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NOTES

Tripropeptin E, a New Tripropeptin Group Antibiotic Produced by *Lysobacter* sp. BMK333-48F3

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Since the late 20th century, the prevalence of infectious diseases caused by the drug-resistant bacteria, especially by the Gram-positive ones has become a serious problem in the medical treatments, rendering the major antimicrobial drugs less active or ineffective against many important bacterial infections[1,2]. Thus the newer drugs effective against the multidrug-resistant bacteria are being requested.

Recently, we have discovered novel compounds designated tripropeptins (TPPs); particularly tripropeptin C (TPPC) and tripropeptin D (TPPD) show the excellent activities against Gram-positive bacteria including both methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE)[3]. The antimicrobial activities of these tripropeptins correlate well to the length of fatty acyl side-chains, indicating that the longer was the more active[3]. Thus, we are greatly interested in the study on the structure-activity relationship regarding fatty acyl side-chains.

The present paper described the isolation of a new type of tripropeptin-antibiotic, tripropeptin E (TPPE), its chemical and physicochemical characterization and also its anti-bacterial activities. Furthermore, the structure-activity relationship of tripropeptin-like lipopeptides will be discussed.

Fermentations were carried out likewise as reported previously[3] except that the culture medium consists of 1.5% glycerol, 1.5% cotton seed meal, 0.3% NaCl, 0.5% sodium L-glutamate monohydrate, 0.2% L-leucine in deionized water (pH 7.4 before sterilization).

A fermentation broth (15 liters) was poured into 15 liters of acetone. Mixing the above suspension well, 30 liters of deionized water and then Diaion HP20 (Mitsubishi Chemical Co., 1 liter wet volume) were added subsequently. The mixture was filtered, and the residues were washed with 3 liters of deionized water and 50% aqueous methanol, successively. The active principles were then eluted with 3 liters of acetone. The acetone eluate was concentrated *in vacuo* to yield a brownish oil (30 g), which was chromatographed on a column of silica gel (1000 ml wet volume) with 3000 ml each of $CHCl_3$: MeOH : H_2O = 10 : 5 : 1 and BuOH : MeOH : H_2O = 4 : 1 : 2 successively. Active fractions eluted with the latter solvent mixture were concentrated *in vacuo* to give a yellowish brown oil (5.1 g). The oil was dissolved in a small volume of 30% aqueous methanol, adjusted to pH 2.6 with 1 M HCl, and was applied to column chromatography using 250 ml wet volume of CHP20P (Mitsubishi Chemical Co.). The elution with stepwise gradients of acetone and H_2O (750 ml each of 12 : 28, 14 : 26, 16 : 24, 18 : 22, 20 : 20, 22 : 18, 23 : 17, 24 : 16, 25 : 15, 26 : 14, 27 : 13 and 28 : 12 V/V), gave active components; Tripropeptin A (45.7 mg, 18 : 22 and 20 : 20), TPPC (918.4 mg, 20 : 20, 22 : 18 and 23 : 17), and a mixture of TPPC and TPPE (288.9 mg, 23 : 17～26 : 14) in the ratio of acetone and H_2O in parentheses.

The mixture of TPPC and TPPE was dissolved in a small volume of distilled water, adjusted to pH 2.0 with 1 M HCl and subjected to column chromatography using 30 ml wet volume of CHP20P (Mitsubishi Chemical Co.). The elution was carried out with stepwise gradients of acetonitrile and H_2O (100 ml each of 30 : 70, 31 : 69, 32 : 68, 33 : 67, 34 : 66, 35 : 65, 36 : 64, 37 : 63, 38 : 62, 39 : 61 and 40 : 60 V/V). The fractions eluted with a mixture of acetonitrile and H_2O (33 : 67 and 34 : 66), and a mixture of acetonitrile and H_2O (36 : 64 and 37 : 63) gave 198.5 mg of TPPC and 17.5 mg of TPPE, respectively.

The chemical structure of TPPE as shown in Fig. 1 was determined on the basis of the spectroscopic and mass spectrometric data. The IR spectrum displayed the characteristic absorption of peptide bonds (1635 and 1538 cm^{-1}) and of lactone linkage (1741 cm^{-1}). The molecular weight of 1181 and the molecular formula of TPPE ($C_{53}H_{87}N_{11}O_{19}$: see Table 1) were suggested by *m/z* 1202.5901 in the HRESI-MS ($[M-2H+Na]^-$, calcd

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Fig. 1. Structure of tripropeptin E.

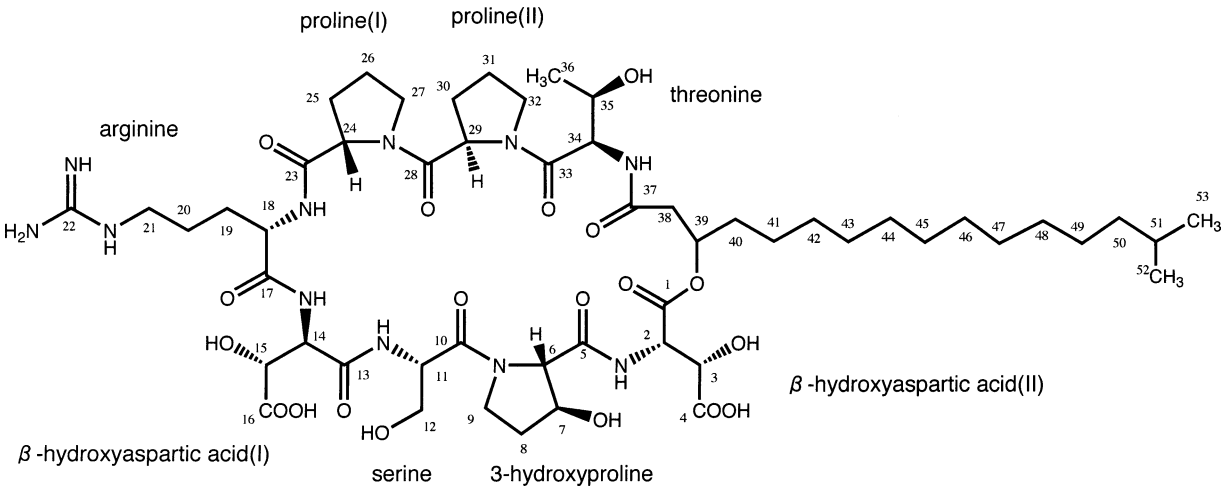


Table 1. Physicochemical properties of tripropeptin E.

Appearance	**colorless powder**
[α]$_D^{24}$ (MeOH)	**-9.4°(c 1.0)**
ESI-MS(*m/z*)	
negative	**1202.6(M-2H+Na)$^-$**
HRFAB-MS(*m/z*)	
found	**1202.5901(M-2H+Na)$^-$**
calcd. for $C_{53}H_{85}N_{11}O_{19}$ Na	**1202.5921**
Molecular formula	**$C_{53}H_{87}N_{11}O_{19}$**
UV λ $_{max}$(MeOH)	**end absorption**
IR υ $_{max}$(KBr)cm^{-1}	**3336, 2925, 1741, 1635, 1538, 1452, 1265, 1201, 1097**
Color Reaction	
positive	**I_2, Rydon-Smith, Sakaguchi**

1202.5921).

The planar structure of TPPE was determined as follows. The connections of all bonds between ^{1}H and ^{13}C signals were well interpreted by DEPT and heteronuclear multiple quantum coherence (HMQC) experiments. The DEPT and HMQC experiments revealed the presence of three methyl carbons, twentyfour methylene carbons, fourteen methine carbons, one sp^2 quaternary carbon and eleven carbonyl carbons in TPPE. The ^{1}H and ^{13}C NMR spectral data of TPPE were shown in Table 2.

The ^{1}H-^{1}H COSY and HMBC spectra of TPPE indicated the presence of one β-hydroxy fatty acid, eight amino acids, one residue each of threonine (Thr), serine (Ser), arginine (Arg) and 3-hydroxyproline (OHPro), and two residues of proline (Pro) and β-hydroxyaspartic acid (β-OHAsp) as shown in Fig. 2.

The amino acid sequence of TPPE was determined by the following correlation from H-2 (δ 4.27) of OHAsp (II) to carbonyl carbon C-5 (δ 170.0) of OHPro, from H-6 (δ 4.40) of OHPro to carbonyl carbon C-10 (δ 170.2) of Ser, from H-11 (δ 4.50) of Ser to carbonyl carbon C-13 (δ 169.7) β-OHAsp (I), from amide proton NH-14 (δ 8.26) of β-OHAsp (I) to carbonyl carbon C-17 (δ 170.0) of Arg, from amide proton NH-18 (δ 7.96) of Arg to carbonyl carbon C-23 (δ 172.4) of Pro (I), from H-27 (δ 3.33) of Pro (I) to carbonyl carbon C-28 (δ 171.3) of Pro (II), from H-29 (δ 4.41) of Pro (II) to carbonyl carbon C-33 (δ 168.3) of Thr, from methine protons H-34 (δ 4.70) and H-39 (δ 4.92) to carbonyl carbon C-37 (δ 169.4) of 3-hydroxy-15-methylhexadecanoic acid as a 3-hydroxy-15-methylhexadecanoyl-Thr-Pro-Pro-Arg-β-OHAsp-Ser-OHPro-β-OHAsp.

Likewise other tripropeptins, we clarified the chemical structure of TPPE as shown in Fig.1. The stereochemistries of the constituent amino acids, determined by Marfey's

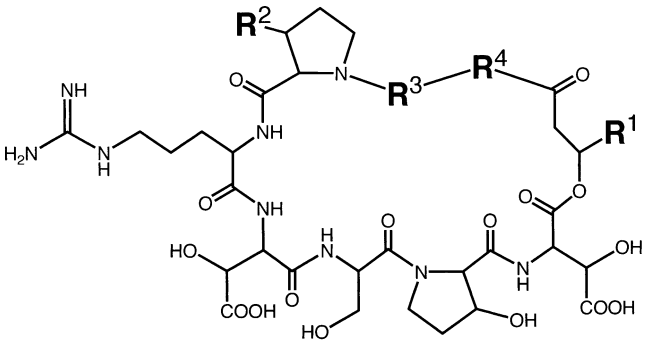
Table 2. ^{13}C and ^{1}H NMR data of tripropeptin E in DMSO-d_6.

position	type	δ C[a]	δ H[b] (multiplicity, J =Hz)
1	>C=O	170.5	
2	>CH-N	57.6	4.27(1H, m)
3	>CH-O	70.4	4.22(1H, m)
4	>C=O	172.9	
5	>C=O	171.2	
6	>CH-N	67.0	4.40(1H, m)
7	>CH-O	69.3	4.39(1H,m)
8	-CH_2-	32.6	1.78(2H, m)
9	-CH_2N<	43.9	3.21(1H, m), 3.36(1H, m)
10	>C=O	170.2	
11	>CHNH-	52.2	H:4.50(1H, m), NH:7.82(1H, d, 8.0)
12	-CH_2O-	60.6	3.47(1H, m) , 3.63(1H, m)
13	>C=O	169.7	
14	>CHNH-	54.9	H:4.56(1H, m), NH:8.26(1H, m)
15	>CH-O	70.4	4.25(1H, m)
16	>C=O	174.0	
17	>C=O	170.0	
18	>CHNH-	52.0	H:4.22(1H, m), NH:7.96(1H, d, 8.0)
19	-CH_2-	29.0	1.56(1H, m), 1.63(1H, m)
20	-CH_2-	24.7	1.32(2H, m)
21	-CH_2NH-	40.2	H:3.05(2H, m), NH:7.82(1H, d, 8.0)
22	-N=C(N-)N-	156.8	
23	>C=O	172.4	
24	>CH-N	59.8	4.83(1H, m)
25	-CH_2-	31.6	2.08(1H, m), 2.13(1H, m)
26	-CH_2-	22.7	1.63(1H, m), 1.82(1H, m)
27	-CH_2-N	46.8	3.33(2H, m)
28	>C=O	171.3	
29	>CH-N	57.8	4.41(1H, m)
30	-CH_2-	32.9	1.63(2H, m)
31	-CH_2-	25.1	2.00(2H, m)
32	-CH_2-N	47.2	3.56(1H, m), 3.69(1H, m)
33	>C=O	168.3	
34	>CHNH-	55.9	H:4.70(1H, dd, 7.0), NH:8.16(1H, d, 8.4)
35	>CH-O	65.7	3.85(1H, m)
36	-CH_3	18.6	0.96(1H, d, 6.6)
37	>C=O	169.4	
38	-CH_2-	39.0	2.33(1H, d, 12.0), 2.81(1H,m)
39	>CH-O	72.8	4.92(1H, m)
40	-CH_2-	32.9	1.63(2H, m)
41	-CH_2-	23.5	1.22(2H, m)
42	-CH_2-	28.7	1.22(2H, m)
43	-CH_2-	28.9	1.22(2H, m)
44	-CH_2-	28.9	1.22(2H, m)
45	-CH_2-	29.1	1.22(2H, m)
46	-CH_2-	29.3	1.22(2H, m)
47	-CH_2-	29.0	1.22(2H, m)
48	-CH_2-	29.0	1.22(2H, m)
49	-CH_2-	26.8	1.12(1H, m), 1.22(1H, m)
50	-CH_2-	38.4	1.13(1H, m), 1.22(1H, m)
51	>CH-	27.4	1.46(1H, m)
52	-CH_3	22.5	0.83(3H, d, 7.0)
53	-CH_3	22.5	0.83(3H, d, 7.0)

[a] 125 MHz, chemical shift in ppm.

[b] 500 MHz, chemical shift in ppm.

Fig. 2. Structures of tripropeptin-like lipopeptides.



TRIPROPEPTINS

A	$R^1=(CH_2)_7CH(CH_3)CH_3$	R^2=H	R^3=Pro	R^4=Thr
B	$R^1=(CH_2)_8CH(CH_3)CH_3$	R^2=H	R^3=Pro	R^4=Thr
C	$R^1=(CH_2)_9CH(CH_3)CH_3$	R^2=H	R^3=Pro	R^4=Thr
D	$R^1=(CH_2)_{10}CH(CH_3)CH_3$	R^2=H	R^3=Pro	R^4=Thr
E	$R^1=(CH_2)_{11}CH(CH_3)CH_3$	R^2=H	R^3=Pro	R^4=Thr
Z	$R^1=(CH_2)_6CH(CH_3)CH_3$	R^2=H	R^3=Pro	R^4=Thr

PLUSBACINS

A_1	$R^1=(CH_2)_{10}CH_3$	R^2=OH	R^3=Ala	R^4=Thr
A_2	$R^1=(CH_2)_9CH(CH_3)CH_3$	R^2=OH	R^3=Ala	R^4=Thr
A_3	$R^1=(CH_2)_{10}CH(CH_3)CH_3$	R^2=OH	R^3=Ala	R^4=Thr
A_4	$R^1=(CH_2)_{12}CH_3$	R^2=OH	R^3=Ala	R^4=Thr
B_1	$R^1=(CH_2)_{10}CH_3$	R^2=H	R^3=Ala	R^4=Thr
B_2	$R^1=(CH_2)_9CH(CH_3)CH_3$	R^2=H	R^3=Ala	R^4=Thr
B_3	$R^1=(CH_2)_{10}CH(CH_3)CH_3$	R^2=H	R^3=Ala	R^4=Thr
B_4	$R^1=(CH_2)_{12}CH_3$	R^2=H	R^3=Ala	R^4=Thr

EMPEDOPEPTIN

$R^1=(CH_2)_{10}CH_3$	R^2=H	R^3=Ser	R^4=Pro

Method were the same as those of the other TPPs[4].

The wide range of antimicrobial activities of TPP A, B, C, D, E and Z were summarized in Table 3. TPPE showed an excellent antimicrobial activity against a broad range of Gram-positive bacteria including MRSA, penicillin-resistant *Streptococcus pneumoniae* (PRSP) and VRE, but showed little activity against Gram-negative organisms. As shown in Table 4, the antimicrobial activities of TPPC, TPPD and TPPE against the recent clinically-isolated *Staphylococcus aureus* strains, both methicillin-susceptible *Staphylococcus aureus* (MSSA) and MRSA, were favorable compared with the currently available antimicrobial agents such as vancomycin, meropenem, *etc.* The orders of activities of TPPs did depend on the tested organisms, TPPD>C>E against *Staphylococcus* and *Streptococcus*, and TPPD>E>C against *Enterococcus*. Before discovery of TPPE, it was likely that antimicrobial activities of TPPs correlate to length of fatty acyl side-chain and that the longer was the more active. However, it was proved that TPPD having the second longest acyl side-chain of these tripropeptins showed the strongest antimicrobial activity.

Discussion

Our present study strongly suggested, as current studies have demonstrated, that the acyl side-chain plays the important roles in antimicrobial activities not only of TPPs but also of other lipopeptides, such as echinocandin-micafungin group[5], daptomycin group (LY146032 and A21978Cs)[6], polymyxins and octapeptins[7].

Tripropeptin-like lipopeptide antibiotics, plusbacins[8,9] and empedopeptin[10,11] were reported, and their structure were shown in Fig. 2. Comparison in antimicrobial activities between TPPs and those tripropeptin-like lipopeptides (data from literatures) suggested that the C-13 fatty acyl chain seems to be the best one for antimicrobial activity. On the other hand, these results also suggested that

Table 3. Antimicrobial activities of tripropeptins.

Test organisms	MIC (μg/ml) TPPA	TPPB	TPPC	TPPD	TPPE	TPPZ
Staphylococcus aureus FDA209P	1.56	0.78	0.39	0.39	0.78	12.5
S. aureus MS9610	6.25	0.78	0.78	0.78	0.78	25
S. aureus No.5(MRSA)	N.T.	1.56	1.56	1.56	0.78	N.T.
S. aureus No.17(MRSA)	N.T.	1.56	1.56	1.56	0.78	N.T.
S. aureus MS16526(MRSA)	3.13	0.78	0.78	1.56	0.78	25
S. aureus TY-04282(MRSA)	6.25	0.78	0.78	0.78	0.78	25
Streptococcus pneumoniae TY-5708(PRSP)[a]	6.25	1.56	0.78	0.78	0.78	25
S. pneumoniae TY-5745(PRSP)[a]	3.13	0.78	0.78	0.78	1.56	12.5
S. pneumoniae TY-5840[a]	6.25	1.56	0.78	0.78	1.56	25
S. pyogenes TY-5727[a]	25	6.25	0.78	1.56	1.56	>50
S. pyogenes TY-5740[a]	1.56	0.78	0.78	0.39	0.78	3.13
S. pyogenes TY-5914[a]	1.56	0.78	0.39	0.39	0.78	3.13
S. pyogenes MH613[a]	1.56	0.78	0.39	0.39	0.78	6.25
S. pyogenes MH624[a]	1.56	0.39	0.39	0.39	0.78	6.25
S. pyogenes MH630[a]	1.56	0.39	0.39	0.39	0.39	6.25
S. pyogenes MH635[a]	3.13	0.78	0.39	0.39	0.78	12.5
S. pyogenes MH759[a]	3.13	0.78	0.39	0.20	0.78	6.25
S. pyogenes MH771[a]	1.56	0.39	0.39	<0.20	0.78	3.13
S. pyogenes Cook(MS-1)[a]	3.13	0.78	0.78	<0.20	0.78	12.5
Enterococcus faecalis JCM 5803[a]	50	25	6.25	3.13	3.13	100
E. faecalis NCTC 12201 (VCM R)[a]	50	12.5	3.13	1.56	1.56	100
E. faecalis NCTC12203 (VCM R)[a]	50	50	6.25	1.56	3.13	>100
E. faecium JCM 5804[a]	50	25	12.5	3.13	6.25	>100
E. faecium NCTC 12202 (VCM R)[a]	50	25	6.25	3.13	3.13	>100
E. faecium NCTC 12204 (VCM R)[a]	50	25	6.25	3.13	3.13	>100
Micrococcus luteus IFO3333	<0.39	<0.20	<0.20	<0.20	0.39	<0.39
M. luteus PCI1001	N.T.	0.20	0.20	0.39	0.20	N.T.
Bacillus subtilis NRRL B-558	N.T.	0.39	0.78	0.78	0.39	N.T.
B. subtilis PCI219	3.13	0.78	0.78	0.78	0.78	25
B. cereus ATCC10702	N.T.	6.25	1.56	1.56	3.13	N.T.
Corynebacterium bovis 1810	1.56	0.20	<0.20	0.20	<0.20	6.25
Escherichia coli NIHJ	>50	>100	>50	>50	>50	>50
Shigella dysenteriae JS11910	>50	>50	>50	>50	>50	>50
Salmonella enteritidis 1891	>50	>50	>50	>50	>50	>50
Serratia marcescens	>50	>50	>50	>50	>50	>50
Pseudomonas aeruginosa A3	>50	>50	>50	>50	>50	>50
Klebsiella pneumoniae PCI602	>50	>50	>50	>50	>50	>50
Mycobacterium smegmatis ATCC607[b]	>50	>50	>50	>50	>50	>50
Candida albicans 3147	>50	>100	>50	>50	>50	>50

N.T.; not tested
Mueller Hinton agar (Difco), 37°C 18 hours, except a and b.
a: Mueller Hinton agar(Difco) + 5% Sheep blood,37°C 18 hours
b: Mueller Hinton agar (Difco), 37°C 42 hours.

Table 4. Antimicrobial activities against *Staphylococcus aureus*.

test sample	MIC (μg/ml) MSSA(12*) range	MIC_{50}	MIC_{90}	MRSA(10*) range	MIC_{50}	MIC_{90}
TPPA	3.13 ~ 12.5	6.25	6.25	1.56 ~ 12.5	6.25	12.5
TPPB	0.78 ~ 3.13	1.56	3.13	0.39 ~ 3.13	3.13	3.13
TPPC	0.39 ~ 0.78	0.78	0.78	0.39 ~ 1.56	0.78	1.56
TPPD	0.39 ~ 0.78	0.78	0.78	0.39 ~ 0.78	0.78	0.78
TPPE	0.39 ~ 0.78	0.78	0.78	0.39 ~ 1.56	0.78	1.56
vancomycin	0.78	0.78	0.78	0.39 ~ 1.56	1.56	1.56
teicoplanin	0.39 ~ 0.78	0.39	0.78	0.20 ~ 1.56	0.78	1.56
meropenem	0.05 ~ 0.10	0.10	0.10	6.25 ~ 50	12.5	50
levofloxacin	0.05 ~ 0.39	0.10	0.20	3.13 ~ >100	25	>100
ofloxacin	0.20 ~ 0.78	0.39	0.39	6.25 ~ >100	100	>100
ampicillin	0.10 ~ 3.13	0.78	3.13	6.25 ~ 50	25	50
arbekacin	0.20 ~ 0.78	0.39	0.78	0.20 ~ 0.39	0.39	0.39
erythromycin	0.10 ~ >100	0.20	12.5	>100	>100	>100
tetracycline	0.20	0.20	0.20	1.56 ~ 50	50	50
fosfomycin	3.13 ~ 50	6.25	25	>100	>100	>100

* tested numbers of clinical isolates in 2002

the straight chain or branched chain structure of the fatty acids did not so much influence on the antimicrobial activities.

These results strongly suggested that so far as tripropeptin-like lipopeptides are concerned, the hydrophilic and hydrophobic balance of the molecule is very important in expressing its antimicrobial activity. It might contribute to bring the molecule to the targeted protein.

Tripropeptins, especially TPPC, D and E showed the excellent antibacterial activity against most clinically important Gram-positive species and so might be one of the new useful drug candidates.

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