Bulgarian Chemical Communications, Volume 52, Issue 2 (pp. 284 - 288) 2020 DOI: 10.34049/bcc.52.2.5209

Synthesis and hydrolytic stability of new analogues of Bactenecin 2A

A. Hristova[1], S. Valcheva-Kuzmanova[2], T. Pajpanova[3*]

[1] *Medical College, Medical University "Prof. Dr. Paraskev Stoyanov",84 Tsar Osvoboditel blvd., 9002 Varna, Bulgaria*
[2]*Department of Pharmacology and Clinical Pharmacology and Therapeutics, Faculty of Medicine, Medical University "Prof. Dr. Paraskev Stoyanov", 55 Marin Drinov str., 9002 Varna, Bulgaria*
[3]*Institute of Molecular Biology "Roumen Tsanev", Bulgarian Academy of Sciences, Acad. G. Bonchev str., block 21, Sofia 1113, Bulgaria*

Received: August 20, 2019; Revised: April 03, 2020

The hydrolytic stability of peptides is one of the most important properties in terms of their application in practice. Pre-information on stability is essential for the pharmacokinetic behavior in the body, the storage conditions, the occurrence of toxic effects associated with their degradation products, and others. We synthesized a series of short linear Bactenecin 2A mimetics containing cationic unnatural amino acids and examined their hydrolytic stability under physiological conditions. The five newly synthesized analogues showed high hydrolytic stability in the acidic region and only some of them were stable in neutral and alkaline environment.

Keywords: antimicrobial peptides, Bactenecin, unnatural amino acids, solid-phase synthesis, hydrolytic stability

INTRODUCTION

Drug resistance of microorganisms is among the serious threats to human health, and in the last few years an incremental trend in the incidence of diseases caused by resistant microorganisms has been observed. That determines the necessity to search for new antibiotics, and to look for substances which are capable of enhancing antibiotic effects, or of reversing microbial resistance, even if they themselves have no antimicrobial effect. Some peptides have been shown to have antimicrobial activity, and/or ability to enhance the effects of antimicrobial agents. Thus, the antimicrobial peptides (AMPs) are some of the most promising candidates for a novel class of antibiotics [1-4].

Cationic antimicrobial peptides (CAMPs), also called cationic host defense peptides, are present in virtually every form of life, from bacteria and fungi to plants, invertebrates, and vertebrates [5]. An under-studied representative of this group is the cyclic cationic peptide Bactenecin isolated from bovine neutrophils. This short peptide ($(R^1L^2C^3R^4I^5V^6V^7I^8R^9V^{10}C^{11}R^{12})$) is composed of 12 amino acid residues including four arginines and two cysteines, and is shown to form a β-turn structure consisting of one disulfide bond [6].

Bactenecin is active against *Escherichia coli* and *Staphylococcus aureus* as it was previously found [7, 8]. Compared to other β-structural natural AMPs, Bactenecin has less pronounced antimicrobial activity, but because of its small size it is used as the basis for creating more effective AMPs [8-11].

The **Bac2A** peptide (Fig. 1) is a linear variant of Bactenecin. In **Bac2A**, two cysteine residues are replaced by two alanine residues. They have an average activity with MICs between 2 and 32 μg/ml for Gram-negative bacteria and between 0.25 and 16 μg/ml for Gram-positive bacteria. Based on the substitution analysis of **Bac2A** (RLARIVVIRVAR-NH_2), different 12mer peptides with superior activity against different human pathogens were developed [12].

On the other hand, there are several limitations to the clinical application of AMPs as therapeutic agents, especially their low bioavailability under physiological conditions due to proteolytic degradation. To overcome this problem and improve their antimicrobial potential, several studies have focused on chemical modification of naturally occurring sequences [13, 14], such as the use of unnatural amino acids, D-form amino acids, fatty acids, or conjugation with antibiotics [15]. A peptidomimetic approach that was applied in our recent studies with significant potential is the use of unnatural amino canavanine, a structural analogue of arginine [16].

In this context, our first objective was to synthesize short model peptide fragments of **Bac2A** (Fig. 1) containing non-protein amino acids canavanine (Cav), as well as S-cysteine sulfonamide ($Cys(SO_2NH_2)$) and D-amino acid S- homocysteine sulfonamide ($hcys(SO_2NH_2)$),

* To whom all correspondence should be sent:
E-mail: tamara@bio21.bas.bg

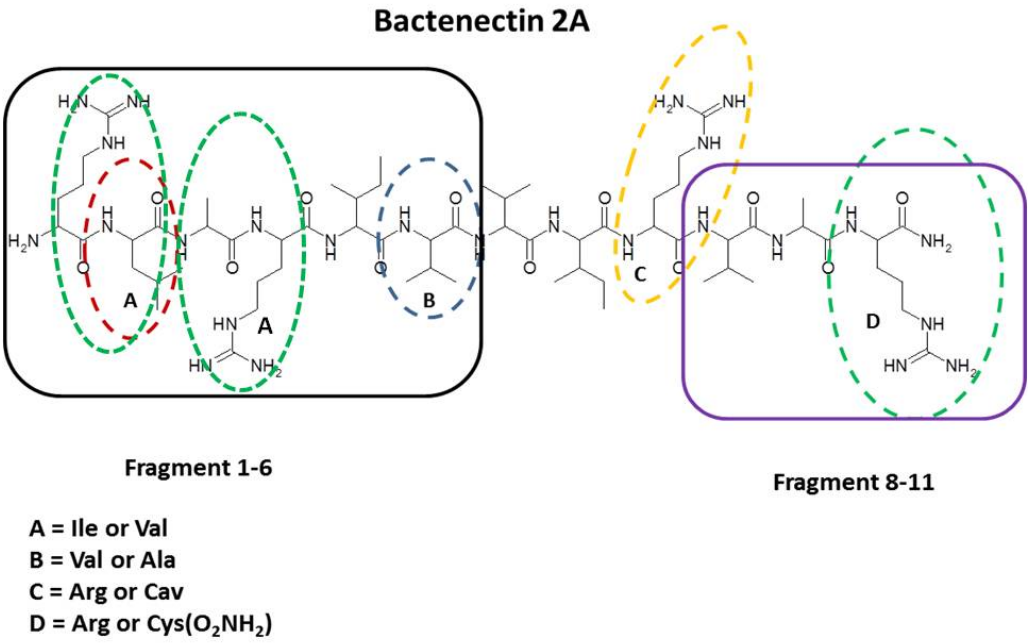


Figure 1. Bac2A and its structural analogues.

for which there is evidence of antimicrobial activity [17-19].

The second aim of this study was to evaluate the hydrolytic stability of newly synthesized Bac2A analogues under physiological conditions such as body temperature of 37° C and pH values of 1.2 (stomach), 7.4 (blood plasma) and 8.5 – 9.0 (small intestine).

EXPERIMENTAL

Peptide synthesis

For the preparation of the desired model peptides, we have referred to the solid phase peptide synthesis method. Reagents, resins and Fmoc-amino acids used in peptide synthesis were purchased from Merck (Darmstadt, Germany) and Iris Biotech GMBH (Germany). Solvents dimethylformamide (DMF) and dichloromethane (DCM) were purchased from Merck (Darmstadt, Germany).

Fmoc-Cav(Boc)-OH, Z-Cys(SO_2NH_2)-OH and Fmoc-hcys(SO2NH2)-OH were prepared according to previously reported methods [19, 20].

Synthesis of all peptides was performed by the conventional manual stepwise Fmoc solid-phase synthesis on 2-chlorotrityl chloride resin with substitution, 1.4 mmol/g. The coupling of each amino acid was performed in the presence of 3 mol excess of Fmoc-amino acid, 3 mol excess of N-hydroxybenzotriazole (HOBt), 3 mol excess of diisopropylcarbodiimide (DIC), and 5 mol excess of diisopropylamine (DIPEA) in DMF. The completion of coupling reactions was monitored by the Kaiser test and the Fmoc groups were removed by adding 25% piperidine in DMF. The peptides were cleaved from the resin and the final deprotection was done in a cocktail containing trifluoroacetic acid (TFA), triisopropylsilane (TIPS), thioanisole, and water (92.5 : 2.5 : 2.5 : 2.5). The crude peptides were precipitated into cold petroleum ether/diisopropyl ether (50:50). Then, the precipitate was dissolved in 10% CH_3COOH and desalted by gel filtration on a Sephadex G25.

Peptide purity

HPLC analysis was performed with LKB Bromma (Sweden) and Waters Alliance® (Waters Corporation, USA) instruments and a variable detector using column: XTerra® MS C18, 3,5µm, 3.0 × 150 mm**;** eluent: ACN/0.05%TFA 5/95 (v/v), flow 0.4 ml/min, 25°C, 220 nm, injected volume 20 µL.

Peptide stability

The hydrolytic stability of the peptides was determined by UV spectroscopy. For stability testing, the concentration $0.5.10^{-4}$ mol/l of each of the peptides (P1 – P5), respectively, was dissolved in 10 ml of buffers: pH=1.2 (0.063 mol/l $HClO_4$); pH=7.4 (0.1 mol/l Na_2HPO_4 + 0.1 mol/l NaH_2PO_4); pH=9.0 (0.1 mol/l $Na_2B_4O_7$). The obtained solution of the peptides was tempered in the incubator ES-20 LKB (Sweden) at 37ºC. The test samples were placed in a Beckman DU 650 spectrophotometer (Beckman Instruments, USA) equipped with a temperature-controlled cell changer; 1 ml quartz cuvettes were used. The decrease in the absorbance

at 220 nm (UV maximum of the peptide) was monitored.

RESULTS AND DISCUSSION

In order to design short model peptide fragments of Bac2A, literature data have been taken into account indicating that the most important structural requirement for high antimicrobial activity is the presence of basic amino acids such as arginine and lysine, at least 3 net charge in each peptide.

It was also shown that changes in any single position of the peptide may affect other residues at all other positions in the parent peptide. Therefore, each single modified peptide variant may lead to different activity and stability [21].

In our study, a substantial difference in obtaining the corresponding **Bac2A** analogue was found primarily in the non-protein amino acid used. Arg at positions **1** and **4**, as well as Val at position **6** were successively or simultaneously replaced with Arg(NO_2) and non-protein amino acids – Cav, Cys(SO_2NH_2) and hcys(SO_2NH_2).

All linear shortened analogues (**P1-P5**) of **Bac2A,** modified at the N-terminus (fragment 1-6) were obtained using essentially the same experimental set-up outlined in Scheme 1.

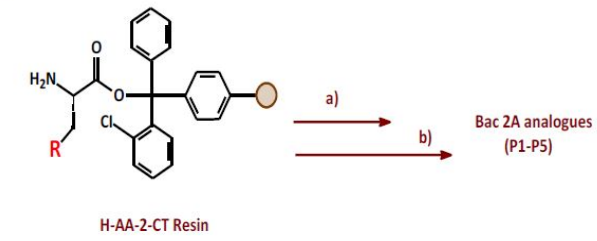
The yields of each peptide were over 60%. HPLC analysis revealed a purity of >95% for all obtained analogues (Fig. 2). The net charge, yield, RP-HPLC purity and molecular weight of Bac 2A linear analogues synthesized in this study are summarized in Table 1.

The hydrolytic stability of peptides is one of the most important properties in terms of their application in practice. Pre-information on stability is essential for the pharmacokinetic behavior in the body, the storage conditions, the occurrence of toxic effects associated with their degradation products, and others.

We examined the hydrolytic stability of the resulting peptide analogues under physiological conditions such as: body temperature of 37° C and physiological pH value of 1.2 (stomach), 7.4 (blood plasma) and 8.5–9.0 (small intestine) using UV-spectrophotometry and RP-HPLC. At defined intervals of time we measured the absorbance at 220 nm. For each of the studied peptides, a plotted time/concentration graph was constructed.

As shown on Fig. 3, at acidic pH, the peptide Cav-Leu-Ala-Arg(NO_2) (**P1**) retained stability for six hours and its concentration was about 95%. By the third hour of the test, the peptide remained stable at both acidic and alkaline pH of nearly 100 %. At alkaline pH, at the end of the sixth hour the concentration of the peptide was about 90 %.

At pH 7.4, P1 retained significantly higher stability within one hour of the test, after which its concentration decreased to 80% at the end of the sixth hour.



P1- Cav-Leu-Ala-Arg(NO_2); P2 - Arg(NO_2)-Leu-Ala-Cav;
P3 - Cys(SO_2NH_2)-Ile-Arg-Val-Ala;
P4 - Cys(SO_2NH_2)-Ile-Arg-Val-Val;
P5 - Arg(NO_2)-Leu-Ala-Arg(NO_2)-Ile-hcys(SO_2NH_2)

Scheme 1. Reagents and conditions: a) 1/ Fmoc-AA-OH, DIC, DIPEA, HOBt, DMF; 2/ 25% piperidine/DMF; b) TFA/ TIPS /H_2O/thioanisol, RT, 1-2 h.

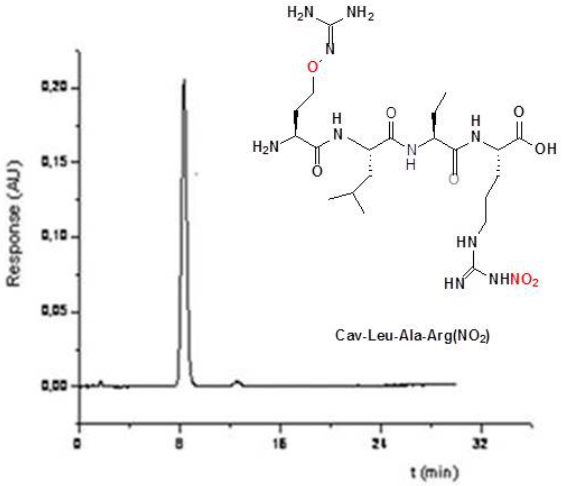


Figure 2. Analytical HPLC of H-Cav-Leu-Ala-Arg(NO_2)-OH (P1); Column: AtlantisTMdC18 (Beckman Ultrasphere®) 4.6 × 150 mm; mobile phase: acetonitrile / deionized water 40/60 (v/v); 25°C, flow rate: 1 ml/min, UV detection – 206 nm.

Table 1. Analytical data on synthesized analogues of Bactenecin 2A.

Code	Net charge	Yield %	Purity %	Molecular formula	MW calculated	MW [M + H^+]
P1	2+	86	99	$C_{20}H_{39}N_{11}O_8$	561.591	562.597
P2	2+	87	99	$C_{20}H_{39}N_{11}O_8$	561.591	562.593
P3	2+	46	96	$C_{23}H_{45}N_9O_8S$	607.720	608.207
P4	2+	77	98	$C_{25}H_{49}N_9O_8S$	635.780	636.805
P5	3+	71	95	$C_{31}H_{60}N_{15}O_{13}S$	882.960	868.937

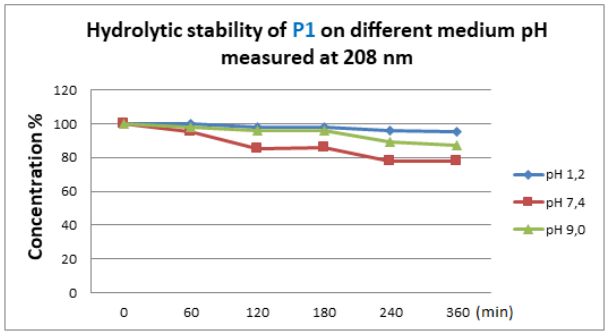


Figure 3. Hydrolytic stability of P1 at different pH measured at 220 nm by UV-spectroscopy.

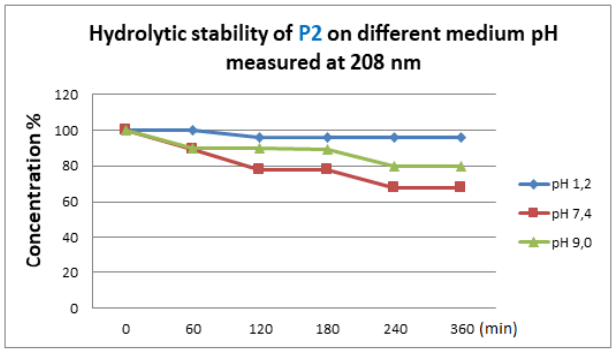


Figure 4. Hydrolytic stability of P2 at different pH measured at 220 nm by UV-spectroscopy.

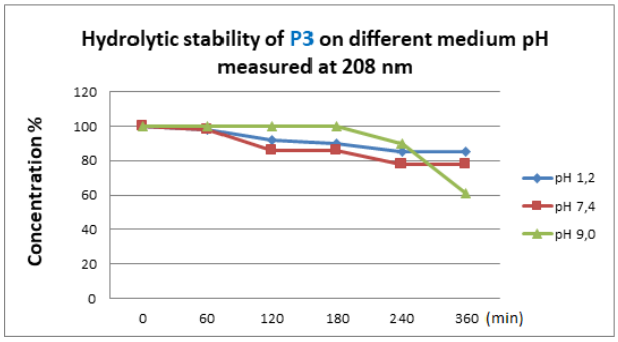


Figure 5. Hydrolytic stability of P3 at different pH measured at 220 nm by UV-spectroscopy.

On Fig. 4, the hydrolytic stability of the Arg(NO_2)-Leu-Ala-Cav (**P2**) peptide is presented. It is evident from the graph that **P2** retained its stability in acidic medium till the end of the sixth hour to a significant extent (98%), even higher than that of P1 (95%). As opposed to **P1**, the concentration of **P2** in alkaline and neutral pH began to decrease within the first hour (90%). The concentration till the end of the sixth hour in neutral medium decreased to 80% and in alkaline environment to 70%.

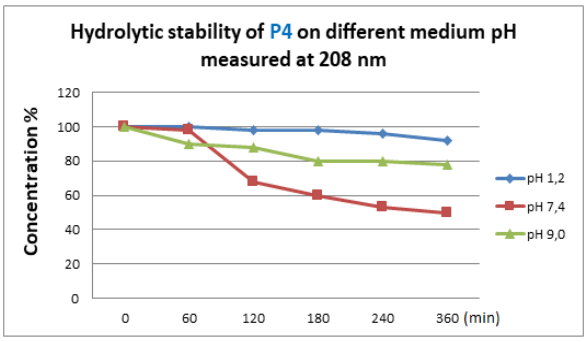


Figure 6. Hydrolytic stability of P4 at different pH measured at 220 nm by UV-spectroscopy.

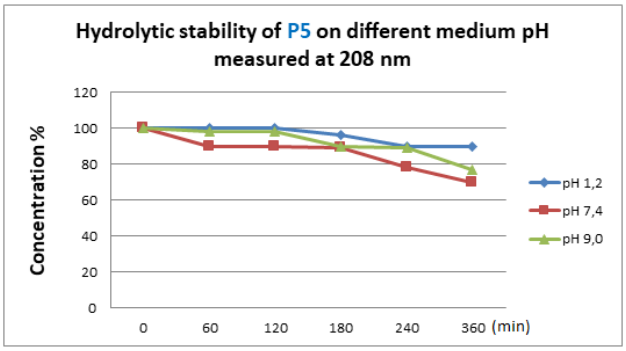


Figure 7. Hydrolytic stability of P5 at different pH measured at 220 nm by UV-spectroscopy.

The peptide Cys(SO_2NH_2)-Ile-Arg-Val-Ala (**P3**) (Fig. 5) retained 100% stability in the three physiological media within the first hour of the assay. In an alkaline environment, it retained its stability by the end of the third hour, then at the fourth hour its concentration was about 84% and at the end of the sixth hour it wass 60%. At acidic pH, at the end of the test the concentration was 83% and at pH 7.4 it was 79%.

Fig. 6 presents the graph showing the stability of the peptide Cys(SO_2NH_2)-Ile-Arg-Val-Val (**P4**). The concentration it retained in acidic medium (93%) till the end of the test was very close to those of **P1** and **P2**. The stability in a neutral buffer medium remained unchanged within the first hour, then sharply dropped and at the end of the sixth hour hardly reached 50%. At pH 9.0, the concentration gradually decreased, being 79% at the end of the study.

The stability of the peptide Arg(NO_2)-Leu-Ala-Arg(NO_2)-Ile-hcys(SO_2NH_2) (**P5**) is presented on Fig. 7.

Like the other four peptides (**P1-P4**), in acidic medium **P5** retained the highest levels of concentration (90%) for a long time. At pH 9.0 the peptide remained unchanged for three hours, after which the concentration was reduced to 79% at the end of the sixth hour. At neutral pH, on the first hour

the concentration of the peptide was 90% and it was maintained until the end of the third hour. At the end of the test the concentration was 75%.

As it was expected, all newly synthesized peptides containing unnatural amino acids exhibited a remarkably high hydrolytic stability. It is noticeable that after six hours of incubation in acidic medium, > 95% of the initial concentration of **P1** and **P2** was still present. The stability profile of all peptides in basic and neutral medium looks similar for the first three hours, even 90% of the initial concentration of **P1** is still present after six hours of incubation. The concentrations of **P4** and **P5** sharply dropped at the end of the six hours and hardly reached 50%. We might note that among all modifications made, introduction of the canavanine residue at position **1** (**P1**) and at position **4** (**P2**) is the most favorable for the hydrolytic stability.

CONCLUSION

In summary, we successfully synthesized 5 novel shortened analogues of Bactenecin 2A that exhibited a high hydrolytic stability in the acidic region. Our results indicated that they were more sensitive to hydrolytic degradation in neutral and alkaline environment in which **P1** showed the highest stability. The incorporation of the unnatural amino acid canavanine into the N-terminal region of Bac 2A may be a useful tool to improve the stability of the shortened linear analogues without a great loss of the activity.

Acknowledgement: *The work was supported by Science Fund, Medical University, Varna, Bulgaria; Project: 17010/2017.*

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