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NOTE

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A New Type of Tripropeptin with Anteiso-branched Chain Fatty Acid from *Lysobacter* sp. BMK333-48F3

Hideki Hashizume, Masayuki Igarashi, Ryuichi Sawa, Hayamitsu Adachi, Yoshio Nishimura, Yuzuru Akamatsu

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Abstract Branched chain amino acids are often utilized as the precursors of many lipid-containing bacterial secondary metabolites. The effect of isoleucine on the composition of the mixture of cyclic lipopeptide antibiotics, tripropeptins from *Lysobacter* sp. BMK333-48F3 was evaluated. As expected, a novel tripropeptin analog with an anteiso-branched fatty acid was produced. The new compound, TPPaiC shows potent antibacterial activity against Gram-positive bacteria including MRSA and VRE. On the other hand, no increase was observed in the production of other tripropeptins by the addition of isoleucine.

Keywords branched fatty acid, biosynthesis, MRSA, VRE, lipopeptide, antibiotics

Introduction

The widespread emergence of drug resistant Gram-positive bacteria, especially, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) is the biggest issues in infectious diseases treatment. Although vancomycin is the antibiotic of last resort for MRSA treatment, recently vancomycin-resistant *Staphylococcus aureus* (VRSA) strains have been isolated clinically [1～4]. On the other hand, *Enterococci* are sensitive to vancomycin but are nevertheless resistant to many other clinically important drugs. The use of avoparcin, structurally related to vancomycin, as a growth enhancer for livestock has resulted in the emergence of VRE, and VRE are now frequently isolated from humans and food animals [5～7]. These facts create the urgent necessity for the development of novel potent anti-MRSA/VRSA/VRE drugs given the limited choice of chemotherapeutic agents for the treatment of MRSA/VRSA/VRE infections [8～11].

Tripropeptins (TPPs), new drug candidates for MRSA/VRSA/VRE infections, were discovered in the course of our screening program [12, 13]. TPPs are cyclic depsipeptides consisting of eight amino acids and an iso-branched chain fatty acid [14]. TPPs show potent antibacterial activities against Gram-positive bacteria including MRSA and VRE. Of these, the major components tripropeptin C (TPPC) and tripropeptin D (TPPD) have emerged as new drug candidates because of their potent antimicrobial activities, excellent therapeutic efficacy in mouse septicemia models and further favorable toxicological profiles [15]. For industrial development, we have achieved the predominant production of TPPC by addition of L- or D-leucine, 4-methyl-2-oxo-pentanoic acid or isovaleric acid to the culture media [16]. Prior to this work, no tripropeptins with linear or anteiso-branched chain fatty acid have been reported.

Based on an analysis of the possible biosynthetic pathway of the fatty acids of tripropeptins in the previous work [16], we have discovered a new type of tripropeptin with anteiso-branched fatty acyl side chain, designated tripropeptin aiC (TPPaiC, Fig. 1) by adding isoleucine to the culture medium. The present paper describes the production of TPPaiC, as well as its physicochemical properties, structure elucidation and biological activities.

H. Hashizume (Corresponding author)**, M. Igarashi, R. Sawa, H. Adachi, Y. Nishimura, Y. Akamatsu:** Microbial Chemistry Research Center, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan, E-mail: hashizumeh@bikaken.or.jp

Results and Discussion

Fermentations were carried out in a similar manner as our previous report [12]. Fermentation for 48 hours at 27°C of *Lysobacter* sp. BMK333-48F3 yields the TPPs as tripropeptin A (20.8 μg/ml), tripropeptin B (25.3 μg/ml), TPPC (150.4 μg/ml), TPPD (25.4 μg/ml) and tripropeptin E (1.1 μg/ml) (Table 1). As shown in the previous work, the addition of L-leucine to the culture medium resulted in the predominant production of TPPC [12]. This result suggested that isoleucine might be incorporated instead of L-leucine. As shown in Table 1, a novel product, TPPaiC was predominatly produced by adding L-isoleucine, and its ratio depended on the quantity of additional L-isoleucine. The production of TPPaiC was observed by addition of no less than 0.5% L-isoleucine to the medium, while 1.2~2.0% of additional L-isoleucine severely inhibited the whole production of TPPs including TPPaiC. Interestingly, both TPPB and TPPD were not detected by addition of no less than 0.5% of L-isoleucine. The highest production of TPPaiC (82.3 μg/ml) was observed by fermentation in the medium with 0.8% L-isoleucine, and the highest content of TPPaiC among TPPs was 85.4% in the culture medium with 1.0% of L-isoleucine. No increase in the overall production of TPPs was observed by the addition of L-isoleucine. On the other hand, when D-isoleucine was added to the culture medium, the growth of producing organism and the production of TPPs were strongly inhibited (Table 1).

TPPaiC was produced in a similar manner as the previous report [12] except that the culture medium consisted of 1.5% glycerol, 1.5% cotton seed meal, 0.3% NaCl, 0.5% sodium L-glutamate monohydrate and 1.0% L-isoleucine in deionized water (pH 7.4 before sterilization).

To a fermentation broth (5.0 liters) were added 100 g of $Al(OH)_3$ and Diaion HP20 (Mitsubishi Chemical Co., 500 ml wet volume), and the mixture was stirred for 5 hours. Then the mixture was filtered, and the residue was washed successively with 2.0 liters of deionized water and 50% aq MeOH. The active principles were then eluted with 2.0 liters of Me_2CO. The Me_2CO eluate was concentrated *in vacuo* to yield a brownish oil (75 g), which was chromatographed on a column of silica gel (500 ml wet volume) successively with 1500 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 concentrated *in vacuo* to give a brownish oil (2.5 g). The oil was dissolved in a small volume of 30% aq MeOH and adjusted to pH 2.6 with 1.0 M HCl, the solution was subjected to column chromatography using 600 ml wet volume of CHP20P (Mitsubishi Chemical Co.). The linear gradient elution with CH_3CN and H_2O (0~540 minutes, $CH_3CN : H_2O = 30 : 70 \sim 50 : 50$, flow rate: 6 ml/minute) gave 53.3 mg of TPPaiC at 240~344 minutes and 143.7 mg of TPPC/TPPaiC crude mixture at 345~404 minutes.

The chemical structure of TPPaiC as shown in Fig. 1 was determined on the basis of spectroscopic and mass spectrometric data. The IR spectrum showed the characteristic absorption of peptide bonds (1635 and 1538 cm^{-1}) and of lactone linkage (1737 cm^{-1}). The molecular formula of TPPaiC (see Table 2) was determined

Table 1 Effect of amino acids addition on TPPs production

Amino acid	Dose (%)	pH at 48 hours	Productivities of TPPs (μg/ml)						% of TPP-aiC
			A	B	C	D	E	aiC	
Control		8.8~9.2	20.8	25.3	150.4	25.4	1.1	N.D.	—
L-leucine	0.2	8.8	19.1	13.7	178.3	15.2	1.7	N.D.	—
	0.5	8.8	18.3	6.3	176.3	8.1	2.4	N.D.	—
	1.0	8.6~8.8	20.6	0.7	181.1	0.5	3.7	N.D.	—
L-isoleucine	0.1	8.6~8.8	19.9	24.4	145.1	25.3	2.2	N.D.	—
	0.5	8.4~8.6	6.6	N.D.	58.6	N.D.	N.D.	53.2	44.9%
	0.8	8.2	5.7	N.D.	10.2	N.D.	N.D.	82.3	83.8%
	1.0	8.0	2.9	N.D.	7.8	N.D.	N.D.	62.5	85.4%
	1.2	7.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	—
	2.0	7.0~7.6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	—
D-isoleucine	0.5	7.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	—
	1.0	7.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	—

N.D.: not detected.

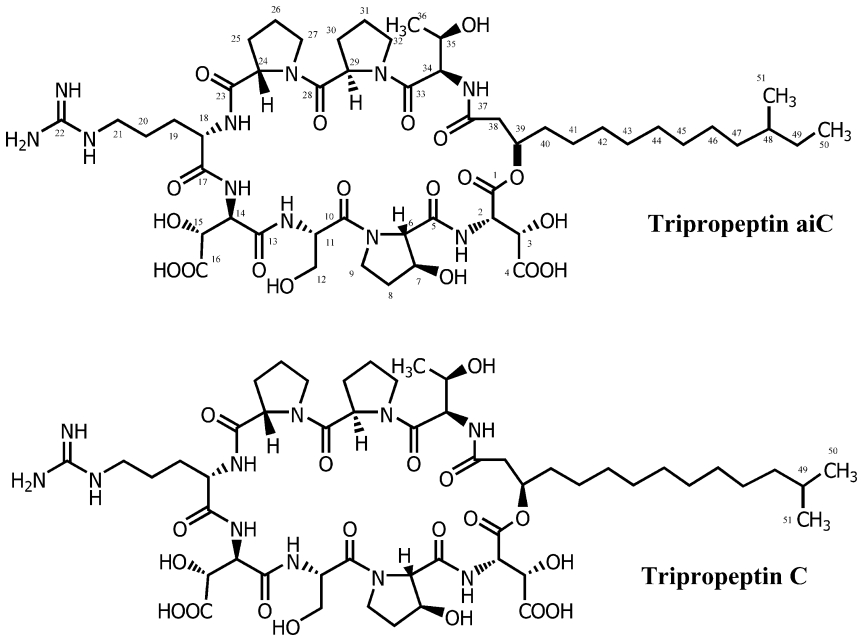

Fig. 1 Structures of tripropeptin aiC and tripropeptin C.

Table 2 Physico-chemical properties of tripropeptin aiC

Appearance	White powder
$[\alpha]_D^{24}$ (MeOH)	−8.8° (*c* 1.0)
ESI-MS (*m/z*)	
Positive	1176 $(M+Na)^+$
Negative	1152 $(M-H)^-$
HRESI-MS (*m/z*)	
Found	1152.5770 $(M-H)^-$
Calcd. for $C_{51}H_{82}N_{11}O_{19}$	1152.5783
Molecular formula	$C_{51}H_{83}N_{11}O_{19}$
UV λ_{max} (MeOH)	End absorption
IR ν_{max} (KBr) cm^{-1}	3370, 2930, 1737, 1635, 1538, 1450, 1263, 1201, 1097
Color reaction positive	I_2, Rydon-Smith, Sakaguchi

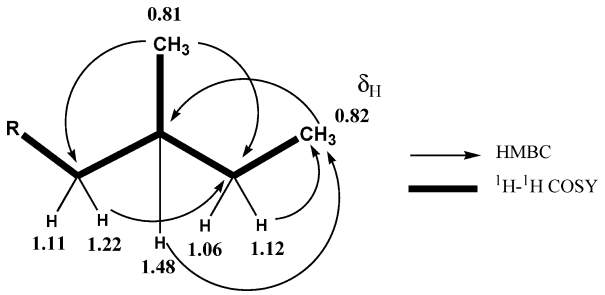

Fig. 2 ^1H-^1H COSY and HMBC experiments of tail structure of tripropeptin aiC in DMSO-d_6.

as $C_{51}H_{83}N_{11}O_{19}$ by the HRESI-MS (*m/z* 1152.5770 $[M-H]^-$, calcd. 1152.5783 as $C_{51}H_{82}N_{11}O_{19}$).

The connections of all bonds between ^1H and ^{13}C signals in the NMR spectra were assigned by DEPT and heteronuclear multiple quantum coherence (HMQC) experiments. The ^1H- and ^{13}C-NMR spectral data of TPPaiC were shown in Table 3. These NMR data of TPPaiC were quite similar to those of TPPC. The ^1H-^1H COSY and HMBC spectra of TPPaiC indicated the presence of one β-hydroxy fatty acid, eight amino acids: one residue of threonine, serine, arginine and 3-hydroxyproline, and two residues of proline and β-hydroxyaspartic acid. The amino acid sequence of TPPaiC was the same as those of other tripropeptins which were determined by the correlation in HMBC experiments. The planar structure of TPPaiC was clarified by analysis of the NMR data, and the structural difference between TPPaiC and TPPC is shown only in the tail structure of the fatty acid. The structure of anteiso-fatty acid chain of TPPaiC was determined by analysis of the NMR spectrum as shown in Fig. 2, which was in accord with the expectations. This is also supported by MSMS fragment patterns of TPPaiC, which coincided with those of TPPC (data not shown). The stereochemistry of constituent amino acids was identified as the same that of TPPs previously reported [14].

The antimicrobial activity of TPPaiC is summarized in Table 4. The MIC were examined by serial agar dilution method using Mueller-Hinton agar (Difco) for bacteria including *Enterococci* and *Staphylococcus aureus* after

Table 3 ^{13}C- and ^{1}H-NMR data of tripropeptin aiC and tripropeptin C in DMSO-d_6

Position	Type	Tripropeptin aiC		Tripropeptin C	
		δ_C[a]	δ_H[b] (multiplicity, J=Hz)	δ_C[a]	δ_H[b] (multiplicity, J=Hz)
1	>C=O	170.6		168.6	
2	>CH–N	57.6	4.27 (1H, m) NH: 7.88 (1H, d, 10.0)	54.8	H: 4.62 (1H, m) NH: 7.80 (1H, d, 10.0)
3	>CH–O	70.5	4.22 (1H, m)	70.0	4.55 (1H, d, 2.4)
4	>C=O	173.3		171.8	
5	>C=O	171.3		169.9	
6	>CH–N	66.9	4.41 (1H, m)	68.8	4.23 (1H, s)
7	>CH–O	69.3	4.39 (1H, m)	72.5	4.26 (1H, d, 3.8)
8	$-CH_2-$	32.6	1.80 (1H, m), 1.89 (1H, m)	32.3	1.75 (2H, m)
9	$-CH_2N<$	43.8	3.21 (1H, m), 3.45 (1H, m)	45.0	3.53 (1H, m), 3.66 (1H, m)
10	>C=O	170.6		167.9	
11	>CHNH–	52.0	H: 4.52 (1H, m) NH: 7.78 (1H, d, 8.0)	53.1	H: 4.58 (1H, m) NH: 7.25 (1H, d, 8.0)
12	$-CH_2O-$	61.0	3.47 (1H, m), 3.65 (1H, m)	61.3	3.53 (2H, m)
13	>C=O	169.7		168.5	
14	>CHNH–	54.9	H: 4.56 (1H, m) NH: 8.26 (1H, m)	56.3	H: 4.64 (1H, m) NH: 8.49 (1H, d, 8.4)
15	>CH–O	70.5	4.24 (1H, m)	70.0	4.5 (1H, d, 2.0)
16	>C=O	173.9		172.9	
17	>C=O	170.1		171.2	
18	>CHNH–	52.0	H: 4.24 (1H, m) NH: 7.93 (1H, d, 8.0)	51.7	H: 4.55 (1H, m) NH: 7.77 (1H, d, 8.6)
19	$-CH_2-$	29.0	1.56 (1H, m), 1.63 (1H, m)	28.9	1.56 (1H, m), 1.63 (1H, m)
20	$-CH_2-$	24.7	1.32 (2H, m)	24.7	1.35 (2H, m)
21	$-CH_2NH-$	40.2	H: 3.05 (2H, m) NH: 7.93 (1H, d, 8.0)	40.3	H: 3.06 (2H, m) NH: 7.62 (m)
22	–N=C(N–)N–	156.9		156.9	
23	>C=O	172.4		172.6	
24	>CH–N	59.8	4.83 (1H, m)	60.6	4.72 (1H, m)
25	$-CH_2-$	31.6	2.08 (1H, m), 2.13 (1H, m)	31.7	1.93 (1H, m), 2.13 (1H, m)
26	$-CH_2-$	22.7	1.69 (1H, m), 1.84 (1H, m)	22.2	1.78 (2H, m)
27	$-CH_2-N$	46.8	3.31 (2H, m)	46.9	3.35 (1H, m), 3.49 (1H, m)
28	>C=O	171.1		172.0	
29	>CH–N	57.8	4.35 (1H, m)	57.9	4.18 (1H, t, 12.4)
30	$-CH_2-$	32.9	1.60 (2H, m)	29.0	1.63 (2H, m)
31	$-CH_2-$	25.1	2.00 (2H, m)	24.4	1.75 (1H, m), 1.85 (1H, m)
32	$-CH_2-N$	47.2	3.54 (1H, m), 3.70 (1H, m)	47.3	3.54 (1H, m), 3.61 (1H, m)
33	>C=O	168.2		169.0	
34	>CHNH–	55.9	H: 4.70 (1H, dd, 7.0) NH: 8.15 (1H, d, 8.4)	56.0	H: 4.52 (1H, d, 7.0) NH: 8.04 (1H, d, 8.4)
35	>CH–O	65.7	3.85 (1H, m)	67.2	3.74 (1H, m)
36	$-CH_3$	18.6	0.95 (3H, d, 6.6)	19.0	0.97 (3H, d, 6.6)
37	>C=O	169.4		169.5	
38	$-CH_2-$	39.2	2.33 (1H, m), 2.82 (1H, m)	40.1	2.28 (1H, d, 12.0), 2.66 (1H, m)
39	>CH–O	72.8	4.92 (1H, m)	72.8	5.06 (1H, m)
40	$-CH_2-$	32.9	1.63 (2H, m)	33.7	1.50 (2H, m)
41	$-CH_2-$	23.5	1.22 (2H, m)	24.1	1.21 (2H, m)
42	$-CH_2-$	28.7	1.22 (2H, m)	29.1	1.21 (2H, m)
43	$-CH_2-$	28.9	1.22 (2H, m)	29.1	1.21 (2H, m)
44	$-CH_2-$	28.9	1.22 (2H, m)	29.1	1.21 (2H, m)
45	$-CH_2-$	29.1	1.22 (2H, m)	29.1	1.21 (2H, m)
46	$-CH_2-$	29.0	1.22 (2H, m)	29.1	1.21 (2H, m)
47	$-CH_2-$	26.0	1.11 (1H, m), 1.22 (1H, m)	26.8	1.13 (1H, m), 1.21 (1H, m)
48	>CH–	32.8	1.48 (1H, m)	38.5	1.13 (1H, m), 1.21 (1H, m)
49	$-CH_2-$	29.0	1.06 (1H, m), 1.12 (1H, m)	27.4	1.49 (1H, m)
50	$-CH_3$	23.0	0.82 (3H, t, 7.0)	22.5	0.83 (3H, d, 7.0)
51	$-CH_3$	11.3	0.81 (3H, d, 7.0)	22.5	0.83 (3H, d, 7.0)

[a] 150 MHz, chemical shift in ppm.
[b] 600 MHz, chemical shift in ppm.

Table 4 Antimicrobial activities of tripropeptin aiC and related compounds

Test organisms	MIC (μg/ml), range			
	Tripropeptin aiC	Tripropeptin C	Vancomycin	Teicoplanin
Staphylococcus aureus (methicillin sensitive), (*n*=10)	0.78～1.56	0.78～1.56	0.39～1.56	0.39～0.78
Staphylococcus aureus (methicillin resistance), (*n*=10)	0.39～3.13	0.39～3.13	0.78～1.56	0.1～1.56
Enterococcus faecium/*faecalis* (vancomycin sensitive), (*n*=2)	6.25～25	6.25～12.5	0.78	0.78
Enterococcus faecium/*faecalis* (vancomycin resistance, vanA), (*n*=4)	6.25～25	6.25～12.5	>100	>100

Mueller Hinton agar (Difco), 37°C, 18 hours.

incubation for 18 hours at 37°C. TPPaiC showed the excellent antimicrobial activity against Gram-positive bacteria including MRSA and VRE, but showed little activity against Gram-negative organisms (data not shown).

Our present study described the production of a new type of tripropeptin with anteiso-branched chain fatty acid by addition of an excess amount of L-isoleucine to the culture medium. In usual fermentation, iso-branched tripropeptins A, B, C, D, E and Z are produced, but any detectable tripropeptins with anteiso-branched chain fatty acid are not produced. Although TPPaiC is produced in dose dependent manner of L-isoleucine in the culture medium, the doses of L-isoleucine showing the predominant production of TPPaiC ranging from 0.8 to 1.0% are relatively close to the growth repression of the producing organism at 1.2%. The producing organism could hardly survive under such conditions with the high dosage of L-isoleucine as no less than 1.0%. These data suggest that TPPs-biosynthetic enzymes show lower affinity to anteiso-branched precursors than to iso-branched counterparts, and/or that the producing organism has a low capacity for supplementation with anteiso-branched precursors, and this could be the reason why TPPs with anteiso-branched fatty acids were not produced under usual fermentation. On the other hand, when D-isoleucine was added to the culture medium, the growth of the producing organism was severely inhibited.

Antibacterial activities of TPPaiC are comparable to those of TPPC. This result indicates that iso- or anteiso-branched chain structure of the constituent fatty acids little influence on the biological activity, although the length of fatty acyl side chains is known to influence the biological activity [13]. These structure-activity relationships are in accord with those of plusbacins from *Pseudomonas* sp., closely related to TPPs in structures [17].

Tripropeptins including TPPaiC also show the potent antimicrobial activity against the multi-drug resistant Gram-positive bacteria, suggesting strongly that TPPs have the different mode of action from the known drugs.

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