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Revised structure of the peptide lactone antibiotic, TL-119 and/or A-3302-B

Yasuo Kitajima[1,*], Michinori Waki, Jun'ichi Shoji[2], Tamio Ueno[3] and Nobuo Izumiya[1]

[1] *Laboratory of Biochemistry, Faculty of Science, Kyushu University 33, Higashi-ku, Fukuoka 812, Japan,* [2] *Shionogi Research Laboratories, Shionogi and Co. Ltd., Fukushima-ku, Osaka 553, Japan* and [3] *Pesticide Research Institute, College of Agriculture, Kyoto University, Sakyo-ku, Kyoto, 606, Japan*

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The peptide lactone antibiotic TL-119 and/or A-3302-B was chemically synthesized in order to confirm the proposed structure. The synthetic compound was different from both natural TL-119 and A-3302-B in their physicochemical properties and in biological activity. Re-examination of the configuration of the constituent amino acid residues in natural TL-119 and/or A-3302-B indicated that natural TL-119 and A-3302-B contains D-aThr instead of the original L-Thr. We tentatively propose a revised structure for TL-119 and/or A-3302-B.

Cyclic peptide; Peptide synthesis; Antibacterial activity; Structure confirmation

1. INTRODUCTION

TL-119 (**1**) is a peptide antibiotic isolated from a strain of *Bacillus subtilis*, which is active against Gram-positive bacteria [1]. Nakagawa et al. proposed a structure (**I**) for TL-119 without describing the stereochemistry of the amino acid residues as shown in Fig. 1A [2]. The structure **I** indicates that the antibiotic is a peptide lactone containing an ester bond and an α,β-dehydroamino acid, 2-amino-2-butenoic acid (ΔAba), at position 7 in the molecule. Two antibiotics, A-3302-A and -B, were also isolated from a strain of *Bacillus subtilis*, and their structures including the stereochemistry of the constituent amino acids were elucidated [3]. One of them, A-3302-B (**2**), was assumed to be identical with TL-119 based on the structural similarity (Fig. 1A) [3].

We were interested in confirming the unique structure **I** proposed for TL-119 and/or A-3302-B by its chemical synthesis, and in studying the structure-activity relationship of the antibiotic by the synthesis of various analogs. Hereafter compound number **3** is used for the compound that has the structure of **I**. In this report we describe the synthesis of **I**, its physicochemical properties and biological activity and compare it with both natural TL-119 and natural A-3302-B.

Correspondence address: M. Waki, Laboratory of Biochemistry, Faculty of Science, Kyushu University 33, Higashi-ku, Fukuoka 812, Japan

* *Present address:* Suntory Institute for Biomedical Research, Shimamoto-cho, Mishima-gun, Osaka 618, Japan

Abbreviations: Abbreviations used are according to IUPAC-IUB Commissions (1984) Eur. J. Biochem. 138, 9–37. Other abbreviations: DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; HOSu, *N*-hydroxysuccinimide; HPTLC, high-performance thin layer chromatography

2. EXPERIMENTAL

2.1. *General*

Specific optical rotations were measured with a Union high-sensitivity polarimeter PM-71. ^{1}H-NMR spectra at 90 MHz were recorded on a Jeol JNM-FX90Q, tetramethylsilane as an internal standard. Mass spectra were taken on a Jeol mass spectrometer Model JMS-01SG-2 with a direct inlet system operating at 75 eV. IR spectra were recorded on a Hitachi infrared spectrophotometer Model 260-10. Circular dichroism (CD) spectra were taken on a Jasco J-600A spectropolarimeter using a cell of 0.1 cm path length. Amino acid analysis was performed with a Yanagimoto amino acid analyzer LC-8A. The modified Manning-Moore method to analyze dipeptide diastereoisomers was carried out with a Hitachi amino acid analyzer KLA-5 with spherical resin under the following conditions: column, 0.6 × 55 cm; buffer, 0.1 M sodium citrate at pH 4.25 and 0.35 M sodium citrate at pH 5.28; flow rate, 30 ml/h; jacket temperature, 55°C.

2.2. *Synthesis of* **3**

The ring part (tetrapeptide lactone) of the precursor was successfully prepared as follows (see Fig. 2). At first an ester bond between the hydroxyl function in Z-L-Thr-OPac and the carboxyl function in Boc-L-Thr(Bzl)-OH was formed by the DCC/4-dimethylaminopyridine method to give an ester **5** in 98% yield. Successive treatment of **5** with HCl in dioxane and coupling with Boc-amino acid by the DCC-HOBt method afforded a compound **6** in good yield. The OPac group of **6** was removed by Zn/AcOH, and then converted to the corresponding *N*-hydroxysuccinimide ester **7**. After deblocking the Boc group of **7** with trifluoroacetic acid (TFA), the resultant active ester trifluoroacetate (**8**·TFA) was subjected to cyclization via peptide bond formation

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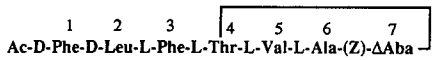
A.



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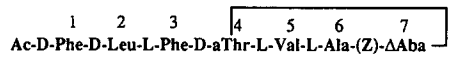

Fig. 1. (A) Structure (**I**) proposed for A-3302-B (**2**) and/or TL-119 (**1**). (B) Revised structure (**II**) proposed for **1** and/or **2**.

under high dilution conditions in pyridine (3 mM). Purification of the crude cyclization product by Sephadex LH-20 afforded a desired cyclic monomer (**9**) (M^+: m/z 596.2694) in 21% yield. The tail part (**10**) of the precursor was prepared by the stepwise elongation method as shown in Fig. 2. Fragment coupling by the DCC-HOBt method of the tail part **10** and the ring part **11**·HCl, which is derived from **9** by catalytic hydrogenation, and subsequent purification on Sephadex LH-20 column gave the stable precursor **4** in 67% yield.

To convert the Thr residue of the precursor **4** into the ΔAba residue, dehydration of **4** was carried out by methanesulfonylation and subsequent β-elimination. The methanesulfonyl (Ms) group was introduced into **4** using MsCl (6 eq.) in pyridine at 0°C for 24 h to give the methanesulfonylated product (**12**) (δ = 3.09; methyl protons of a Ms group) in 80% yield. Subsequent treatment of **12** with diethylamine (2 eq.) in DMF at room temperature for 6 h afforded the desired peptide lactone **3** in 31% yield after decantation with MeOH and a mixture of DMF-EtOAc. Yields and physical constants of the synthetic intermediates are shown in Table I. The other synthetic intermediates were also characterized by elemental analysis and/or ^{1}H-NMR. The synthetic compound **3** gave a single spot on HPTLC using 3 solvent systems, satisfactory elemental analysis (Table II) and the expected molecular ion peak (M^+: m/z 803) by EI-MS analysis.

Table II

Physicochemical properties of the synthetic and natural products

Compound	M.p. (°C)	$[\alpha]_D$/° (DMSO)	R_f^1	R_f^2	R_f^3
Natural **1**	>250	−8.7	0.55	0.30	0.34
Natural **2**	289–294 (dec)	−2.4	0.56	0.30	0.34
Synthetic **3**	277–293 (dec)	−68.6	0.45	0.20	0.01

HPTLC solvent systems; R_f^1, $CHCl_3$-MeOH (9:1, v/v); R_f^2, $CHCl_3$-MeOH (15:1, v/v); R_f^3, EtOAc-EtOH (10:1, v/v). Elemental analysis of **3**; Found: C, 61.95; H, 7.12; N, 11.80. Calculated for $C_{42}H_{57}O_9N_7 \cdot 1/2H_2O$: C, 62.05; H, 7.19; N, 12.06

2.3. *Antibacterial assay*

The antibacterial activity of both the natural and the synthetic compounds was evaluated by the usual agar dilution method, and the activity was expressed as minimum inhibitory concentration (MIC, μg/ml).

3. RESULTS AND DISCUSSION

In the synthesis of **I** we first focused on the synthesis of a stable precursor peptide, [L-Thr^7]-**I** (**4**) containing a L-Thr residue in place of (Z)-ΔAba at position 7 in **3**, because the Thr residue might be converted to (Z)-ΔAba residue by a dehydration reaction [4]. The compound **4** was successfully synthesized by the fragment coupling of the tail part **10** with the ring part **11**·HCl as shown in Fig. 2. To convert the Thr residue of the precursor **4** into a ΔAba residue, dehydration of **4** was attempted by usual tosylation and subsequent β-elimination with base. However, the tosylation did not proceed perhaps because of the sterically hindered

Table I

Yields and physical constants of synthetic intermediates

Compound	% Yield	R_f[a]	M.p. (°C)	$[\alpha]_D^{20}$/°	Formula	Found (%) (Calculated (%))		
						C	H	N
4	67	0.64 (1)	275–277	−56.3 (*c* 0.5, DMF)	$C_{42}H_{59}O_{10}N_7 \cdot H_2O$	59.90 (60.06	7.31 7.32	11.61 11.67)
5	98	0.82 (2)	oil	–	$C_{36}H_{42}O_{10}N_2$		–	
6	74	0.76 (2)	72–74	+8.6 (*c* 1, EtOAc)	$C_{44}H_{56}O_{12}N_4$	63.25 (63.45	6.93 6.78	6.84 6.73)
7	100	0.65 (1)	oil	–	$C_{40}H_{53}O_{13}N_5$			
8·TFA	100	0.10 (1)	oil	–	$C_{35}H_{45}O_{11}N_5 \cdot TFA$			
9	21	0.69 (1)	90–95	−63.7 (*c* 1, EtOAc)	$C_{31}H_{40}O_8N_4 \cdot H_2O$	60.75 (60.57	6.79 6.89	9.37 9.11)
10	57	0.49 (3)	209–212	+7.9 (*c* 1, DMF)	$C_{26}H_{33}O_5N_3$	66.45 (66.79	6.94 7.11	9.00 8.99)
11·HCl	98	0.23 (1)	oil	–	$C_{16}H_{28}O_6N_4 \cdot HCl$		–	
12	80	0.75 (1)	170–180	–	$C_{43}H_{61}O_{12}N_7S$		–	

[a] Values in parentheses indicate TLC solvent systems; 1, $CHCl_3$-MeOH (5:1, v/v); 2, $CHCl_3$-MeOH (9:1, v/v); 3, $CHCl_3$-MeOH-AcOH (50:10:2, v/v). All the compounds except **8**·TFA, **9** and **11**·HCl were also characterized by NMR

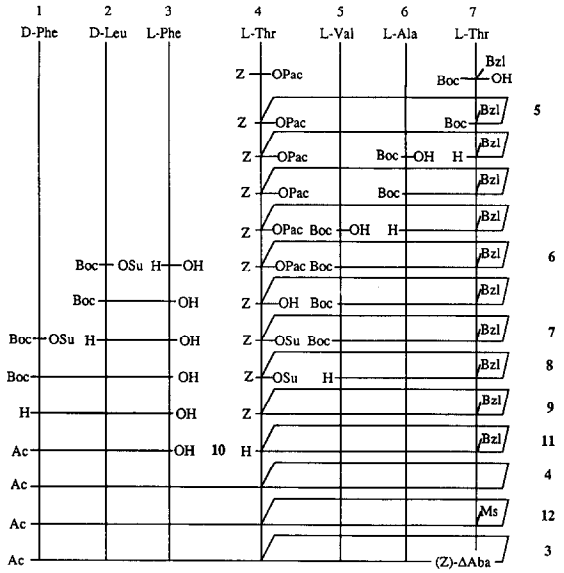

Fig. 2. Synthetic scheme for precursor **4** and **3**.

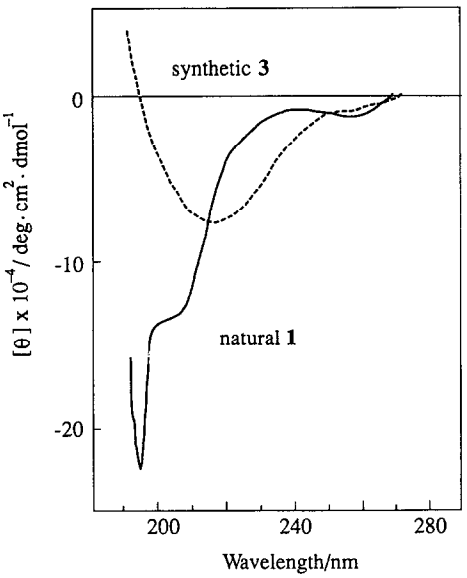

Fig. 3. CD spectra of synthetic **3** and natural **1** in trifluoroethanol.

nature of **4** and/or *p*-toluenesulfonyl chloride. Then introduction of a less sterically hindered Ms group to the hydroxyl function of **4** was attempted using MsCl in pyridine. The methanesulfonylated product **12** was obtained in satisfactory yield. Finally the desired peptide lactone **3** was obtained by the treatment of **12** with diethylamine. The structure of synthetic **3** was confirmed by elemental analysis and by EI-MS.

Then the physicochemical properties of the synthetic **3** and natural products, **1** and **2**, were compared. Some of them are listed in Table II. However, both the melting point and the specific optical rotation of **3** were different from those reported for the natural ones. The behavior of **3** on HPTLC was also different from those of **1** or **2**. But each of the natural products, **1** and **2**, showed identical behavior on the HPTLC. Quantitative amino acid analysis of the acid hydrolysate of the synthetic and natural products reveal that all the products have the same amino acid constituents in identical ratios as shown in Table III. These results caused some doubts to arise concerning the proposed structure **I** for **1** and/or **2**. Therefore, the synthetic and natural products were subjected to the following spectroscopic analyses.

The IR spectra (KBr) of synthetic **3** and natural **1** suggested that both compounds are peptides containing a lactone or an ester linkage: 3290 (N-H); 1660, 1640 (amide I C=O); 1540 (amide II C=O); 1735 (synthetic) and 1725 cm^{-1} (natural) (ester or lactone C=O). However, the IR absorption bands of synthetic **3** in the fingerprint region (1500–650 cm^{-1}) showed small but distinct differences from those of natural **1**.

The ^{1}H-NMR spectra (90 MHz; DMSO-d_6) of **3** and **1** indicated the presence of a (Z)-ΔAba residue and an acetyl group in both compounds: γ-CH_3 protons and a vinyl proton on the side chain of the ΔAba residue (δ = 1.65 (3H, d, J = 7.2 Hz) and 6.75 ppm (1H, q, J = 7.3 Hz) for **3**, and 1.64 (3H, d, J = 7.3 Hz) and 6.70 ppm (1H, q, J = 7.3 Hz) for **1**, respectively) and CH_3 protons in acetyl group (d = 1.72 ppm (3H), s) for **3** and 1.78 ppm (3H, s) for **1**). But some changes in the NMR spectral patterns of **3** and **1** were observed in the methyl and methylene region (0–2 ppm).

Significant differences were observed in the CD spectra (Fig. 3) and the antimicrobial activities (Table IV) of synthetic and natural products, showing that **3** has a different backbone conformation from that of **1** in trifluoroethanol and no biological activity, whereas

Table III

Amino acid composition of acid hydrolysates of synthetic **3** and natural **1** and **2**

Amino acid	3	1	2
Thr	0.99 (1)	0.98 (1)	1.15 (1)
Ala	1.00 (1)	1.00 (1)	1.00 (1)
Val	1.07 (1)	1.09 (1)	1.09 (1)
Leu	0.93 (1)	1.00 (1)	0.98 (1)
Phe	2.02 (2)	2.20 (2)	2.11 (2)
NH_3	1.38 (1)	1.12 (1)	0.65 (1)

Table IV

Antibacterial activity of the synthetic and natural products

Strain	Synthetic	Natural	
	3	1	2
Bacillus subtilis PCI 219	>50	12.5	12.5
Staphylococcus aureus FAD 209 JC-1	>50	1.6	1.6
Escherichia coli NIHJ JC-2	>50	>50	>50
Klebsiella pneumoniae	>50	>50	>50

The values are MIC (μg/ml).

Appendix

Physical constants of synthetic intermediates

Compound	M.p. (°C)	$[\alpha]_D^{20}$/°	Formula	Found (%) (Calculated (%))		
				C	H	N
Z-L-Thr-OPac	126–129	−22.1 (*c* 1, MeOH)	$C_{20}H_{21}O_6N_1$	64.66 (64.88	5.81 5.70	3.81 3.77)
Z-L-Thr[Boc-L-Ala-L-Thr(Bzl)-]-OPac[a]	oil		$C_{39}H_{47}O_{11}N_3$			
Z-L-Thr[Boc-L-Val-L-Ala-L-Thr(Bzl)-]-OH	84	+15.9 (*c* 1, EtOAc)	$C_{36}H_{50}O_{11}N_4$	60.40 (60.49	7.14 7.05	7.97 7.84)
Boc-D-Leu-L-Phe-OH	oil		$C_{20}H_{30}O_5N_2$			
HCl·H-D-Leu-L-Phe-OH	183–188	−16.6 (*c* 1, MeOH)	$C_{15}H_{22}O_3N_2 \cdot HCl \cdot 1/5H_2O$	56.76 (56.58	7.51 7.41	8.64 8.80)
Boc-D-Phe-D-Leu-L-Phe-OH	164–166	+24.4 (*c* 1, MeOH)	$C_{29}H_{39}O_6N_3 \cdot H_2O$	64.02 (64.07	7.38 7.60	7.80 7.73)
HCl·H-D-Phe-D-Leu-L-Phe-OH	224–227	+15.6 (*c* 1, MeOH)	$C_{24}H_{31}O_4N_3 \cdot HCl$	62.20 (62.34	6.94 6.98	9.08 9.10)

[a] Satisfactory NMR data were obtained

natural **1** and **2** exhibited strong inhibitory activities against Gram-positive bacteria.

However, synthetic **3** gave an almost identical EI-mass spectrum to that of natural **1**: a molecular ion peak at m/z 803 and major fragment ion peaks at m/z 703, 632, 614, 533, 450, 303, 190 and 162. The fragmentation pattern in the mass spectrum of **3** supported that synthetic **3** has the same structure **I** except for the stereochemistry of that proposed for **1** and/or **2** [2,3].

The above results imply that the differences between the synthetic and natural products in their physicochemical and biological properties except for the mass spectra might be brought about by their conformational differences possibly caused by the presence of some amino acid residues with a different configuration. Therefore, re-examination of the configuration of the constituent amino acid residues in natural products seemed to be necessary. The configuration of the constituent amino acids in natural **1** and **2** was determined by the modified Manning-Moore method [5]. Coupling of acid hydrolysate of **1** with Z-L-Leu-OSu and subsequent hydrogenolysis gave a mixture of dipeptides (L-Leu-amino acid), which was analyzed by an amino acid analyzer. The results of the chromatogram indicate the presence of L-Ala, L-Val, D-Leu, L-Phe and D-Phe in **1** and **2** as reported before [3] (data not shown). The expected L- or D-Thr could not be detected, however, the presence of D-aThr in **1** and **2** was confirmed. α-Aminobutyric acid (Aba) was also detected in the acid hydrolysate of hydrogenated material of natural **1**.

From all of the results obtained and other reports [2,3], we tentatively propose a revised structure (**II**) for natural TL-119 (**1**) and/or A-3302-B (**2**), which contains a D-aThr residue in place of the original L-Thr residue at position 4, as shown in Fig. 1B. It may be that A-3302-A [3] (which is different from A-3302-B in that it has a propionyl group instead of an acetyl group at the N-terminus) also contains a D-aThr in the cyclic part. It is also noteworthy that many cyclic peptides isolated from microorganisms have a D-amino acid residue in the cyclic part. Further confirmation of the revised structure **II** for **1** and/or **2** by its chemical synthesis is now in progress.

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