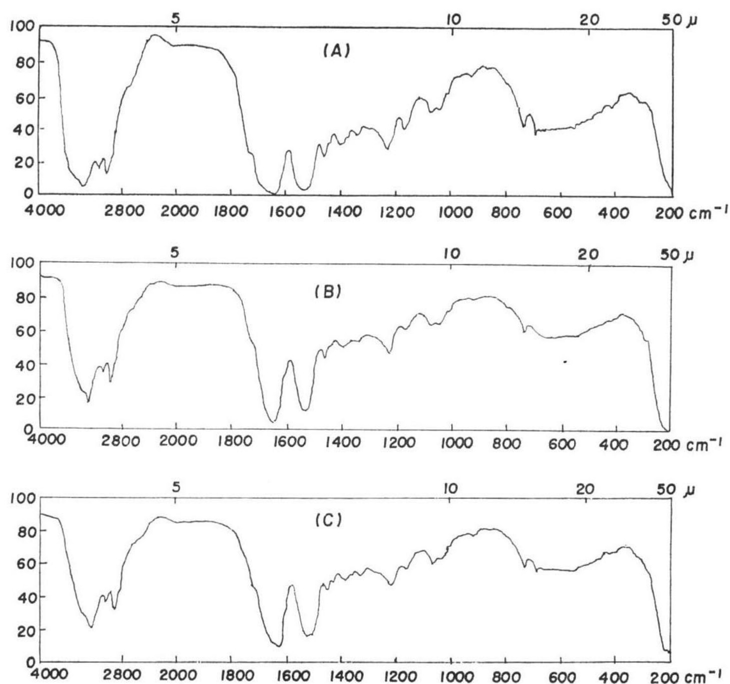

Table 4. Amino acid analysis of tridecaptins A, B and C.

	Ser	Glu	Gly	Ala	Val	aIle	Ile	Phe	Trp	Dab	Amm
Tridecaptin A											
(μmoles/mg)	0.88	0.59	0.57	0.52	1.11	0.43		0.52	0.43	1.78	0.22
(moles/mole)	(2)	(1)	(1)	(1)	(2)	(1)		(1)	(1)	(3)	
Tridecaptin B											
(μmoles/mg)	1.35	0.58	1.10		0.88	0.35	0.39		0.47	1.76	0.27
(moles/mole)	(3)	(1)	(2)			(3)			(1)	(3)	
Tridecaptin C											
(μmoles/mg)	1.29	0.59	0.62		1.35	0.12		0.47	0.61	1.61	0.44
(moles/mole)	(3)	(1)	(1)		(3)			(1)	(1)	(3)	

with constant boiling hydrochloric acid containing 4% thioglycolic acid at 110°C for 48 hours. The hydrolyzates were analyzed with an automatic amino acid analyzer Hitachi KLA–5. The amino acids found are listed in Table 4. This means that they are tridecapeptides, tridecaptin A is a single entity but tridecaptin B is heterogeneous with respect to Val, aIle and Ile, and tridecaptin C is also heterogeneous with respect to Val and aIle.

The ethereal extracts of the hydrolyzates were methylated and analyzed with gas chromatography. β-Hydroxy anteisononanoic acid was detected from tridecaptin A, anteisononanoic acid from tridecaptin B, and β-hydroxy isodecanoic acid and β-hydroxy anteisoundecanoic acid from tridecaptin C. These fatty acids have already been reported as constituents of acyl peptide antibiotics originated from the genus *Bacillus* in our previous papers[4,5,6]. The detailed data of the identification will be presented in succeeding reports on structural studies[7].

Biological Properties

Tridecaptins A, B and C are active against Gram-negative and Gram-positive bacteria as shown

Table 5. Antimicrobial spectra of tridecaptins A, B and C.

Test organism	MIC (mcg/ml)		
	A	B	C
Bacillus subtilis PCI 219	12.5	12.5	6.25
Staphylococcus aureus FDA 209P JC–1	50	25	12.5
Staphylococcus aureus Smith	50	25	12.5
Staphylococcus aureus 80257	50	25	12.5
Streptococcus pyogenes C–203	50	25	12.5
Diplococcus pneumoniae	>50	50	25
Escherichia coli NIHJ JC–2	6.25	12.5	6.25
Escherichia coli EC–14	3.13	6.25	6.25
Escherichia coli 80750	6.25	6.25	3.13
Klebsiella pneumoniae	6.25	12.5	6.25
Salmonella typhimurium	6.25	12.5	6.25
Pseudomonas aeruginosa Ps–24	50	50	25
Proteus vulgaris CN–329	50	>50	>50
Proteus mirabilis PR–4	>50	>50	>50

Obtained by the usual agar dilution method.

Table 6. Therapeutic efficacy and toxicity in ICR mice.

	ED_{50} (mg/kg×2, sc)*		LD_{50} (mg/kg, ip)
	E. coli EC–14	*K. pneumoniae*	
Tridecaptin A	0.41	5.5	*ca.* 25
Tridecaptin B	0.44	2.7	50~100
Tridecaptin C	1.4	17	50~100

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

in Table 5. The antibiotics showed also therapeutic effect to ICR mice infected with *Escherichia coli* or *Klebsiella pneumoniae* when administered subcutaneously. It should be noticed that the ED_{50} values and the LD_{50} values are different to some extent between these analogous antibiotics (Table 6).

Discussion

A number of peptide antibiotics have been isolated from the genus *Bacillus*. Of these, none of structure similar to the tridecaptin antibiotics have been reported. Antibiotic BN–109[8] bears some resemblance to the tridecaptin antibiotics with respect to the amino acid residues but apparently differs in molecular size. BN–109 is assumed to be an acyl hexadecapeptide from the reported amino acid composition. Mycobacillin[9] is a tridecapeptide, but it has a non-acylated cyclic structure. Consequently we consider the tridecaptin antibiotics to be a new class of antibiotics from the genus *Bacillus*.

While tridecaptin A is a single entity, tridecaptin B is a complex of acyl peptides; it is heterogeneous with respect to Val, aIle and Ile residues. Tridecaptin C is also a complex of acyl peptides differing in both the fatty acyl residue as well as the peptide portion. Further resolution of these peptide complexes is now under our investigation.

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