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Tripropeptins, Novel Antimicrobial Agents Produced by *Lysobacter* sp.

I. Taxonomy, Isolation and Biological Activities

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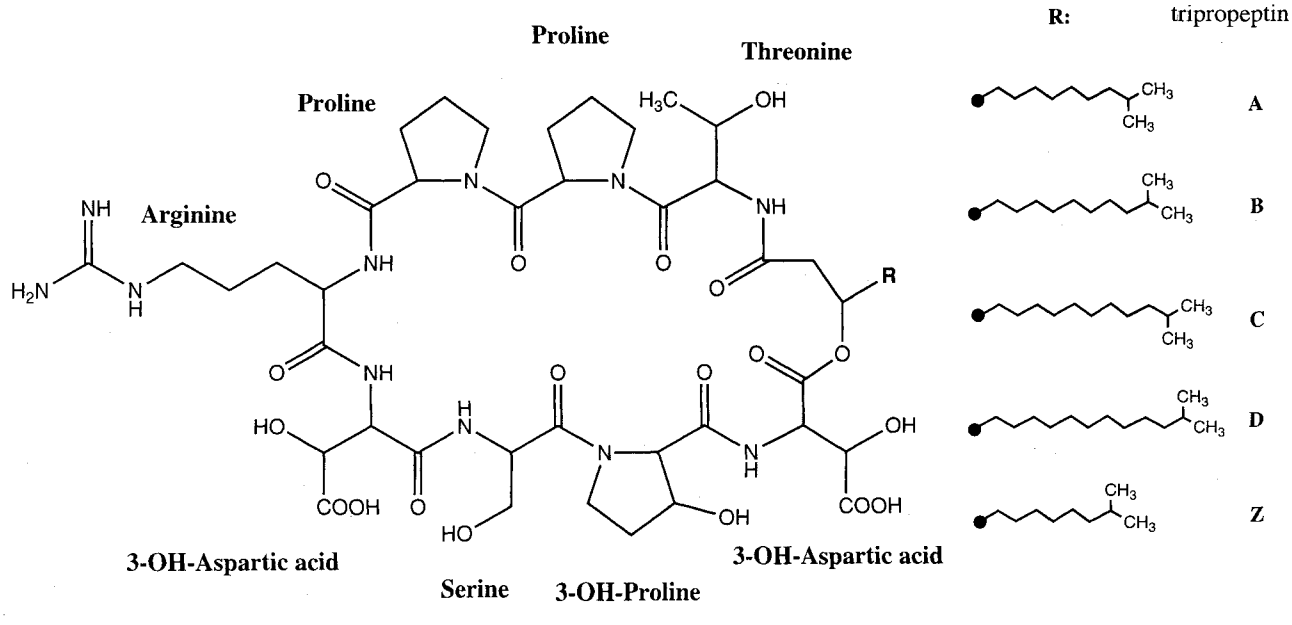
Peptide antibiotics tripropeptins A, B, C, D and Z were isolated from cultured cells and broth of *Lysobacter* sp. The differences among these components are in the lengths of the alkyl side chain. Tripropeptins are active against Gram-positive bacteria including MRSA *in vitro*. Bactericidal activity of tripropeptin C disappeared in the simultaneous presence of chloramphenicol, a bacteriostatic agent.

In our screening program for new antibiotics that are active against drug-resistant bacteria, we discovered novel compounds, tripropeptins (Fig. 1), produced by a species of *Lysobacter*[1,2] (BMK333-48F3). These antibiotics were isolated from *Lysobacter* cultures grown at 27°C for 2 days. We describe here the taxonomy of the producing organism, fermentation conditions, isolation procedures and some characteristics of the antibiotics. Structural studies will be reported in papers to follow.

Fig. 1. Structure of tripropeptins.



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Materials and Methods

The Antibiotic-producing Organism

The antibiotic-producing organism was isolated from a soil sample collected at Naha, Okinawa prefecture, Japan, and was deposited at the National Institute of Advanced Industrial Science and Technology, under the accession number of FERM BP-7477.

Fermentation

A seed culture of the tripropeptins-producing organism was prepared by transferring a loopful of surface growth from an agar slant into a 500-ml volume baffled Erlenmeyer flask containing 110 ml of a culture medium consisting of 1.5% glycerol, 1.5% cotton seed meal, 0.3% NaCl, 0.5% sodium L-glutamate monohydrate and deionized water to volume (pH 7.4 before sterilization). The inoculated medium was incubated on a rotary shaker (180 rpm) at 27°C for 1 day (seed culture). A 2% transfer was made from the seed culture to 450 Erlenmeyer flasks of the same model, each containing 110 ml of the same medium. The inoculated medium (in the 450 flasks) was incubated likewise for 2 days. The contents of the flasks were combined (fermentation broth) and submitted to isolation procedures of the antibiotics.

Antibacterial Activity

Antibacterial activities of the fractions containing tripropeptins were assayed using a cup or paper-disk diffusion method against *Staphylococcus aureus* Smith (*S. aureus* Smith) and *Micrococcus luteus* IFO3333. Minimum inhibitory concentrations (MICs) of tripropeptins against a wide range of bacteria and yeast were determined using the serial agar dilution method; Mueller Hinton agar (Difco) was used for bacteria except *Enterococci* for which Mueller Hinton agar (Difco) containing 5% sheep blood was used.

Bactericidal Activity of Tripropeptin C

Four tenth ml of a seed culture of *S. aureus* Smith in a nutrient medium was transferred to L-shaped tubes, each containing 8 ml of the same medium, and incubated at 37°C with shaking until an optical density (OD) at 600 nm (using a COLEMAN spectrometer) reached approximately 0.1. Tripropeptin C and/or chloramphenicol, at appropriate dilutions, were added to the cultures (referred to as 0 minute). Incubation was continued and at 30, 60, 120 and 180 minutes of incubation, OD at 600 nm were measured and a 100 μl sample of each culture was taken and diluted with 9.9 ml of ice cold sterile saline. After serial 1/10 dilutions, 100 μl portions were spread on nutrient agar plates, which were incubated at 37°C for 18 hours, and numbers of colonies (CFU) were counted.

Results and Discussion

Taxonomy

Physiological characteristics of strain BMK333-48F3 are shown in Table 1. The organism is an aerobic, Gram-negative, non-sporulating rod (0.5~0.8 μm×1.6~2.0 μm) bacterium. It has no flagella and exhibits a spreading growth on the agar surface due to gliding motility. Fruit bodies are not produced. G+C mol% in DNA is 70.5%.

Optimum growth temperature ranges 24~30°C. Some acids were generated from glucose, D-fructose, maltose and trehalose, while gas was not generated. Neither acid nor gas was generated from D-xylose, D-mannose, D-galactose, sucrose, arabinose, D-sorbitol, D-mannitol, inositol, glycerol or starch.

From descriptions in the BERGEY's Manual of Systematic Bacteriology[2], these characteristics indicate that the organism belongs to the Genus *Lysobacter*.

Isolation of the Antibiotics

A fermentation broth (50 liters) was centrifuged and the cells were separated from the supernatant. The cells were extracted with 10 liters of methanol and the extract was concentrated *in vacuo* leaving an oily residue. The residue was combined with the supernatant and the mixed solution was applied to a Diaion HP20 column (Mitsubishi Chemical Co., 6 liters wet volume). The column was washed with 18 liters of deionized water and 18 liters of 50% aqueous methanol. Active principles were then eluted with 18 liters of 65% aqueous acetone. The aqueous acetone eluate was concentrated *in vacuo* to yield a brown oil (30 g), which was chromatographed using a silica gel column (1500 ml wet volume) developed successively with 4500 ml each of $CHCl_3$: MeOH : H_2O/10 : 5 : 1 and BuOH : MeOH : H_2O/4 : 1 : 2. Active fractions, eluted with the latter solvent mixture, were collected and concentrated *in vacuo* to give a yellowish brown oil (10.8 g). The oil was dissolved in a small volume of 50% aqueous methanol, adjusted to pH 2.6, and subjected to column chromatography using 250 ml wet volume of CHP20P (Mitsubishi Chemical Co.), eluted with stepwise gradients of acetone : H_2O (750 ml each of 4 : 16, 7 : 13, 8 : 12, 9 : 11, 10 : 10, 11 : 9 and 12 : 8 v/v). Active components (their designated numbers printed in boldface) and their dry weights and ratios of acetone : H_2O

Table 1. Taxonomic data of strain BMK333-48F3.

Characterization		Characterization	
Gram stain	-	Decomposition of glucose	+
Motility	-	D-mannose	-
Gliding	+	maltose	+
Fruit bodies	-	sucrose	-
G+C contents	70.5%	D-galactose	-
Peptonization of milk	+	D-fructose	+
Coagulation of milk	+	arabinose	-
Chitin degradation	+	inositol	-
Catalase	+	D-mannitol	-
Oxidase	+	trehalose	+
Urease	-	D-sorbitol	-
Indole test	-	D-xylose	-
H_2S test	-	cellobiose	-
Nitrate reduction	+	glycerol	-
Denitrification	-	starch	-
Methyl red test	-	salicin	-
Voges-Proskauer test	-	lactose	-
Citrate utilization	+	Acid fast	-
Hydrolysis of Casein	+	Pigments	+
Esclin	+	pH range for growth	6.0-9.0
Gelatin liquefaction	+	Temperature range for growth	20-37°C
O/F test	oxidation	Optimum growth temperature	24-30°C

(in parentheses) are as follows; **5** (65.7 mg, 8 : 12), **1** (189.7 mg, 9 : 11), **2** (210.6 mg, 9 : 11 and 10 : 10), **3** (986.6 mg, 9 : 11 and 10 : 10) and **4** (50.1 mg, 10 : 10, 11 : 9 and 12 : 8). Tripropeptins were successfully separated into these components using the CHP20P column chromatography as described above but thin-layer chromatographical methods with different solvent systems tested so far failed to separate them. Tripropeptins are soluble in methanol, water and dimethyl sulfoxide, while insoluble in acetone, ethyl acetate and chloroform.

Biological Activities

As shown in Table 2, tripropeptin C strongly inhibited growth of Gram-positive bacteria including MRSA, but was not active against Gram-negative bacteria, *Mycobacterium* and *Candida*.

Table 3 shows antimicrobial activities of tripropeptin A, B, C, D and Z and, for comparison, arbekacin, against Gram-positive and a few Gram-negative bacteria. Among tripropeptins, antimicrobial activities paralleled the lengths of side chains, the longer the more active. Vancomycin-resistant enterococci were as sensitive to tripropeptins as were the vancomycin-sensitive counterparts.

Tripropeptin C acted as a bactericidal agent against *S. aureus* Smith and the bactericidal activity was abolished by the simultaneous presence of chloramphenicol, as shown in Fig. 2. Tripropeptin C at 2.5 μg/ml was a marginal concentration to inhibit the increase in the turbidity of *S. aureus* Smith in a shaken culture (Fig. 2-a; results at other concentrations are not shown). Although the turbidity of the culture remained almost the same for 3 hours after the addition of tripropeptin C, numbers of viable cells (CFU) in the suspension dropped rapidly with incubation time (Fig. 2-b). Interestingly, the simultaneous presence of 2.5 μg/ml of chloramphenicol, a bacteriostatic agent, abolished the bactericidal activity of tripropeptin C (Fig. 2-b).

Tripropeptin C did not show an acute toxicity in mice at a dose of 300 mg/kg when administered intravenously.

Discussion

Tripropeptins are compared with known antibiotics of bacterial origin in view of structure and activity, as follows. Empedopeptin[3,4] produced by *Empedobacter* sp., another inhibitor of Gram-positive bacteria, has the same amino acid residues as those of tripropeptins, but the amino acid sequence of empedopeptin is different from that of tripropeptins. Furthermore, a tripropeptin has a branched

Table 2. Antimicrobial activities of tripropeptin C.

Test organisms	MIC(μg/ml)
Staphylococcus aureus FDA209P	0.78
S. aureus Smith	0.78
S. aureus MS9610	0.78
S. aureus No.5(MRSA)	0.78
S. aureus No.17(MRSA)	3.12
S. aureus MS16526(MRSA)	0.78
S. aureus TY-04282(MRSA)	1.56
Micrococcus luteus IFO3333	<0.2
M.luteus PCI1001	<0.2
Bacillus subtilis NRRL B-558	0.39
B. cereus ATCC10702	6.25
Corynebacterium bovis 1810	0.39
Escherichia coli NIHJ	>100
Shigella dysenteriae JS11910	>100
Salmonella enteritidis	>100
Proteus mirabilis IFM OM-9	>100
Serratia marcescens	>100
Pseudomonas aeruginosa A3	>100
Klebsiella pneumonie PCI602	>100
Mycobacterium smegmatis ATCC607[a]	100
Candida albicans 3147	>100
Enterococcus faecalis JCM 5803[b]	12.5
Enterococcus faecium JCM 5804[b]	12.5
E. faecalis NCTC 12201 (VCM R)[b]	6.25
E. faecium NCTC 12202 (VCM R)[b]	12.5
E. faecalis NCTC12203 (VCM R)[b]	25
E. faecium NCTC 12204 (VCM R)[b]	12.5

Mueller Hinton agar (Difco), 37°C 18 hours, except a and b.
a: 37°C 42 hours.
b: Mueller Hinton agar(Difco) + 5% Sheep blood, 37°C 18 hours

Table 3. Antimicrobial activities of tripropeptins.

test organism \ MIC(μg/ml)	tripropeptin					arbekacin	vancomycin	gentamicin
	A	B	C	D	Z			
Staphylococcus aureus FDA 209P	1.56	1.56	1.56	0.39	12.5	0.10	0.39	<0.2
S. aureus Smith	0.78	1.56	1.56	0.39	6.25	0.10	0.39	<0.2
S. aureus MS9610	6.25	3.13	1.56	0.78	25	1.56	0.39	<0.2
S. aureus MS16460(MRSA)	6.25	6.25	3.13	1.56	50	6.25	N.D.	>800
S. aureus MS16497(MRSA)	6.25	3.13	1.56	0.78	25	0.39	N.D.	0.78
S. aureus MS16526(MRSA)	3.13	3.13	1.56	0.78	25	25	0.78	>800
S. aureus TY-00933(MRSA)	6.25	3.13	3.13	0.78	25	3.13	0.78	>100
S. aureus TY-03454(MRSA)	6.25	3.13	1.56	0.78	25	0.39	0.39	100
S. aureus TY-03456(MRSA)	6.25	3.13	3.13	0.78	25	1.56	0.78	>100
S. aureus TY-04282(MRSA)	6.25	3.13	1.56	0.78	25	0.39	0.39	0.06
Enterococcus faecalis JCM5803*	50	25	12.5	3.13	100	12.5	0.78	N.D.
Enterococcus faecium JCM5804*	50	25	12.5	6.25	>100	12.5	0.78	N.D.
E. faecalis NCTC 12201 VCM R*	50	12.5	6.25	3.13	100	100	>400	N.D.
E. faecium NCTC 12202 VCM R*	50	25	12.5	6.25	>100	50	>400	N.D.
E. faecalis NCTC 12203 VCM R*	50	50	25	6.25	>100	50	400	N.D.
E. faecium NCTC 12204 VCM R*	50	25	12.5	6.25	>100	25	400	N.D.

medium: Mueller-Hinton agar except *: Mueller-Hinton agar+5% Sheep blood
N.D.; not determined

Fig. 2-a. Effect of tripropeptin C (TPPC) and/or chloramphenicol (CP) on cell growth.

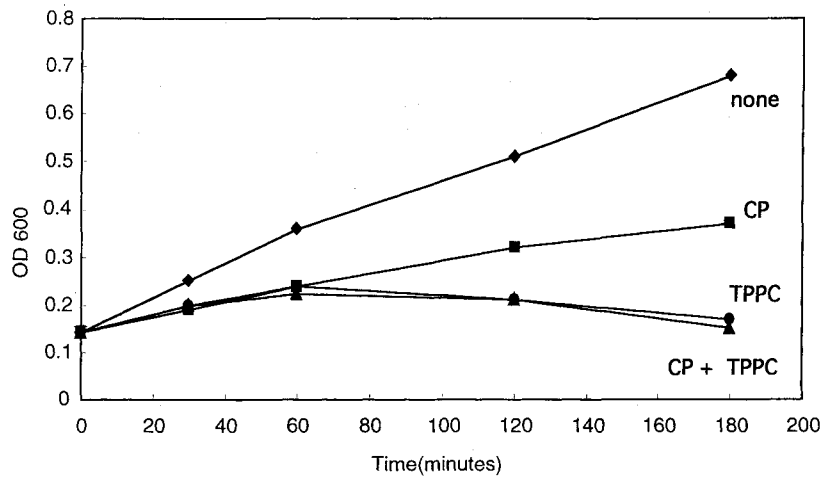
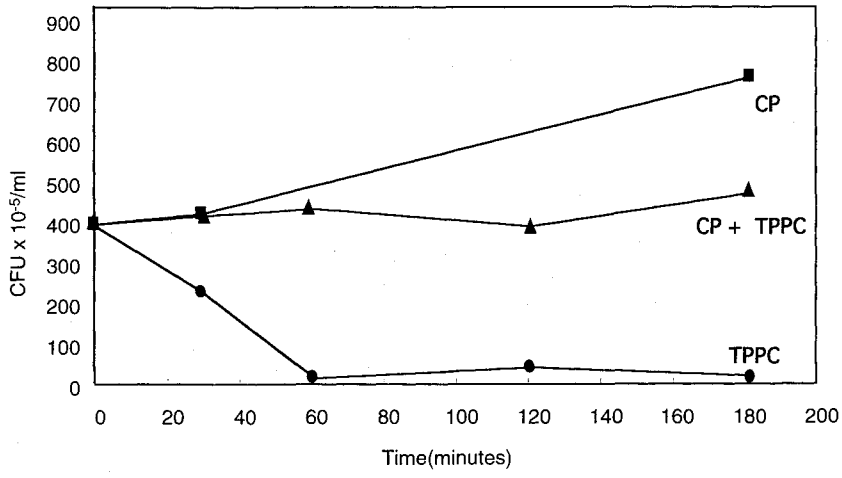


Fig. 2-b. Effect of tripropeptin C (TPPC) and/or chloramphenicol (CP) on numbers of viable cells.



Chloramphenicol was added 10minutes prior to tripropeptin C addition.
Concentrations of both agents are 2.5μg/ml.

Abbreviations: CP, chloramphenicol ; TPPC, tripropeptin C

alkyl chain while empedopeptin has a linear alkyl chain. Plusbacins[5,6] produced by *Pseudomonas* sp., seem to be the closest in their structures to tripropeptins, differing only in the amino acid composition, and also active against Gram-positive bacteria.

The antagonism between tripropepin C and chloramphenicol in respect to cell killing may be explained as follows. It is possible that tripropeptin C inhibits synthesis of the cell envelope but allows the increase in the intracellular contents, leading the cells to burst. Reversible inhibition by chloramphenicol of protein synthesis seems to protect cells from bursting. MAKI H. *et al.* recently reported that plusbacin A_3 inhibited peptidoglycan synthesis[7].

Acknowledgement

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