Synthesis and Antimicrobial Activity of Bactenecin and Its Analogs

Yoshiki UCHIDA* and Mitsuno SHINDO
Laboratory of Food Hygienics, Department of Food Sciences,
Faculty of Liberal Arts, Osaka Shoin Women's College,
Hishiyanishi, Higashi-Osaka 577
(Received August 26, 1991)

Synopsis. Bactenecin and four analogs were synthesized by Merrifield's solid phase method. The antimicrobial activity and conformation of the synthetic peptides were studied by the microplate dilution method and CD spectroscopy, respectively. The results suggest that the original sequence and cyclic structure are important for the expression of the potent antimicrobial activity of bactenecin. The CD spectrum of bactenecin revealed a profile corresponding to that of the random coil conformation.

Neutrophilic leukocytes of higher animals are major constituents of host defense systems against invading microorganisms. The cytoplasmic granules of neutrophils contain a group of highly cationic polypeptides which exert potent bactericidal activity in vitro.[1,2] In 1988, a novel antibacterial peptide, bactenecin, was isolated by Romeo et al. from bovine neutrophils.[3] The peptide has the amino acid sequence, H–Arg–Leu–Cys–Arg–Ile–Val–Val–Ile–Arg–Val–Cys–Arg–OH,[4] and contains a cyclic structure formed by a disulfide bond between the two cysteine residues. This dodecapeptide was reported to exhibit antibacterial activity against *E. coli* and *S. aureus*. The mechanism of action may involve processes of an interaction with and a translocation through the bacterial membranes. Conformation analysis of bactenecin using a computer modeling system suggested that the peptide chain exhibits an antiparallel extended structure forming a γ turn at residue 7.[3]

Studies of the chemical synthesis and the structure-function relationship of bactenecin have not yet been the subject of any other reports. In the present investigation, in order to clarify the biological effects of the cyclic structure and the peptide chain length of bactenecin, we synthesized bactenecin and its four analogs, and subjected them to biological assay. Furthermore, to obtain a better understanding of the structure-function relationship of the synthetic peptides, the conformations of the peptides were studied by circular dichroism (CD) spectroscopy.

Bactenecin and the analogs shown in Fig. 1 were synthesized by the solid phase technique,[5] using a manual apparatus. All coupling reactions were carried out by the dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) method according to the program shown in Table 1. The protective groups used were the *t*-butoxycarbonyl (Boc) group for the α-amino function, and the *p*-methoxybenzyl (MBzl) and *p*-toluenesulfonyl (Tos) groups for the Cys and Arg side chains, respectively. N^{α}–Boc deblocking was achieved with trifluoroacetic acid (TFA). The synthetic products, except for the linear analog **4**, were then subjected to air oxidation under extremely dilute conditione to allow slow formation of the disulfide bond. The crude peptide was passed through a Sephadex G-25 column, followed by preparative reversed-phase high-performance liquid chromatography (HPLC). The purity of

Table 1. Program for Solid Phase Peptide Synthesis

	Reagent	Operation	Mix time/min
1	CH_2Cl_2	6×Wash	1
2	TFA (33% in CH_2Cl_2)	Prewash	2
3	TFA (33% in CH_2Cl_2)	Deprotection	30
4	CH_2Cl_2	3×Wash	1
5	CH_2Cl_2 : DMF (1 : 1)	3×Wash	1
6	CH_2Cl_2	3×Wash	1
7	TEA (10% in CH_2Cl_2)	3×Neutralization	1
8	CH_2Cl_2	3×Wash	1
9	CH_2Cl_2 : DMF (1 : 1)	3×Wash	1
10	Boc–Amino Acid and HOBt (2.5 mmol equiv each) in CH_2Cl_2 : DMF (1 : 1)	Mix	5
11	DCC in CH_2Cl_2	Coupling	180
12	CH_2Cl_2 : DMF (1 : 1)	3×Wash	1
13	CH_2Cl_2	2×Wash	1
14	MeOH	2×Wash	1
15	CH_2Cl_2	3×Wash	1

H-Arg-Leu-Cys-Arg-Ile-Val-Val-Ile-Arg-Val-Cys-Arg-OH — Bactenecin

1 H-Leu-Cys-Arg-Ile-Val-Val-Ile-Arg-Val-Cys-Arg-OH — Bactenecin (2-12)

2 H-Cys-Arg-Ile-Val-Val-Ile-Arg-Val-Cys-Arg-OH — Bactenecin (3-12)

3 H-Arg-Leu-Cys-Arg-Ile-Val-Val-Ile-Arg-Val-Cys-OH — Bactenecin (1-11)

4 H-Arg-Leu-Ala-Arg-Ile-Val-Val-Ile-Arg-Val-Ala-Arg-OH — [$Ala^{3,11}$]-Bactenecin

Fig. 1. Amino acid sequences of bactenecin and its synthetic analogs.

Table 2. Physical Properties and Yields of Synthetic Peptides

Analog	$[\alpha]_D^{17}$/° (c=0.3, 2 M AcOH)	Retention time/min	R_f^I	R_f^{II}	Yield/%
Bactenecin	−16.7	18.0	0	0.41	17.7
1	+15.6	19.7	0	0.48	15.1
2	−3.3	11.5	0	0.40	12.6
3	+12.5	18.7	0	0.45	15.2
4	−62.5	7.7	0	0.44	36.4

Table 3. Amino Acid Analyses of Synthetic Peptides

Analog	Found (Calcd)						
	Ala	$(Cys)_2$	Val	Ile	Leu	Arg	NH_3
Bactenecin	—	0.94(1)	2.87(3)	1.98(2)	1.10(1)	4.10(4)	0.30(0)
1	—	0.91(1)	2.87(3)	1.93(2)	1.08(1)	3.20(3)	0.35(0)
2	—	0.96(1)	2.86(3)	1.92(2)	—	3.26(3)	0.50(0)
3	—	1.00(1)	2.84(3)	1.89(2)	1.11(1)	3.16(3)	0.89(0)
4	2.09(2)	—	2.86(3)	1.98(2)	1.11(1)	3.96(4)	0.54(0)

Table 4. Antibacterial Activity of Synthetic Bactenecin and Its Analogs

Organism	MIC/μg ml^{-1}				
	Bactenecin	**1**	**2**	**3**	**4**
B. subtilis IFO 3513	3.13	3.13	6.25	6.25	25
B. megaterium ATCC 19213	1.56	3.13	6.25	3.13	12.5
S. aureus IFO 12732	3.13	3.13	6.25	6.25	12.5
S. epidermidis IFO 12993	3.13	3.13	6.25	6.25	25
S. faecalis IFO 3989	3.13	6.25	12.5	6.25	100
E. coli IFO 12734	25	50	100	50	>100
S. typhimurium IFO 12529	12.5	25	100	25	>100
K. pneumoniae IFO 3317	50	100	>100	>100	>100
S. marcescens IFO 3046	>100	>100	>100	>100	>100
P. aeruginosa IFO 3080	25	100	>100	50	>100

the synthetic peptides was assessed by analytical HPLC, high-performance thin-layer chromatography (HPTLC) and amino acid analysis. These data are presented in Tables 2 and 3. The peptides were considered to be appropriately pure for bioassay and CD spectral measurement.

The minimum inhibitory concentrations (MIC), i.e., the minimum amounts of the peptides necessary for complete inhibition of the growth of various kinds of bacteria, are shown in Table 4. The strains used in this study were five kinds each for gram positive (upper section in Table 4) and gram negative (lower section in Table 4) bacteria, respectively. The synthetic bactenecin tested here showed high antimicrobial activity against gram positive bacteria such as *B. subtilis, B. megaterium,* and *S. aureus*, but slightly lower activity against gram negative bacteria such as *E. coli* and *P. aeruginosa.* The characteristics of the antimicrobial activity of the synthetic bactenecin were similar to those of naturally occurring bactenecin, which was active against *S. aureus* (a gram positive bacterium) rather than *E. coli* (a gram negative one). The synthetic bactenecin showed MIC against *E. coli* and *S. aureus* of 25 and 3.13 μg ml^{-1}, respectively. This potency was exactly the same as that of the natural one, as reported by Romeo et al.[3] The analogs **1** and **3**, which lack an Arg residue at the N- or C- terminal in comparison with bactenecin, possessed activity against all microorganisms tested except for *S. marcescens* and/or *K. pneumoniae.* On the other hand, the activity of analog **2**, in which the N-terminal two amino acid residues of bactenecin are eliminated, was reduced against gram positive bacteria. Furthermore, in the case of analog **2**, bacteriostatic effects against gram negative bacteria such as *K. pneumoniae* and *P. aeruginosa* were absent. The replacement of Cys residues at positions 3 and 6 with Ala moieties brought about a further decrease of the antibacterial activity. The activity of analog **4**, especially, against gram negative bacteria was absent. The order of antimicrobial potency of the synthetic peptides prepared in this study was bactenecin >**1**>**3**>**2**>**4**. These results indicate that the original chain length and a cyclic structure are necessary for the antimicrobial activity of bactenecin.

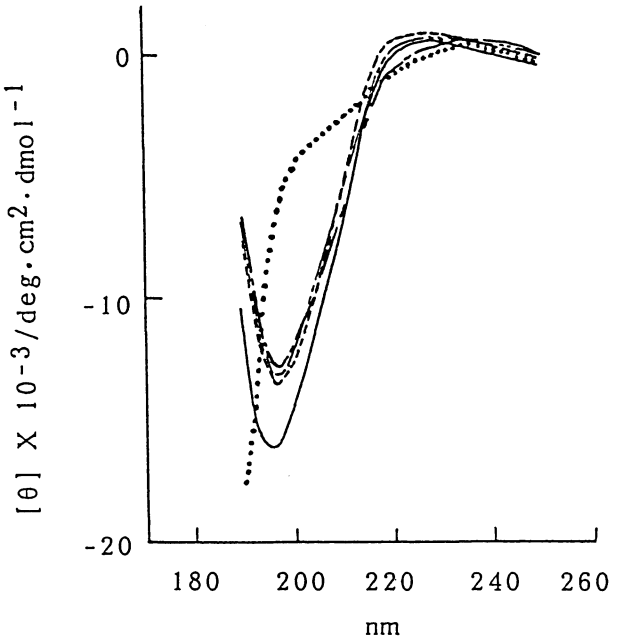

Fig. 2. CD spectra of synthetic peptides in H_2O.
——; Bactenecin, ----; **1**, —·—; **2**, —··—; **3**, ·····; **4**.

CD spectra of the synthetic peptides bactenecin and analogs **1**—**4** in H_2O are shown in Fig. 2. Bactenecin exhibited a weak band near 220 nm and a strong negative band near 198 nm. These bands were responsible for those of a random coil conformation.[6] The CD spectra of analogs **1**—**3** were found to be similar to that of bactenecin, although their troughs (198 nm) were shallow. The antibacterial activity of analogs **1**—**3** was less than that of bactenecin. Furthermore, the CD spectrum of analog **4** was very different from that of bactenecin and the lowest bacteriostatic activity was observed in this analog. These results suggest that the steady random coil-like structure observed in the CD

spectrum of bactenecin itself is important for the expression of the potent antimicrobial activity of this agent.

Experimental

Optical rotations were measured on a JASCO DIP-370 Polarimeter. HPTLC was carried out on silica-gel plates (Merck, NJ, USA). The following solvent systems were used and allowed to ascend for 10 cm: R_f^I, *n*-BuOH : AcOH : H_2O (4 : 1 : 5, upper phase); R_f^{II}, *n*-BuOH : pyridine : AcOH : H_2O (4 : 1 : 1 : 2). The peptides on silica-gel plates were detected with ninhydrin and chlorine-*o*-tolidine reagents. The CD spectra were obtained by use of a JASCO spectropolarimeter, model J-500A, using a 0.5 mm quartz cell. The CD spectroscopy of bactenecin and the analogs was carried out with aqueous solutions at a concentration of 10^{-4} M. Analytical HPLC was performed on a μBondasphere 5μ C18 column (3.9×150 mm) by gradient elution with the following solvent system: A, 20 mM phosphate buffer (pH 3.0) and B, CH_3CN. A linear gradient from 15% B to 25% over 15 min, at a flow rate of 1.0 ml min^{-1}, was used and the eluate was monitored at 210 nm. Amino acid analyses of samples previously hydrolyzed with 6 M HCl (1 M=1 mol dm^{-3}) (110 °C, 48 h) were performed on a Hitachi L-8500 Amino Acid Analyzer.

Peptide Synthesis. The peptide used in this study was synthesized by Merrifield's solid phase method starting from Boc-Arg(Tos) or Boc-Cys(MBzl) chloromethyl-resin (Watanabe Chemical Industries, Ltd., Hiroshima, Japan). For the incorporation of each N^{α}-Boc-protected amino acid, the program shown in Table 1 was used. The amino acid derivatives (Peptide Institute, Inc., Osaka, Japan) were dissolved in dichloromethane (CH_2Cl_2), except for Boc-Arg(Tos)-OH, which was dissolved in *N*,*N*-dimethylformamide (DMF), and coupled by the DCC method for 3 h. A 2.5-fold excess of the amino acid derivative was used for all couplings. One equivalent of HOBt was used as an additive to facilitate the repetitive coupling reaction, using the program without step 1—3. Completion of the coupling reaction of each amino acid was checked by Kaiser's ninhydrin test[7] and, if necessary, was repeated by the same method. After repetition of the coupling two or three times, the unreacted amino groups were blocked completely by acetylation, using an excess of acetic anhydride (5 equiv of resin used) in the presence of pyridine (1 equiv) in a mixture of DMF and CH_2Cl_2 (1 : 1) for 20 min. The Boc group was removed by treating the resin with 33% TFA in CH_2Cl_2 for 2 and 30 min at room temperature. Ten percent TEA in CH_2Cl_2 was used for neutralization (1 min, 3 times). After final coupling, the protected peptide was cleaved from the resin and deprotected by treatment with anhydrous hydrogen fluoride (HF)[8] containing 10% anisole at 0 °C for 60 min. After evaporation of HF, the peptide-resin mixture was washed with ethyl acetate and then the peptide was extracted with 2 M AcOH. The crude peptides, except for the linear analog **4**, were subjected to air oxidation at 3 L $mmol^{-1}$ of peptide concentration in 0.04 M ammonium acetate (pH 6.8) at 4 °C for 3 d.

Purification. The crude material was gel-filtered on a Sephadex G-25 column (23×400 mm), eluting with 2 M AcOH. Further purification was carried out by preparative reversed-phase HPLC (System 600E, Waters, MA, USA) on a column (19×150 mm) of μBondasphere 5μ C_{18}, which was eluted with a CH_3CN-0.1% TFA solvent system at a flow rate of 7.0 ml min^{-1}. Eluate was monitored at 210 nm, and each peptide emerged at 40—60 min by isocratical elution with 19—24% of the CH_3CN content of the solvent system. The desired fraction was applied to a Sephadex G-25 column eluted with 2 M AcOH and the product was lyophilized.

Bacteria. *Bacillus subtilis* IFO 3513, *Bacillus megaterium* ATCC 19213, *Staphylococcus aureus* IFO 12732, *Staphylococcus epidermidis* IFO 12993, *Streptococcus faecalis* IFO 3989, *Escherichia coli* IFO 12734, *Salmonella typhimurium* IFO 12529, *Klebsiella pneumoniae* IFO 3317, *Serratia marcescens* IFO 3046 and *Pseudomonas aeruginosa* IFO 3080 were grown overnight at 37 °C on nutrient agar medium and harvested in sterile saline. Densities of bacterial suspensions were determined at 600 nm, using a standard curve relating absorbance to number of colony forming units (CFU).

Assay of Antimicrobial Activity. MIC of the synthetic peptides against ten bacterial strains were assayed by the microplate dilution method as follows: 100 μl of serial dilution of analogs was added to a mixture of 10 μl of bacterial suspension (approximately 10^6 CFU ml^{-1}) and 90 μl of Mueller-Hinton broth (Difco Laboratories, MI, USA) in each well of a flat-bottomed microplate (Corning Laboratory Sciences Company, II, USA). The highest peptide concentration tested was 100 μg ml^{-1}. The plates were then incubated overnight at 37 °C for MIC evaluation. Controls were run by replacing the peptide solution with sterile saline alone. MIC was expressed as the lowest final concentration (μg ml^{-1}) at which no growth was observed.

The authors are grateful to Dr. Saburo Aimoto and Mr. Hironobu Hojo of the Institute for Protein Research, Osaka University, for their helping the preparation of peptides.

References

1) R. Gennaro, L. Dolzani, and D. Romeo, *Infect. Immun.*, **40**, 684 (1983).
2) R. Marzari, B. Scaggiante, B. Skerlavaj, M. Bittolo, R. Gennaro, and D. Romeo, *Infect. Immun.*, **56**, 2193 (1988).
3) D. Romeo, B. Skerlavaj, M. Bolognesi, and R. Gennaro, *J. Biol. Chem.*, **263**, 9573 (1988).
4) Amino acids, peptides and their derivatives mentioned in this paper are of L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **139**, 9 (1984).
5) R. B. Merrifield, *Adv. Enzymol.*, **32**, 221 (1969).
6) W. C. Johnson, Jr., *Annu. Rev. Biophys. Chem.*, **17**, 145 (1988).
7) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
8) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).