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Antimicrobial Peptides Containing Arginine

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Abstract—Tetradecapeptides $(RLARLAR)_2$, *D*-$(RLARLAR)_2$, $(RLARLAA)_2$, and $(RLGRLGR)_2$ were synthesized by a solid phase method using Fmoc-amino acids. The antibacterial activity of the synthesized peptides was studied against *Escherichia coli* cells. The minimum inhibitory concentration (MIC) was, correspondingly, 3, 1, 3, and 12 μM, which is comparable with MIC of such natural antimicrobial peptides as temporin, magainin, and dermaseptin. It was found that all of the synthesized peptides have no effect on human erythrocytes and rat thymocytes. The peptides form α-helices in 30% trifluoroethanol and in 2.5 mM SDS, which have amphipathic structure.

Key words: antimicrobial peptides, amphipathic peptides, solid phase synthesis

At present more than a hundred natural antimicrobial peptides isolated from different animals are known. The analysis of their structures has shown that practically all of them are amphipathic substances with both hydrophobic (leucine, alanine, isoleucine) and positively charged amino acids (mainly lysine) [1].

External bacterial membranes are negatively charged whereas membranes of animal cells are mostly neutral [2]. Antimicrobial peptides are positively charged; therefore, they interact predominantly with bacterial membranes, remaining nontoxic for eukaryotic cells. Interacting with a bacterial membrane, antimicrobial peptides disrupt its structure and alter its permeability [3]. As a result of the universal mechanism of action, antimicrobial peptides are destructive for microorganisms that have developed resistance to various antibiotics. Most antimicrobial peptides, when interacting with the membrane, form amphiphilic α-helices, i.e., α-helices which have positively charged amino acids (lysine or arginine) on one side and hydrophobic amino acids on the other (leucine, phenylalanine, isoleucine, etc.). However, some of the antimicrobial peptides have also a β-structure [4].

The synthesis of antibacterial peptides from *D*-amino acids has been described recently. These peptides have been shown to have the same activity as that of the corresponding *L*-peptides [5, 6]. However, in contrast to *L*-peptides, *D*-peptides are perfectly resistant to the action of proteases [5]. The knowledge of the structure-and-function dependence of antimicrobial peptides would allow constructing model peptides consisting of a minimal set of hydrophobic and positively charged amino acids [7].

This paper is devoted to the synthesis and analysis of antimicrobial amphipathic tetradecapeptides containing arginine as a positively charged amino acid and leucine as a hydrophobic amino acid. The peptides also contain alanine and glycine.

MATERIALS AND METHODS

Amino acid derivatives, alkoxybenzyl polymer, and TBTU from Novabiochem (Switzerland) were used. An LKB chromatograph (Sweden) was used for HPLC experiments, Separon C-18 columns (Czechia) being used for analytical chromatography experiments, and Octadecyl Si100 3 μ, 22.0 × 250 mm for preparative chromatography. Mass-spectroscopy experiments were performed on a Vision 2000 instrument (Great Britain) and electron microphotography on a JEM 100C electron microscope (Japan). A Jasco G600 spectropolarimeter (Japan) was used to obtain CD spectra.

Abbreviations: Fmoc) 9-fluorenylmethoxycarbonyl; HEPES) N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); PMC) 2,2,5,7,8-pentamethylchromansulfonyl; TBTU) 2-benzotriazolyl-1,1,3,3-tetramethyluronium tetrafluoroborate; MIC) minimal inhibiting concentration.

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Peptide synthesis. Solid phase synthesis of peptides was done on alkoxybenzyl polymer with hydroxyl group content of 1.05 mmol/g using a standard technique [8].

The synthesized peptides were purified using reverse-phase HPLC in an acetonitrile concentration gradient in water from 5 to 100% with the eluent expenditure of 0.8 ml/min. The eluent absorption was recorded at 226 nm.

The peptides synthesized were characterized by the results of analytical HLPC, mass-spectroscopy, and amino acid analysis (Table 1).

Antimicrobial activity of peptides. To test the antimicrobial activity of the peptides, a highly sensitive method of inhibiting the growth region was used. The inhibition of the growth region was tested on strain D21 *E. coli* cells defective in lipopolysaccharide coat components [9].

Bacterial cells were grown overnight at 37°C in 5-ml volume of 3% tryptic soybean hydrolyzate (TSB) solution. To obtain cells of mid-logarithmic phase of growth, 10 μl of the night culture were added to 5-10 ml of fresh growth TSB solution, and the mixture was incubated for 2.5 h at 37°C. Bacteria were precipitated by 10-min centrifugation at 2000 rpm at 4°C; then they were washed twice with cold phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4), pH 7.4, and resuspended in 1 ml of the same buffer. The optical density (OD) was measured at the wavelength of 620 nm and the colony formation units (CFU) were counted using the known empirical ratio:

$$CFU = 25 \cdot 10^7 \, OD_{620}.$$

The aliquot containing $5 \cdot 10^6$ CFU was added to 10 ml of the phosphate buffer containing 3 mg TSB, 0.02% Tween-20, and 1% of melted agarose. After quick stirring, the warm mixture was poured in a Petri dish (the layer thickness being about 1 mm) and placed on a flat surface to cool. Then 3-mm perforations were made in the agarose medium. Ten microliters of peptide solution of a fixed concentration (C) were added to each perforation and incubated for 2 h at 37°C. After termination of the incubation, the dish was covered with a top layer of agar consisting of 6% TSB and 1% agarose. Bacteria were grown for 18 h. The diameters of clear regions (d_c, mm) around the holes were measured with a metric-scale magnifier, and the perforation diameter (d_0) was subtracted from that of the region. The minimal inhibiting concentration C_{MIC} (μM) was determined from the dependence:

$$C_{MIC} = C \times e^{\frac{d-d_o}{k}}.$$

However, it is more convenient to determine C_{MIC} graphically from the following dependence:

$$d_c - d_o = f(\log C),$$

where $\log C = \log C_{MIC}$ at $d_c - d_o = 0$.

Hemolytic activity of peptides [10]. Erythrocytes of fresh blood from a healthy donor (with Rhesus-positive factor) were precipitated by 10-min centrifugation at 1000 rpm. Then they were washed thrice with cold physiological solution, resuspended by dilution with physiological solution to obtain 20% (v/v) erythrocyte suspension, and preincubated at 37°C for 15 min. The suspension was diluted to 10% by addition of physiological solution containing different concentrations of the synthetic peptide and incubated in Eppendorf vials at 37°C for 60 min.

After incubation, an equal volume of the cold physiological solution was added and centrifuged at 10,000 rpm for 10 min. The supernatant was carefully collected with a micropipette and its optical density was measured on a spectrophotometer at 414 nm. To estimate the hemolysis G (%), the following formula was used:

$$G = [(A_{pep} - A_{sol})/(A_{100} - A_{sol})] \times 100,$$

where A_{100} is the absorption of the erythrocyte suspension in which 100% hemolysis has occurred, A_{sol} and A_{pep} are

Table 1. Retention times of peptides under conditions of analytical HPLC, their molecular masses, mass-spectroscopy data (MS), and amino acid analysis (estimated values are given in parentheses)

Peptide	Retention time, min	Molecular mass	Amino acid analysis			
			Leu	Arg	Ala	Gly
$(RLARLAR)_2$	16.5	1690.9 (1692.1)	4 (4)	5.9 (6)	4.1 (4)	
D-$(RLARLAR)_2$	16.5	1690.9 (1692.1)	—	—	—	—
$(RLGRLGR)_2$	15.2	1637.0 (1636.0)	4 (4)	5.8 (6)		4.2 (4)
$(RLARLAA)_2$	18.7	1522.1 (1520.9)	4 (4)	3.9 (4)	6.1 (6)	

absorptions of a clear solution and the erythrocyte suspension solution of a given peptide concentration, respectively.

To achieve 100% hemolysis, physiological solution containing 0.2% Triton X-100 was added to the erythrocyte suspension.

Determination of thymocyte survival. Thymocytes were isolated from female rats (140-160 g) [11]. Cells (10^7/ml) were incubated in the RPMI-1640 medium containing 10% embryonic serum, 10 mM HEPES, and 10 mg/ml gentamicin for 24 h at 37°C. Then the peptide solution was added, the mixture was kept for 20 min, and thymocyte survival (B, %) was determined using the formula $B = N/N_0 \times 100$, where N is the number of live cells in 1 ml affected by the peptide and N_0 is the number of live cells in 1 ml of the initial solution. Killed cells were stained with 0.04% solution of Trypan Blue (Serva, Germany).

Peptide proteolysis. Three milligrams of the peptide were dissolved in 3 ml of 10 mM phosphate buffer, pH 7.4, and 30 μl of trypsin solution (1 mg/ml) (Merck, Germany, EC 3.4.21.4) or chymotrypsin (Serva) was added to the mixture. For analysis, 1 μl of solution was taken and added to 10 μl of 0.1 N HCl. HPLC experiments were performed on a Separon C18 column, the acetonitrile gradient in water being from 5 to 100% (0.1% trifluoroacetic acid), with detection at 226 nm.

Electron microscopy. Strain D21 *E. coli* cells (10^6 cells/ml) were incubated for 120 min in LB solution containing the peptide of a given concentration, and then they were centrifuged for 10 min at 2000*g*. The precipitate was suspended in phosphate buffer (10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4). A drop containing the bacteria was layered on a copper mesh coated with a carbon film which was then contrasted with 2% solution of phosphotungstic acid, pH 6.8. The prepared mesh was analyzed in a JEM 100C electron microscope.

RESULTS AND DISCUSSION

The amino acid sequence of tetradecapeptides was chosen so that the corresponding α-helices had amphipathic structure. This is clearly seen on helical wheel diagrams [12] showing the α-helix from its end. Figure 1 presents helical wheel diagrams of two peptides $(RLARLAR)_2$ and $(RLARLAA)_2$. As seen, the tetradecapeptides form α-helices in which positive charges group on one side of the helix and hydrophobic charges on the other. Exactly the same pattern can be observed on the helical wheel diagrams of natural antimicrobial peptides, e.g., magainin [13].

The peptides were synthesized by a solid phase method. The Fmoc group was used as a temporary protection of the α-amino group, and the side function of arginine was protected by the PMC group removed with trifluoroacetic acid. TBTU was the condensing agent. The obtained peptides are listed in Table 1.

Antimicrobial activity of the synthesized peptides was studied on strain D21 *Escherichia coli* cells deficient in lipopolysaccharide coat components used for testing antimicrobial peptides. The antimicrobial activity was determined by measuring the dimensions of regions that inhibit *E. coli* growth in a thin agar layer [13]. It was found

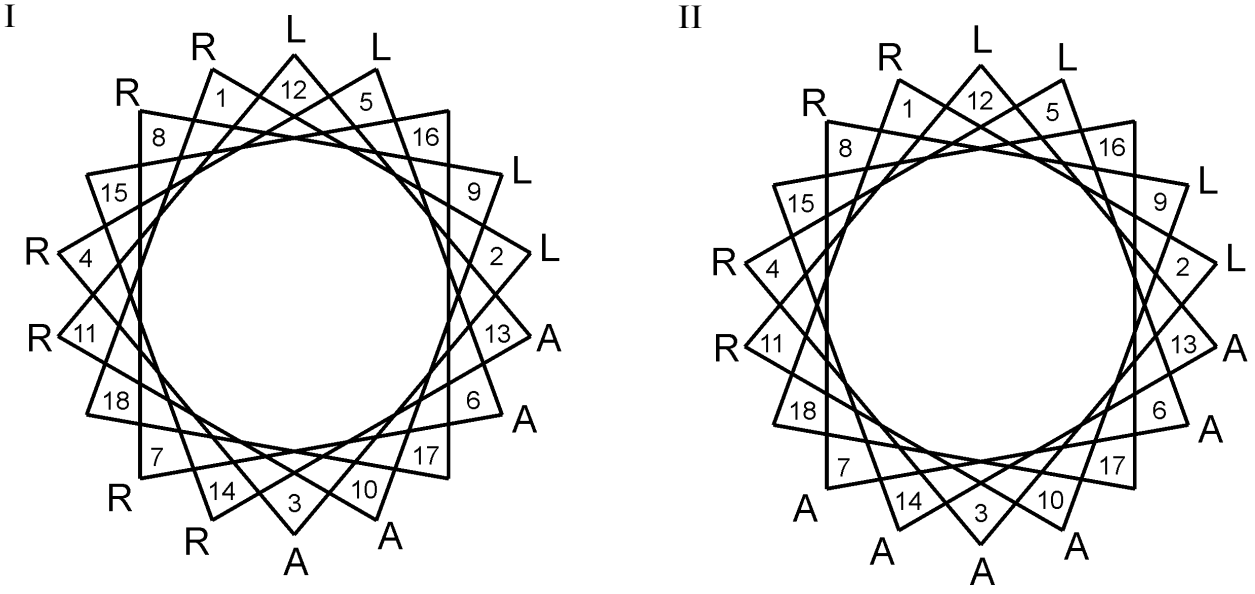

Fig. 1. Helical wheel diagrams of α-helices RLARLARRLARLAR (I) and RLARLAARLARLAA (II).

Table 2. Minimal inhibiting concentration of peptides for *E. coli* (MIC), hemolysis of human erythrocytes, and survival of rat thymocytes in the presence of peptides

Peptide	Positive charge	MIC, μM	Hemolysis at 40 μM, %	Survival of thymocytes at 25 μM, % (360 min)
$(RLARLAR)_2$	6	3	0	100
D-$(RLARLAR)_2$	6	<1	0	100
$(RLARLAA)_2$	4	3	0	100
$(RLGRLGR)_2$	6	12	0	100
Magainin [5]	4	4		

that all the peptides synthesized had antimicrobial activity comparable with that of natural peptides, the highest activity being observed for *D*-$(RLARLAR)_2$ (Table 2). At the same time, none of the peptides destroyed either erythrocytes or thymocytes even at concentrations exceeding greatly MIC. Electron microscopy can give the clearest impression on the action of synthesized peptides on *E. coli* (Fig. 2). When bacteria are affected by peptide *D*-$(RLARLAR)_2$ at concentrations lower than MIC, defective formations on the external membrane can be seen (Fig. 2b). When the peptide concentration exceeds MIC, the membrane is completely destroyed and the bacteria are killed (Fig. 2c).

We also studied the influence of proteolytic enzymes—trypsin and chymotrypsin—on the synthesized antibacterial peptides. As should be expected, the peptides formed of *L*-amino acids are quite readily cleaved with both trypsin and chymotrypsin, whereas *D*-peptide $(RLARLAR)_2$ is resistant to the influence of these enzymes (Fig. 3). Hence, it can be concluded that *D*-peptides can be not only of pure scientific interest.

All the synthesized peptides in an aqueous solution have no fixed secondary structure: only a minimum at 195 nm is seen in CD spectra (curves *1*, Fig. 4). However,

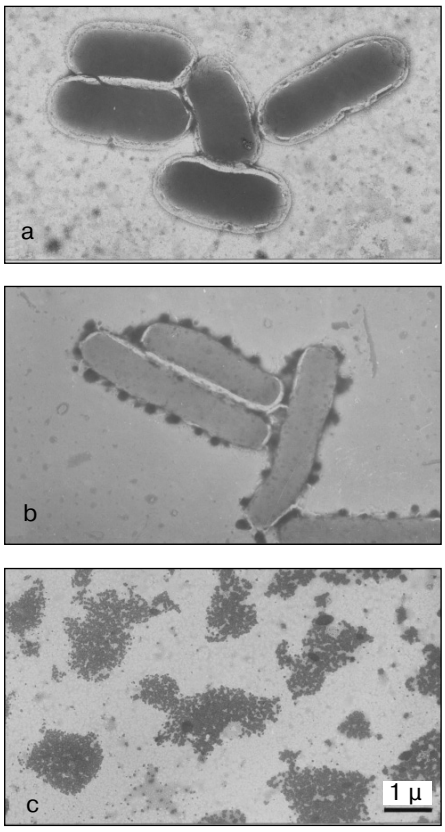

Fig. 2. Electron microphotograph of *E. coli* made by a JEM 100C electron microscope. Negative contrasting was done with 2% phosphotungstic acid. a) Intact *E. coli* cells; b) *E. coli* in the presence of peptide *D*-$(RLARLAR)_2$ at 0.5 MIC; c) *E. coli* in the presence of peptide *D*-$(RLARLAR)_2$ at 3.0 MIC.

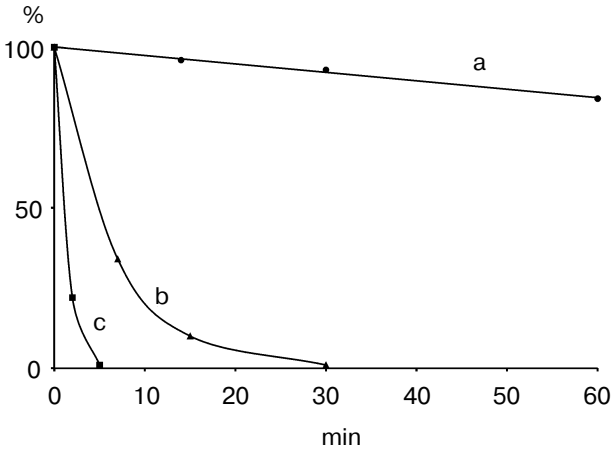

Fig. 3. Proteinase influence on peptides at the enzyme/peptide ratio 1 : 100. a) *D*-$(RLARLAR)_2$ in the presence of trypsin; b) $(RLARLAR)_2$ in the presence of chymotrypsin; c) $(RLARLAR)_2$ in the presence of trypsin.

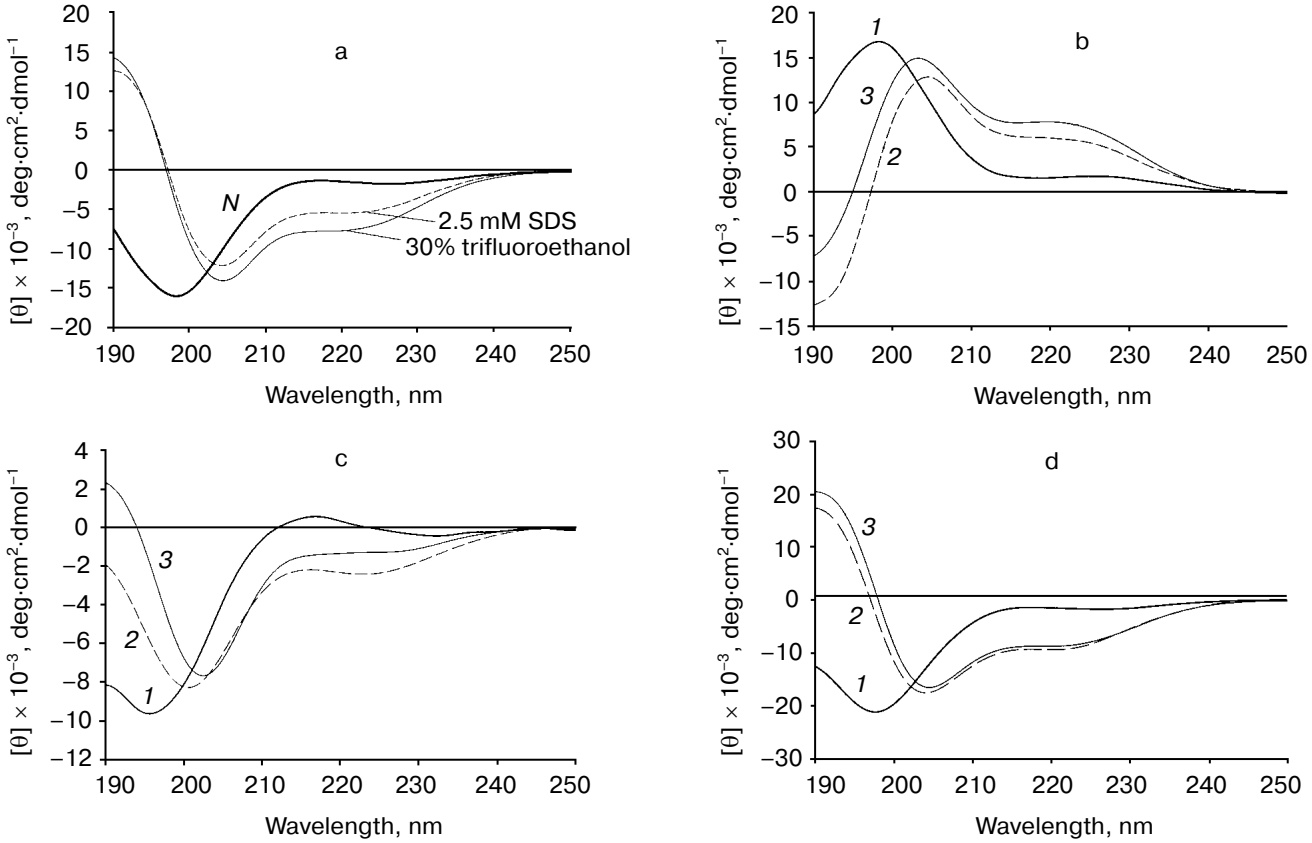

Fig. 4. CD spectra: *1*) in 10 mM Na_2HPO_4; *2*) in 2.5 mM SDS in 10 mM Na_2HPO_4; *3*) in 30% trifluoroethanol in water. a) RLARLAR-RLARLAR; b) *D*-(RLARLARRLARLAR); c) RLGRLGRRLGRLGR; d) RLARLAARLARLAA.

in trifluoroethanol and SDS solutions they can form α-helices, which is indicated by the appearance of a minimum at 220 nm and a shift of the minimum from 195 to 205 nm (curves *2* and *3*, Fig. 4). This suggests that when bacteria interact with membranes they also form α-helices necessary for bactericidal activity of the peptides.

Thus, amphipathic peptides constructed in this way have a rather high antimicrobial activity and are promising for further studies in the line of obtaining new antimicrobial preparations.

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