Antimicrobial Activity and Conformation of Tachyplesin I and Its Analogs[1)]

Hirokazu TAMAMURA,[a] Rie IKOMA,[a] Makoto NIWA,[b] Susumu FUNAKOSHI,[a] Tsukasa MURAKAMI,[c] and Nobutaka FUJII*,[a]

Faculty of Pharmaceutical Sciences, Kyoto University,[a] Sakyo-ku, Kyoto 606, Japan, Department of General Education, Osaka Institute of Technology,[b] Asahi-ku, Osaka 535, Japan, and Osaka City Institute of Public Health and Environmental Sciences,[c] Tennoji-ku, Osaka 543, Japan. Received October 12, 1992

We investigated the structure–antimicrobial activity relationship of tachyplesin I (T-I). Even when Lys[1] and Trp[2] were both deleted from the N-terminal end of T-I, the antimicrobial activity against gram-negative bacteria was not decreased. But as Lys[1] and Trp[2] were deleted one by one, the antimicrobial activity against gram-positive bacteria and antiviral activity were gradually decreased. Deletion of two disulfide bridges caused a significant decrease in all activities. The circular dichroism (CD) spectra revealed that the analogs containing the two disulfide bidges took a β-sheet structure and that the analogs without the disulfide bridges took a random coil conformation. These results suggest that the β-sheet structure maintained by two disulfide bridges plays an important role in the antimicrobial activity of T-I.

Keywords tachyplesin I; antimicrobial activity; structure–activity relationship; circular dichroism; solution structure; β-sheet structure

Tachyplesin I is a 17-residue antimicrobial peptide isolated from acid extracts of membrane of Japanese horseshoe crab (*Tachypleus tridentatus*) hemocytes.[2)] The primary structure and the disulfide array of tachyplesin I (Fig. 1) were elucidated by chemical analysis,[2)] followed by chemical synthesis.[3)] Tachyplesin I was shown to inhibit the growth of both gram-negative and -positive bacteria at low concentrations and to form a complex with bacterial lipopolysaccharide (LPS), causing precipitation in gel diffusion tests.[2)] Murakami *et al.*[4)] also reported that tachyplesin I displayed direct virus-inactivating activity against vesicular stomatitis virus (VSV) and influenza virus. This property suggests the important role of tachyplesin in the self-defense system of the marine invertebrate against microbial invaders. In the present study, in order to investigate the biological effects of the bicyclic structure and the peptide chain length of tachyplesin I, analog peptides were synthesized by the 9-fluorenylmethyloxycarbonyl (Fmoc)-based solid-phase method and subjected to biological assay. We undertook a conformation study of these analog peptides in aqueous solution by CD spectroscopy.

T-I was previously synthesized.[3)] T-I analogs prepared for this study (*i.e.*, des-Lys[1]–T-I, des-[Lys[1],Trp[2]]–T-I, 4Ala-T-I and 4Cys(Acm)–T-I (Fig. 1)) were similarly synthesized using the Fmoc-based solid-phase method. Fmoc-amino acid derivatives bearing side chain protecting groups based on *tert*-butyl alcohol were employed together with Arg(Mtr) and Cys(Acm). Each peptide was cleaved from the resin by thioanisole-mediated deprotection with TMSOTf.[5)] The crude peptides, except for the linear analogs (4Ala–T-I and 4Cys(Acm)–T-I), were treated with

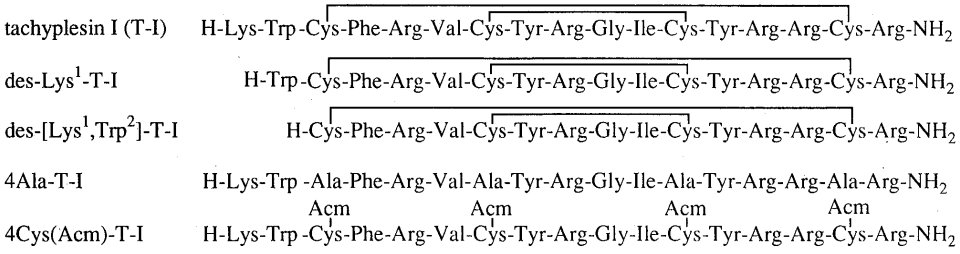

Fig. 1. Amino Acid Sequences of Tachyplesin I and Its Synthetic Analogs

TABLE I. Antibacterial Activity of T-I and Its Analogs

Bacteria strain	T-I		Des-Lys[1]–T-I		Des-[Lys[1],Trp[2]]–T-I		4Ala–T-I		4Cys(Acm)–T-I	
	MIC	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*
Gram (−)										
Salmonella typhimurium LT2(S)	3.13	3.13	6.25	6.25	6.25	12.5	50	50	12.5	25
Salmonella typhimurium 1102(Re)	1.56	12.5	6.25	6.25	3.13	6.25	12.5	12.5	6.25	12.5
Escherichia coli K12	3.13	6.25	6.25	6.25	3.13	6.25	25	25	12.5	50
Gram (+)										
Staphylococcus aureus 209P	12.5	>12.5	25	>25	50	50	>50	>50	25	>50

MIC or *MBC*: μg/ml.

silver trifluoromethanesulfonate (AgOTf)[6] to remove the Acm groups from the Cys residue. After treatment with DTT, each reduced peptide was subjected to air-oxidation at pH 7.5 to establish the disulfide bonds. Each peptide was purified by high-performance liquid chromatography (HPLC). Each synthetic peptide was proved to be a monomer by fast atom bombardment-mass spectrometry (FAB-MS). The position of the disulfide linkages in each analog, except for the linear analog, was determined from the amino acid compositions of the peptides derived from tryptic digest of the intact peptide, as in the case of T-I.[2]

The minimum inhibitory concentrations (*MIC*) and the minimum bactericidal concentrations (*MBC*) of tachyplesin I and its analogs against several bacterial strains were determined by the microplate culture method[2] (Table I). The strains used in this study were *Salmonella typhimurium* strains LT2 (S) and SL1102 (Re) and *Escherichia coli* K12 (gram-negative bacteria) and *Staphylococcus aureus* strains 209P (gram-positive bacteria). In the antimicrobial assay against strains of gram-negative bacteria, des-Lys^1–T-I and des-[Lys^1,Trp^2]–T-I possessed almost the same potency as T-I. 4Ala–T-I and 4Cys(Acm)–T-I, which contained Ala or Cys(Acm) instead of Cys at four modification sites, exhibited very low activity. However, against a strain of gram-positive bacteria, T-I exhibited the most potent activity. The activity of des-Lys^1–T-I and des-[Lys^1,Trp^2]–T-I was reduced in that order. Bacteriostatic effects of 4Ala–T-I and 4Cys(Acm)–T-I were very weak.

Among the viruses which were previously tested [VSV, influenza virus (A/Yamagata/120/86, H1N1) herpes simplex virus (HSV)-1 (HF), HSV-2 (UW268), adenovirus-1 (Ad.7), reovirus-2 (Jones) and poliovirus (Sabin)], VSV was the most susceptible to inactivation by T-I.[4] Consequently we examined the antiviral activities of T-I and its analogs against VSV. The log $TCID_{50}$ (50% tissue culture infective dose) of VSV treated with each peptide is shown in Table II. T-I showed the most potent antiviral activity, and 4Ala–T-I was the least potent. This potency decreases in the order T-I > des-Lys^1–T-I > des-[Lys^1,Trp^2]–T-I, 4Cys(Acm)–T-I > 4Ala–T-I, which is compatible with that of the antimicrobial activity against gram-positive bacteria.

CD spectra of tachyplesin I and its analogs in aqueous solution are shown in Fig. 2. T-I exhibited a strong negative band near 210 nm and a strong positive band near 198 nm. These bands are due to a β-sheet structure.[7] This result is compatible with the nuclear magnetic resonance (NMR) data; Kawano *et al.* revealed by two-dimensional NMR that tachyplesin I formed a rigid antiparallel β-sheet structure connected by a β-turn.[8] The CD spectra of des-Lys^1–T-I and des-[Lys^1,Trp^2]–T-I were similar to that of tachyplesin I and these analogs were also considered to form a β-sheet structure. These three peptides exhibited a strong positive band near 228 nm and this band was thought to be due to a β-turn.[7] On the other hand, the CD spectra of 4Ala–T-I and 4Cys(Acm)–T-I were very different from that of T-I. These two analogs exhibited a strong negative band near 198 nm. This band is due to a random coil conformation.[7]

These results suggest that for the expression of the potent antimicrobial activity against gram-negative bacteria, the rigid antiparallel pleated β-sheet structure maintained by the two disulfide bridges is essential, but the two amino acids (Lys^1,Trp^2) at the N-terminal end are not necessary, whereas for the expression of the potent antimicrobial activity against gram-positive bacteria and antiviral activity, this β-sheet structure and the original chain length are indispensable.

Table II. Antiviral (Anti-VSV) Activity of T-I and Its Analogs

	T-I	Des-Lys^1–T-I	Des-[Lys^1, Trp^2]–T-I	4Ala–T-I	4Cys(Acm)–T-I
log $TCID_{50}$	5.75	6.00	6.25	6.50	6.25
Δ	1.75	1.50	1.25	1.00	1.25

Δ = log $TCID_{50}$ treated with PBS control (7.50) − log $TCID_{50}$ treated with each peptide.

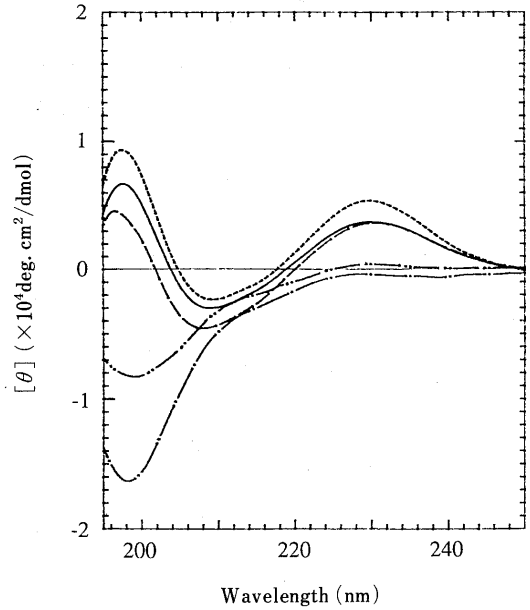

Fig. 2. CD Spectra of T-I and Its Analogs in H_2O

T-I (——), des-Lys^1–T-I (- - - - -), des-[Lys^1,Trp^2]–T-I (— —), 4Ala-T-I (—·—), 4Cys(Acm)-T-I (—· ·—).

Tachyplesin I strongly binds to LPS and significantly inhibits the LPS-mediated activation of factor C, the initiation factor in the limulus clotting cascade, consequently expressing the antimicrobial activity against gram-negative bacteria.[2] The approximate distance between the two phosphate groups of the 1—6 diglucoside-1,4′-diphosphate glycolipid, a major constituent of LPS[9] is 6—8 Å, so that two pairs of guanidino groups of arginine side chains (Arg^5 and Arg^{14}, Arg^{15} and Arg^{17}) can be adapted to interact with those phosphate groups, causing cross linking of LPS.[10] The rigid antiparallel β-sheet structure is thought necessary for fixing the direction of these two pairs of arginine side chains. Furthermore, it is supposed that this amphiphilic β-sheet peptide binds to the membrane to trigger the antimicrobial activity.[11]

In this study, the biological effects of the bicyclic structure and the peptide chain length of tachyplesin I were disclosed. These results will aid in making clear the molecular mechanism of the antimicrobial activity of tachyplesin I.

Experimental

Syntheses of T-I Analog Peptides T-I was previously synthesized.[3] T-I analogs prepared for this study (*i.e.*, des-Lys[1]–T-I, des-[Lys[1],Trp[2]]–T-I, 4Ala–T-I and 4Cys(Acm)–T-I (Fig. 1)) were similarly synthesized using the Fmoc-based solid-phase method according to the principle of Sheppard *et al.*[12] Fmoc-amino acid derivatives bearing protecting groups based on *tert*-butyl alcohol were employed, together with Arg(Mtr) and Cys(Acm), and condensed successively using DIPCDI in the presence of HOBt on the DMBHA resin.[13] Each protected peptide was cleaved from the corresponding resin and deprotected by treatment with 1 M TMSOTf–thioanisole/TFA in the presence of *m*-cresol (100 eq) and EDT (300 eq) at 4 °C for 2 h.[5] Each resin was removed by filtration, and ice-chilled dry ether was added to precipitate each product. After being washed with ice-chilled dry ether, the peptide was dissolved in 4 N AcOH. The crude material was gel-filtered on a Sephadex G-10 column, and eluted with 4 N AcOH. The crude peptide, except for the linear analogs (4Ala–T-I and 4Cys(Acm)–T-I), was treated with AgOTf (80 eq) in TFA in the presence of 1% anisole at 4 °C for 1 h.[6] Ice-chilled dry ether was added to the reaction mixture to precipitate each product. After being washed with ice-chilled dry ether, the product was dissolved in 4 N AcOH, and DTT (80 eq) was added. The mixture was stirred at room temperature overnight and gel-filtered on a Sephadex G-10 column with 4 N AcOH, followed by air oxidation at 0.1 mg/ml peptide concentration in 0.05 M ammonium acetate (pH 7.5) at 4 °C for 5 d. Finally, the crude peptide was purified by HPLC on an Asahipak ODP-90 column (21.5 × 300 mm), which was eluted with a gradient of CH_3CN (20—40%, 40 min) in aqueous 0.1% TFA at the flow rate of 7 ml/min. The eluate corresponding to the main peak was collected and the solvent was removed by lyophilization to give a white fluffy powder. Optical rotations of T-I analogs in 1 N AcOH were measured with a JASCO DIP-360 digital polarimeter, and FAB mass spectra were recorded on a VG Analytical ZAB-SE instrument (Table III).

Bacterial Strains and Culture Media *Salmonella typhimurium* strains LT2 (S) and SL1102 (Re), *Escherichia coli* K12, *Staphylococcus aureus* strains 209P were used. Heart infusion broth (Difco Laboratories, Detroit, U.S.A.) was used for subculture of the bacteria. For the growth inhibition tests of the bacteria, a synthetic Jarvis's medium (abbreviated as JY medium)[14] supplemented with 2 mg/ml of yeast extract (Difco Laboratories) was employed.

Antibacterial Activity *MIC* and *MBC* were determined as follows.[2] Twenty μl of serial dilutions of T-I or its analogs was added to mixtures of 20 μl of bacterial suspension (approximately 10^6/ml) and 160 μl of JY medium in each well of a flat-bottomed microplate (The Data Packing Corp., Cambridge, MA). After incubation at 37 °C for 18—20 h, absorbance at 550 nm was read on a microplate reader (Corona MTV-100P) taking the absorbance of a well filled with uninoculated media as a reference. *MIC* was expressed as the lowest final concentration (μg/ml) at which no growth was observed. *MBC* was expressed as the lowest final concentration (μg/ml) at which bacteria are killed.

TABLE III. Optical Rotations and FAB Mass Spectra of T-I Analogs

	Des-Lys[1]–T-I	Des-[Lys[1], Trp[2]]–T-I	4Ala–T-I	4Cys(Acm)–T-I
$[\alpha]_D^{20}$ (°)	0	−6.5	−38.3	−38.5
FAB-MS *m/z*	2135.1	1948.9	2139.3	2551.0
(Calcd) $[M+H]^+$	(2134.0)	(1947.9)	(2138.2)	(2550.3)

Virus Inactivation[4] VSV was supplied by Dr. K. Yamazaki of Osaka Prefectural Institute of Public Health. T-I and its analogs (125 μg/ml, final) diluted with PBS (pH 7.0) were incubated with VSV at 37 °C for 120 min in a CO_2 incubator. PBS was used as a control. The cytopathic effect $TCID_{50}$ was determined in 96-well microtiter plates.

CD Spectroscopy The CD spectra were obtained using a JASCO spectropolarimeter, model J-720, having a 10 mm cell. The concentration of tachyplesin I and its analogs in water was 10^{-5} M.

Acknowledgements The authors are grateful to Prof. Sadaaki Iwanaga, Kyushu University, for valuable discussions. Our thanks are also extended to Drs. Hirokazu Tanaka and Nobuharu Shigematsu, Tsukuba Research Laboratory, Fujisawa Pharmaceutical Co., Ltd., for measurement of FAB mass spectra.

References and Notes

1) Amino acids used here are of the L-configuration. The following abbreviations are used: Acm = acetamidomethyl, Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl, TMSOTf = trimethylsilyl trifluoromethanesulfonate, DTT = dithiothreitol, TCID = tissue culture infective dose, DIPCDI = 1,3-diisopropylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, DMBHA = 2,4′-dimethoxybenzhydrylamine, TFA = trifluoroacetic acid, EDT = 1,2-ethanedithiol, PBS = phosphate-buffered saline.
2) T. Nakamura, H. Furunaka, T. Miyata, F. Tokunaga, T. Muta, S. Iwanaga, M. Niwa, T. Takao, and Y. Shimonishi, *J. Biol. Chem.*, **263**, 16709 (1988).
3) K. Akaji, N. Fujii, F. Tokunaga, T. Miyata, S. Iwanaga, and H. Yajima, *Chem. Pharm. Bull.*, **37**, 2661 (1989).
4) T. Murakami, M. Niwa, F. Tokunaga, T. Miyata, and S. Iwanaga, *Chemotherapy*, **37**, 327 (1991).
5) N. Fujii, A. Otaka, O. Ikemura, K. Akaji, S. Funakoshi, Y. Hayashi, Y. Kuroda, and H. Yajima, *J. Chem. Soc., Chem. Commun.*, **1987**, 274.
6) N. Fujii, A. Otaka, T. Watanabe, A. Okamachi, H. Tamamura, H. Yajima, Y. Inagaki, M. Nomizu, and K. Asano, *J. Chem. Soc., Chem. Commun.*, **1989**, 283.
7) C. T. Chang, C.-S. C. Wu, and J. T. Yang, *Anal. Biochem.*, **91**, 13 (1978).
8) K. Kawano, T. Yoneya, T. Miyata, K. Yoshikawa, F. Tokunaga, Y. Terada, and S. Iwanaga, *J. Biol. Chem.*, **265**, 15365 (1990).
9) C. Galanos, O. Luderitz, E. T. Rietschel, O. Westphal, H. Brade, L. Brade, M. Freudenberg, U. Schade, M. Imoto, H. Yoshimura, S. Kusumoto, and T. Shiba, *Eur. J. Biochem.*, **148**, 1 (1985).
10) H. Tamamura, M. Kuroda, M. Masuda, A. Otaka, S. Funakoshi, H. Nakashima, N. Yamamoto, M. Waki, A. Matsumoto, N. Fujii, J. M. Lancelin, D. Kohda, S. Tate, and F. Inagaki, A comparative study of the solution structures of tachyplesin I and a novel anti-HIV synthetic peptide, T22 ([Tyr[5,12],Lys[7]]-polyphemusin II), determined by nuclear magnetic resonance, *Biochim. Biophys. Acta*, in press.
11) K. Matsuzaki, M. Fukui, N. Fujii, and K. Miyajima, *Biochim. Biophys. Acta*, **1070**, 259 (1991).
12) A. Dryland and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, **1986**, 125.
13) S. Funakoshi, E. Murayama, L. Guo, N. Fujii, and H. Yajima, *J. Chem. Soc., Chem. Commun.*, **1988**, 382.
14) F. G. Jarvis, M. T. Mesenko, and K. E. Tibba, *J. Bacteriol.*, **80**, 673 (1960).