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Branched Antimicrobial Peptides

A. Yu. Khrushchev[a,b], I. A. Kashparov[a], L. V. Klimenko[a], and Yu. V. Mitin[a,1]

[a] *Institute of Protein Research, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia*
[b] *Faculty of Chemistry, Krasnoyarsk State University, pr. Svobodnyi 79, Krasnoyarsk, 660041 Russia*

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Abstract—Branched peptides E(RLAR)$_2$, E[E(RLAR)$_2$]$_2$, and E(KLAR)$_2$, E[E(KLAR)$_2$]$_2$ were synthesized on the basis of tetrapeptides RLAR and KLAR and glutamic acid bis(pentafluorophenyl) ester. Their minimal antimicrobial concentrations were shown to decrease along with increase in branching, achieving 12 μM for *Escherichia coli* cells, which is comparable to antimicrobial activities of temporin, magainin, and dermaseptin. The branched peptides were found not to act on human erythrocytes.

Key words: antimicrobial peptides, branched peptides, dendrimers, pentafluorophenyl esters

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INTRODUCTION

Last decade is characterized by an elevated interest in antimicrobial peptides.[2] This is particularly caused by that the problem of resistance of pathogenic microorganisms to many antibiotics, such as penicillins, tetracyclins, sulfonamide preparations, etc., has become aggravated. Even the bacteria resistant to all types of antibiotics have appeared [1, 2].

At the same time, antimicrobial peptides that exist in nature already for many millions years perfectly defend animals and plants. They kill bacteria quickly and effectively, which does not allow bacteria to select mutants resistant to such peptides [3].

Several hundreds of antimicrobial peptides isolated from various animals and plants are currently known [3–5]. Bacterial membranes differ from the membranes of animals and plants, which are mainly neutral, by the negatively charged phospholipid groups on their outer sides [4]. It is for this reason that antimicrobial peptides preferably interact with bacterial membranes and remain nontoxic to animal and human cells [6]. Antimicrobial peptides favorably differ from various antibiotics by their universal mechanism of action on bacterial membranes [7, 8]. The majority of antimicrobial peptides form α-helical or β-structure [9]; some antimicrobial peptides with unusual spatial structures also exist [3]. This work is devoted to the synthesis of branched peptides containing positively charged amino acids (arginine and lysine), and to study of their antimicrobial properties.

[1] Corresponding author; phone/fax: +7 (495) 632-7871; e-mail: mitin_y@mail.ru.
[2] Abbreviations: MIC, minimal inhibiting concentration.

RESULTS AND DISCUSSION

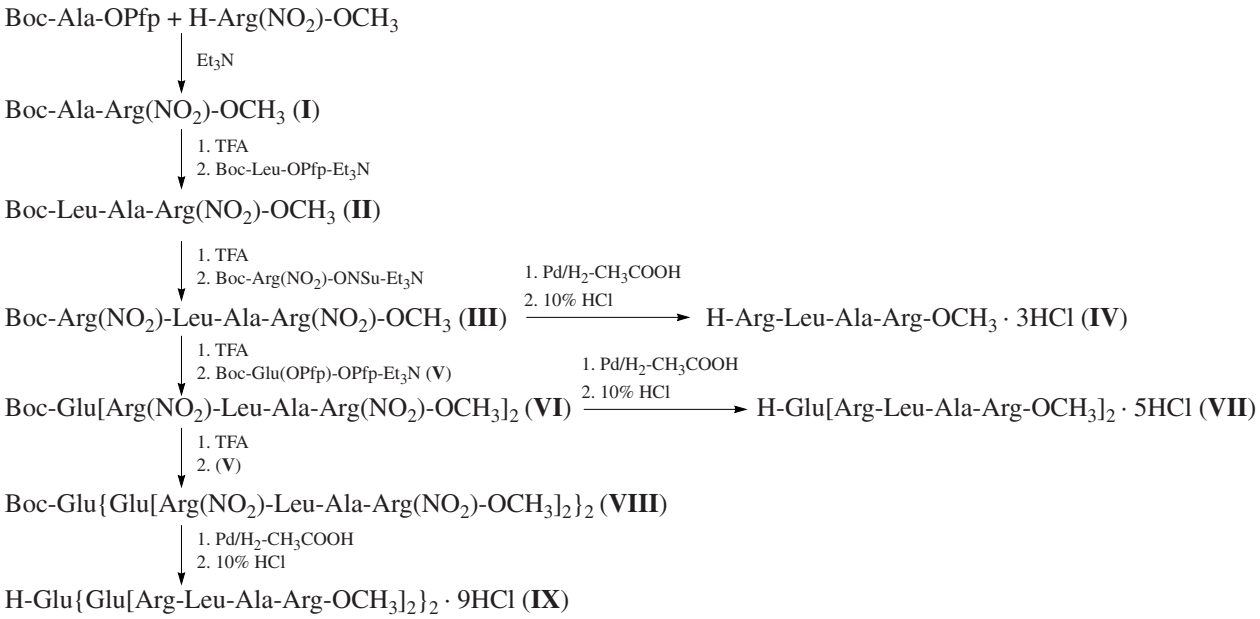
We comparatively analyzed amino acid sequences of various antibacterial peptides and came to the conclusion that the common feature for many of them is a tetrapeptide motif in which two positively charged residues, Lys or Arg, are adjacent to a pair neutral, frequently hydrophobic, residues (Table 1). The generality of this structural motif allowed us to presume that such sites are functionally significant and can determine the antibacterial activity. Within the framework of this presumption, we chose the elementary model peptide sequences RLAR and KLAR when designing new branched peptides with potential antimicrobial activity. They meet the above mentioned criterion as the base structural units. We decided to apply glutamic acid for the formation of branchings, because it easily form active bisesters directly useful for thesynthesis of dendrimers.

Table 1. Tetrapeptide blocks containing positively charged amino acids in the structures of natural antimicrobial peptides [4, 5]

Antimicrobial peptide	Tetrapeptide sites
Cecropin A	KLFK, KIEK
Magainin 2	KFLH, HSAK, KFGK
Pexagenan	KFLK, KFGK, KILK
Deramaseptin	HAGK
Buforin II	RSSR, RVHR, RLLR
Bactenecin I	RLCR, RVCR
Tanatin	RTGK, KCQR

Linear extending of peptide chain up to the stage of tetrapeptide (**III**) was carried out with the help preliminarily obtained pentafluorophenyl esters of Boc-protected amino acids, except for the Boc-Arg(NO_2)- residue for which a mild activation through the formation of more stable *N*-succinimide ester is preferable (scheme). The Boc group of tetrapeptide (**III**) was removed, and the resulting trifluoroacetate directly brought into the reaction with dipentafluorophenyl ester of Boc-glutamic acid (**V**) in the presence of triethylamine. The inclusion of two tetrapeptide molecules with the formation of dendrimer (**VI**) proceeded smoothly and in a high yield. The branched peptide (**VI**) after Ain-deprotection was repeatedly brought into reaction with derivative (**V**) and the dendrimer of the second level (**VIII**) was obtained.

Scheme of synthesis of branched peptides



The completely protected peptides (**III**), (**VI**), and (**VIII**) were deprotected in two steps. First, the constant protective groups were removed by a catalytic hydrogenation in the presence of palladium, and then Boc-groups, by treatment with 10% hydrochloric acid.

Similarly, there were synthesized peptides (**X**) and (**XI**), lysine analogues of tetrapeptides (**III**) and (**IV**), and also the corresponding branched peptides (**XII**)–(**XV**):

Boc-Lys(Z)-Leu-Ala-Arg(NO_2)-OCH_3 (X)

H-Lys-Leu-Ala-Arg-OCH_3 · 3HCl (XI)

Boc-Glu[Lys(Z)-Leu-Ala-Arg(NO_2)-OCH_3]$_2$ (XII)

H-Glu[Lys-Leu-Ala-Arg-OCH_3]$_2$ · 5HCl (XIII)

Boc-Glu{Glu[Lys(Z)-Leu-Ala-Arg(NO_2)-OCH_3]$_2$}$_2$ (XIV)

H-Glu{Glu[Lys-Leu-Ala-Arg-OCH_3]$_2$}$_2$ · 9HCl (XV)

The resulting hydrochlorides (**IV**), (**VII**), (**IX**), (**XI**), (**XIII**), and (**XV**) were purified with the help of preparative HPLC and tested for antimicrobial activity toward *Escherichia coli* (strain D21) cells with the defective components of lipopolysaccharide envelop, which makes this strain a convenient model for testing antimicrobial peptides.

The results of testing showed that minimal microbe-inhibiting concentration of the peptides synthesized grows along with increase in their branching and reaches 12–15 μM for peptides (**XIII**) and (**XV**) whose molecules contain four tetrapeptide fragments (Table 2). These values are comparable to the antimicrobial activities of such natural antimicrobial peptides,

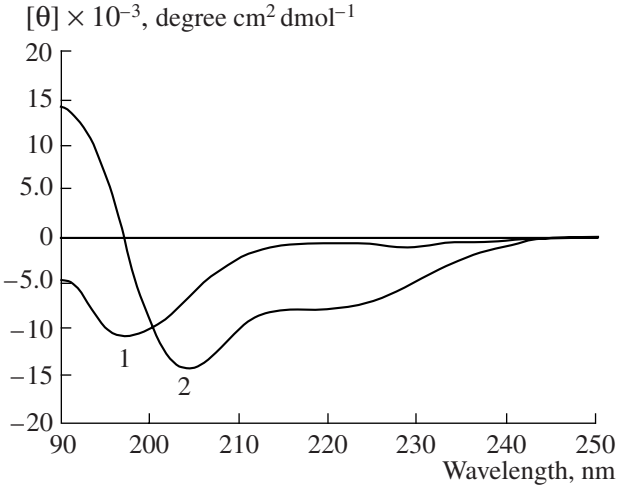
Table 2. Branched antimicrobial peptides

Peptide	Structure (methyl ester)	Molecular mass (ESI MS*)	MIC (*E. coli*), μM
(**IV**)	RLAR	529	Inactive
(**VII**)	$E(RLAR)_2$	1168	250
(**IX**)	$E[E(RLAR)_2]_2$	2447	12
(**XI**)	KLAR	501	Inactive
(**XIII**)	$E(KLAR)_2$	1112	Inactive
(**XV**)	$E[E(KLAR)_2]_2$	2336	15

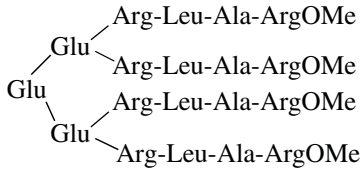
Note: * The values of molecular masses calculated on the basis of registered *m/z* values for the series of multicharged ions in the corresponding ESI mass spectra are given.

as temporin (12 μM) and magainin (4 μM) [5, 6]. From the point of view of potential biomedical use of the peptides synthesized by us, it was also important to estimate their toxic properties, first of all hemolytic activity. We established that none of the branched peptides caused a hemolysis of human erythrocytes within the range of peptide concentration from 20 up to 200 mM. This means that, at working concentrations, the branched antimicrobial peptides do not exert any hemolytic properties.

It is known that many linear antimicrobial peptides form in solution α helices in which positive charges are concentrated on one side of them and the hydrophobic residues, on another [10, 11]. It was assumed that exactly such a structure provides their antimicrobial activity [5]. Therefore, was of interest to demonstrate the basic structural difference of the branched peptide from the natural α-helical antimicrobial peptides. To this end, CD spectra of peptide (**IX**) and its linear analogue RLARLARRLARLAR [7] constructed from same tetrapeptide blocks were registered. One can see from the figure that no minimum in the area of 220 nm characteristic of the CD spectrum of linear peptide capable of forming α helix is seen in the CD spectrum of peptide (**IX**). The results confirms that, in this case, we deal with a new class of antimicrobial peptides not capable of formation of α-helical structure and, nevertheless, possessing a high activity and also a favorable combination of antimicrobial activity and toxicity.



1 – Branched peptide (**IX**)



2 – Linear peptide

Arg-Leu-Ala-Arg-Leu-Ala-Arg-Arg-Leu-Ala-Arg-Leu-Ala-Arg

CD spectra of branched peptide (**IX**) and its linear analogue described earlier [7]. The scheme of synthesis of branched peptides.

EXPERIMENTAL

We used in this work amino acid derivatives from Reanal and Serva. Pentafluorophenyl esters were obtained by the known procedures [12]. The peptides synthesized were purified by the reversed-phase HPLC in a gradient of acetonitrile concentration in water from 5 to 10%, eluate absorption was registered at 226 nm. CD spectra were measured on a Jasko-600 (Japan) spectrophotometer at peptide concentrations of 1.0–1.5 mg/ml in 30% trifluoroethanol. Mass spectra were registered on a ThermoFinnigan ESI MS spectrometer LCQDecaXP (Germany).

Parameters of antimicrobial activity (MIC) for peptides (**X**)–(**XV**) were determined (Table 2) with the use of *E. coli* strain D21 cells according to the technique developed earlier [10].

Hemolytic activity was estimated by the procedure [13].

Synthesis of Peptides

Boc-Ala-Arg(NO_2)-OCH_3 (I). Triethylamine (1.4 ml, 10 mmol) was added to a solution of Boc-Ala-OPfp (1.78 g, 5 mmol) and HCl · H-Arg(NO_2)-OCH_3 (1.5 g,5.5 mmol) in 30 ml acetonitrile, the mixture was kept for 3 h at room temperature and evaporated in a vacuum. Ethyl acetate (50 ml) was added to the residue, and the solution was washed with 10% $NaHCO_3$, water, saturated solution of $KHSO_4$, and water. The residue after ethyl acetate distillation off was triturated with hexane (2 × 50 ml) to give 1.8 g (89%) of viscous oil, R_f 0.57 (chloroform–methanol, 9 : 1).

Boc-Leu-Ala-Arg(NO_2)-OCH_3 (II). A solution of dipeptide (**I**) (0.87 g, 2.15 mmol) in 5 ml of TFA was kept for 10 min at room temperatureand evaporated in a vacuum of water-jet pump. The residue was dissolved in acetonitrile (25 ml), and triethylamine was added to neutral reaction. Boc-Leu-OPfp (0.85 g, 2.14 mmol) and triethylamine to pH ≈ 9 were added to the solution. The mixture was kept for 3 h at room temperature and treated as described in the synthesis of dipeptide. Ethyl acetate was distilled off, and the residue was triturated with ether. The resulting tripeptide (**II**) was crystallized from ethyl acetate–hexane to yield 0.86 g (78%), mp 104–106°C, R_f 0.50 (9 : 1 chloroform–methanol).

Boc-Arg(NO_2)-Leu-Ala-Arg(NO_2)-OCH_3 (III). Boc-group of tripeptide (**II**) (1.09 g, 2.11 mmol) was removed with the help of TFA, the residue was dissolved in DMF (20 ml), triethylamine was added to neutral reaction, and then Boc-Arg(NO_2)-ONSu [14] (1.04 g, 2.5 mmol) and triethylamine to pH ≈ 9. The solution was kept for 4 h at room temperature and evaporated in a vacuum. The residue was dissolved in boiling acetonitrile (5 ml), ethyl acetate was added to turbidity, and the solution was kept in a refrigerator for 20 h. Yield of amorphous product (**III**) was 0.68 g (45%), R_f 0.85 (on alumina in 95 : 5 chloroform–methanol system).

Boc-Glu(OPfp)-OPfp (V). Pentafluorophenol (3.9 g, 21 mmol) was added to a solution of Boc-Glu-OH (2.47 g, 10 mmol)) in acetonitrile (20 ml), the solution was cooled with ice water and DCC (4.5 g, 22 mmol) was added. The mixture was kept for 16 h at 4–5°C, filtered, and evaporated in a vacuum. The residue was crystallized from ethyl acetate–hexane to get diester (**V**); yield 3.6 g (88%), mp 128–132°C. R_f 0.65 (9 : 1 : 10 chloroform–methanol–hexane).

Boc-Glu[Arg(NO_2)-Leu-Ala-Arg(NO_2)-OCH_3]$_2$ (VI). TFA (5 ml) was added to tetrapeptide (**III**) (0.6 g, 0.84 mmol), the mixture was kept for 10 min, evaporated in a vacuum, the residue was dissolved in acetonitrile (15 ml), and triethylamine was added to neutral reaction. Then diester (**VI**) (0.24 g, 0.42 mmol) and triethylamine to pH ≈ 9 were added to the reaction mixture. The solution was kept for 3 h at room temperature and evaporated in a vacuum. The residue was washed with ethyl acetate, filtered, the crystals were washed with ethyl acetate and ether. Yield of (**V**) 0.55 g (92%), amorphous, R_f 0.52 in 8 : 2 : 0.2 chloroform–methanol–hexane system.

Boc-Glu{Glu[Arg(NO_2)-Leu-Ala-Arg(NO_2)-OCH_3]$_2$}$_2$ (VIII). Nonapeptide (**VI**) (0.30 g, 0.21 mmol) was deprotected with the help of TFA, TFA was deleted in a vacuum, the free peptide was dissolved in DMF (5 ml), triethylamine was added to neutral reaction, then ester (**V**) (0.06 g, 0.1 mmol) and triethylamine to pH ≈ 9 were added and the mixture also left overnight. Water was added to the solution, crystals were filtered, and washed with ester. Yield of (**VIII**) 0.23 g (79%), R_f 0.30 and minors 0.10 and 0.36 in 8 : 2 : 0.2 chloroform–methanol–hexane system. In addition, (**VIII**) was chromatographed in the same system on a preparative Merck plate (Cat. Number 11798.0001), yield 0.08 g (28%), R_f 0.30.

Boc-Lys(Z)-Leu-Ala-Arg(NO_2)-OCH_3 (X). Tripeptide (**II**) (0.39 g, 0.75 mmol) was deblocked with the help TFA. After the TFA removal in a vacuum, the residue was dissolved in DMF (10 ml), triethylamine was added to neutral reaction and, then, Boc-Lys (Z)-OPfp (0.41 g, 0.75 mmol) and triethylamine to pH ≈ 9. The solution was kept for 4 h at room temperature, ethyl acetate (50 ml) was then added and the reaction was treated as described in the synthesis of dipeptide. The resulting tetrapeptide was crystallized from ethyl acetate–hexane; yield 0.51 g (88%), mp 114–116°C, R_f 0.50 (95 : 5 chloroform–methanol).

Boc-Glu[Lys(Z)-Leu-Ala-Arg(NO_2)-OCH_3]$_2$ (XII) was obtained as nonapeptide (**VI**) starting from (**X**) (0.50 g, 0.64 mmol) and active ester (**V**) (0.185 g, 0.32 mmol); yield 0.47 g (92%); R_f 0.10 in 95 : 5 chloroform–methanol and 0.71 in 9 : 1 chloroform–methanol.

Boc-Glu{Glu[Lys(Z)-Leu-Ala-Arg(NO_2)-OCH_3]$_2$}$_2$ (XIV) was synthesized similarly to (**VIII**) starting from nonapeptide (**XII**) (0.49 mmol) and ester (**V**) (0.24 mmol). The resulting product was dissolved in hot acetonitrile and crystallized at cooling; yield 0.32 g (40%); R_f 0.45(9 : 1 chloroform–methanol).

H-Arg-Leu-Ala-Arg-OCH_3 · 3HCl (IV), H-Glu[Arg-Leu-Ala-Arg-OCH_3]$_2$ · 5HCl (VII), H-Glu{Glu[Arg-Leu-Ala-Arg-OCH_3]$_2$}$_2$ · 9HCl (IX), H-Lys-Leu-Ala-Arg-OCH_3 · 3HCl (XI), H-Glu[Lys-Leu-Ala-Arg-OCH_3]$_2$ · 5HCl (XIII), and H-Glu{Glu[Lys-Leu-Ala-Arg-OCH_3]$_2$}$_2$ · 9HCl (XV). Deblocking of the protected peptides (**III**), (**VI**), (**VIII**), (**X**), (**XII**), and (**XIV**) was carried out in two stages by standard procedures: nitro and benzyloxycarbonyl groups were deleted by catalytic hydrogenation over Pd in acetic acid, and Boc-group by treatment with 10% HCl for 1 h [15]. The peptides were purified by preparative HPLC and characterized by ESI MS. The molecular masses of the peptides, calculated from the registered *m/z* values of series of multicharged ions are given in Table 2.

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