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Temporin A and its retro-analogues: synthesis, conformational analysis and antimicrobial activities

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Abstract: Temporin A (TA) is a hydrophobic peptide isolated from the skin of the European red frog *Rana temporaria*. Strong antimicrobial activity against gram-positive cocci and Candida, as well as its small molecular weight (10–13 aa residues), makes TA an interesting antimicrobial compound. However, its synthesis is rather problematic. Here, the synthesis of two retro-analogues of TA – retro-TA and (6-1)(7-13)-TA – is reported. The synthesis of retro-TA was performed without any problems, while during the synthesis of (6–1)(7–13)-TA problems similar to those encountered during the synthesis of TA were faced. Antimicrobial assays showed minimal inhibitory concentration (MIC) values of retro-TA to be, in most cases, only one dilution higher than those of original TA, but still remained relatively low. An analysis of the circular dichroism spectra of the peptides shows that TA and (6-1)(7-13)-TA adopt an α-helical structure in a hydrophobic environment, while retro-TA forms mainly unordered conformation under both hydrophobic and hydrophilic conditions. One can postulate that differences in conformation of the peptide chain might be responsible for the lower antimicrobial activity of retro-TA as compared to that of the parent molecule. In any case, retro-TA can be interesting owing to its simple and nonproblematic synthesis. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptides; peptide synthesis; circular dichroism

INTRODUCTION

During the past decades, in spite of the substantial progress in medical sciences, there has been a constantly growing number of antibiotic-resistant microbes. Actually, in the age of opportunistic infections, resistant strains of bacteria and fungi are the third most important cause of human death worldwide [1]. This emerging problem forces us to look for new antimicrobial agents.

Antimicrobial peptides seem to be a reasonable alternative for conventional antibiotics. Actually, several peptides and peptide-based compounds are passing clinical trials [2]. Another problem is concerned with the preservation of drugs and cosmetics. The same substances have been used for decades, while it is known that they can be responsible for some undesired side effects [3]. In this field also antimicrobial peptides seem to be applicable. One of them is a small group of ten antibiotic peptides called *temporins*. These peptides were isolated from the skin of the European red frog *Rana temporaria* [4]. They are composed of 10–13 amino acid residues. The primary structure of temporins is highly variable, but the majority of them contain a single basic residue (arginine or lysine). The common feature of all temporins is the α-amidation on the *C*-terminus of the molecule [5].

Temporin A (TA) is the most well known among the temporins. It is a strongly hydrophobic, 13-amino-acid peptide (FLPLIGRVLSGIL-HN_2) that exhibits antibacterial (mainly against gram-positive cocci) and antifungal activities (against yeast-like *Candida albicans*). The *N*-terminal residue, Arg7, and two Ile residues (5 and 12) are described as the critical ones responsible for the antibacterial activity [6]. TA exerts its antimicrobial activity by its ability to form a transmembrane pore via a barrel-stave mechanism or to form a 'carpet' on the membrane surface via the 'carpet-like' model [7]. Mangoni *et al.* reported that the ability of temporins to destroy microbial cells is independent of membrane composition, since they lysed artificial vesicles built from zwitterionic and acid phospholipids as well [8]. However, the antimicrobial property does not stem from strict chiral interactions between particular amino acids and microbial membranes because TA, built from all-D enantiomers, retains its lytic activity [6]. Interestingly, the replacement of isoleucine by leucine residues in native TA (TA L512) caused an increase in the antimicrobial potential of the compound [6]. The original TA

Abbreviations: CFU, colony forming units; CMC, critical micelle concentration; MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; Nsc, 2-(4-nitrophenylsulfonyl)ethoxycarbonyl; TA, temporin A.

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as well as its TA L512 analogue did not show toxic effect toward keratinocytes cultured *in vitro* at concentrations that are totally bactericidal, and therefore are promising molecules against multiresistant bacterial infections [9].

One of the major limitations in the investigation of TA is a problem with its synthesis. *Harjunpää et al.* compared two methods of chemical synthesis (Fmoc and Nsc chemistry) of this peptide. In both cases, an HPLC chromatogram of the crude product contained several peaks with comparable intensity [10]. The research conducted by our group has confirmed that obtaining TA under standard conditions of synthesis poses serious synthetic problems, which significantly increase the cost of the synthesis of this substance.

This work describes the synthesis of TA and its two retro-analogues (retro-TA and (6-1)(7-13)-TA) designed as reasonable alternatives for this problematic peptide. Moreover, we present the antimicrobial properties and structural research of the original TA and its analogues.

MATERIALS AND METHODS

Antimicrobial Agents

The peptides were assembled by solid-phase procedures on polystyrene AM-RAM resin (0.66 mmol/g, Rapp Polymere, Germany) using 9-fluorenylmethoxycarbonyl (Fmoc) methodology [11]. The Fmoc group of each amino acid was deprotected by 20% piperidine in DMF in two steps of 2 and 20 min. The coupling reactions were carried out with a 4-fold excess of Fmoc-AA in DMF in presence of Triton X-100 using diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) as coupling reagents, for 2 h. Completion of the coupling reaction was monitored by the chloranil test [12]. If the chloranil tests were positive, the resin was washed and coupled again using 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (Fmoc-AA/HOBt/TBTU/DIEA, 1 : 1 : 1 : 2) until no free amino groups were detected. The peptide chains were cleaved from the resin using a trifluoroacetic acid (TFA)–triisopropylsilane (TIS)–water (95 : 2.5 : 2.5) mixture for 2 h. The cleaved crude peptides were concentrated, precipitated with diethyl ether and lyophilized. The crude peptides were purified by reversed-phase HPLC on a Knauer K501 two-pump system with Kromasil C8 column 10 × 250 mm (5 μm particle diameter, 100 Å pore size), employing acetonitrile–water mixture (containing 0.1% TFA) as eluent at a flow rate 5 ml/min and absorbance at 226 nm. The fractions, with purity greater than 95%, were pooled together and liophilized. The peptides were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

Organisms and Antimicrobial Assay

The following strains were tested: *Bacillus subtilis* ATCC 9372, *Enterococcus hirae* ATCC 10541, *Rhodococcus equi* ATCC 6939, *Staphylococcus epidermidis* ATCC 14 990, *Staphylococcus aureus* ATCC 6538, *Staphylococcus saprophyticus* ATCC 20 229, *Escherichia coli* ATCC 8739, *Proteus vulgaris* NCTC 4635, *Pseudomonas aeruginosa* ATCC 9027, *Serratia marcescens* ATCC 274, *Stenotrophomonas maltophila* ATCC 12 714, *C. albicans* ATCC 10 231 and *Aspergillus niger* ATCC 16 404. All microorganisms were from the Polish Collection of Microorganisms (Polish Academy of Sciences, Institute of Immunology and Experimental Therapy, Wrocław, Poland).

MIC was determined using a microbroth dilution method with either the Mueller–Hinton (MH) broth or the Sabouraud Dextrose broth (Becton Dickinson, Le Pont de Claix, France) and an initial inoculum of 10^5–10^6 CFU/ml. Polypropylene 96-well plates (Nunc GmbH & Co. KG, Germany) were incubated for 18 h at 37 °C in air for bacteria and 72 h at 25 °C for fungi. MIC was taken as the lowest drug concentration at which a noticeable growth was inhibited. MBC was taken as the lowest concentration of each drug that resulted in more than 99.9% reduction of the initial inoculum. The experiments were performed in triplicate.

CD Experiments

For CD measurements, TA, retro-TA and (6–1)(7–13)-TA solutions were prepared by weight from lyophilized material. CD spectra were obtained at room temperature on an automated Jasco J-20 spectropolarimeter equipped with a program made by Medson (Poland). Quartz cells of 1.5 mm were used. The results were plotted as the mean residue ellipticity $[\Theta]_r$ (degree × cm^2 × $dmol^{-1}$). Trifluoroethanol (TFE) was of spectroscopic quality, and phosphate buffers were prepared from the purest reagents. 1-Dodecylsulfate sodium salt (SDS) was of analytical grade. The peptide samples (0.1 mM) were prepared in H_2O–TFE solutions (10–80 vol % TFE), in phosphate buffers at pH 3, 5 and 7 and in the presence of SDS micelles. The CMC value was 20 mM in each sample. The circular dichroism curves were analyzed by the deconvolution program SELCON3 [13].

RESULTS

Synthesis

The purpose of the study was to synthesize TA and its two retro-analogues, to estimate their antimicrobial activity and to perform conformational analysis by circular dichroism. The peptides were synthesized by the solid-phase methodology using the Fmoc/tBu strategy and polystyrene AM-RAM resin. Most difficulties were encountered during the synthesis of TA. Coupling of Arg7 and Leu2 were especially problematic, and in both cases acylation had to be repeated three times. Moreover, coupling of Ile5 and Leu4 required a double-repeated reaction. The synthesis of retro-TA was performed without any problems. There was no need to repeat any of the coupling reactions within the whole peptide. During the synthesis of (6–1)(7–13)-TA, the most difficult reactions were those of the addition of Arg7 and Leu3, which had to be repeated three times, while the addition of Leu5 and Ile2 was repeated twice. After detachment from the resin, all three peptides had comparable purity, in the range of 60–70%. Subsequent

purification with preparative HPLC resulted in a purity of over 97%.

Antimicrobial Assay

The antimicrobial potential of the peptides was determined using a microbroth dilution method in polypropylene 96-well plates. As expected on the basis of previous microbiological studies, TA and its analogues exerted noticeable cytocidal activity toward gram-positive bacteria and fungi, while their activity toward gram-negative bacteria was rather poor (Table 1). TA and (6–1)(7–13)-TA were the most active compounds. However, retro-TA was less active only by one dilution in the majority of cases, which does not exclude this molecule as an interesting and applicable antimicrobial agent. It should be emphasized that the effective concentration of retro-TA against gram-positive bacteria did not exceed 32 μg/ml, with the exception of *E. hirae*, which was also relatively resistant for TA and (6–1)(7–13)-TA.

Conformational Studies

The CD spectra of synthetic TA, retro-TA and (6–1)(7–13)-TA were recorded in H_2O–trifluoroethanol (TFE), phosphate buffers and phosphate buffers/SDS micelles. These experiments have shown that in the case of TA and (6–1)(7–13)-TA an increase in TFE concentration (Figure 1) caused a progressive change from an unordered conformation (44 and 32%, respectively) to an α-helical structure (43 and 47%, respectively). The maximal effect was observed already with 30% TFE. With retro-TA, upon increasing the TFE concentration, a change from β-sheet structure (33%) to random conformation (43%) (Figure 1) took place. The CD spectra of all the peptides, recorded in phosphate buffers, display a minimum around 200 nm (Figure 2) and an overall shape that suggest the lack of any preferential conformation(s) under these conditions. As shown in Figure 3, the CD spectra of TA and (6–1)(7–13)-TA in the presence of SDS micelles have two minima at 208 nm and around 220 nm, respectively, indicating the presence of a helical structure under these conditions. A different behavior was observed for retro-TA, in which an unordered structure predominated (Figure 3) in the presence of SDS micelles. The shapes of the CD spectra (in SDS) of all peptides were independent of the pH value.

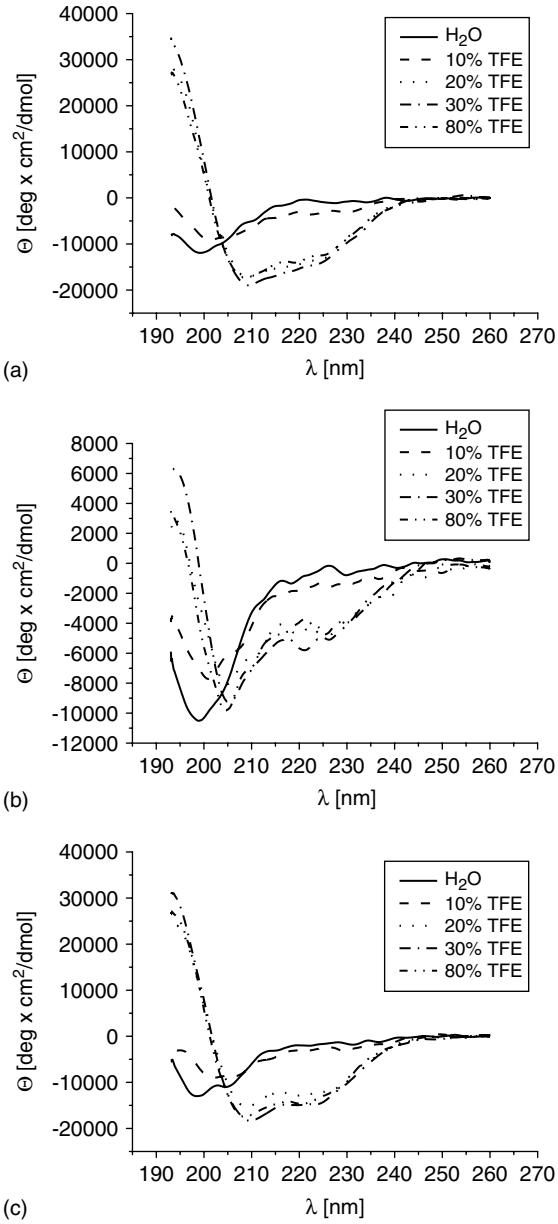

Figure 1 CD spectra of 0.1 mM: (a) TA, (b) retro-TA and (c) (6-1)(7-13)-TA in 10–80% H2O–trifluoroethanol solutions. (H_2O-solid, 10%-dash, 20%-dot, 30%-dash dot and 80%-dash dot dot).

DISCUSSION

When considering the possible introduction of TA analogues to industry, cost effectiveness is one of the most important criteria. From the industrial point of view, the most attractive compounds are those of short sequence and that are easy to synthesize. Temporins themselves form the group of the smallest antimicrobial peptides in nature. Since large-scale production of peptides utilizes automatic synthesis, which utilizes much more solvents and reagents as compared to the manual process, every additional reaction and additional time spent for the process increase the overall costs of production. The synthesis of TA and (6–1)(7–13)-TA is accompanied by problems at the same amino acid residues, so we feel that it is due to the specific conformation and intramolecular interaction in the growing peptide chain.

Other investigators have found that the net positive charge of the molecule is an important factor in

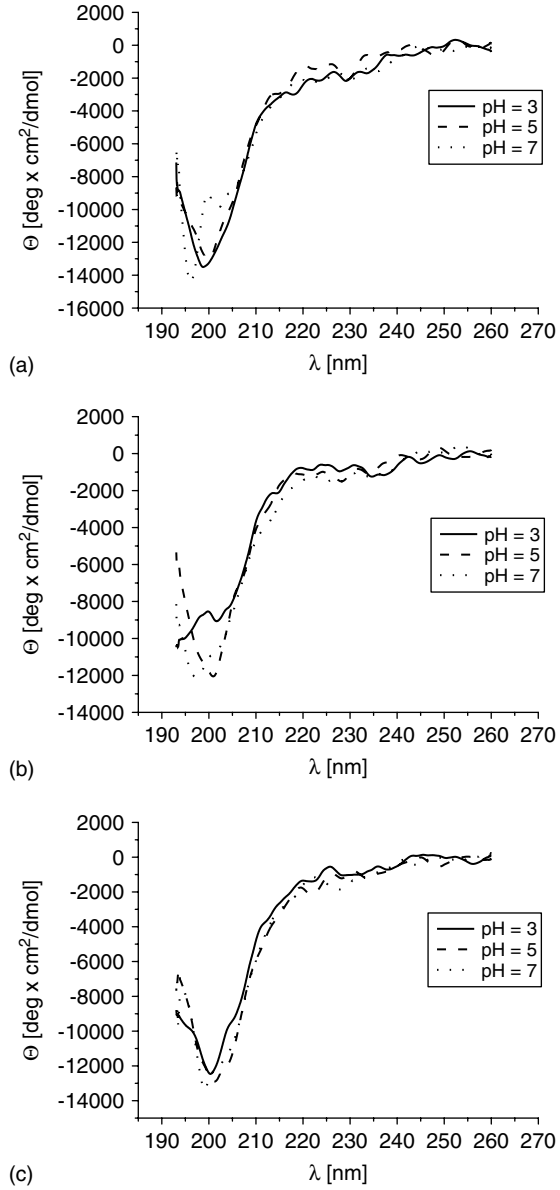

Figure 2 CD spectra of 0.1 mM: (a) TA, (b) retro-TA and (c) (6-1)(7-13)-TA in phosphate buffers of pH 3 (solid), 5 (dash) and 7 (dot).

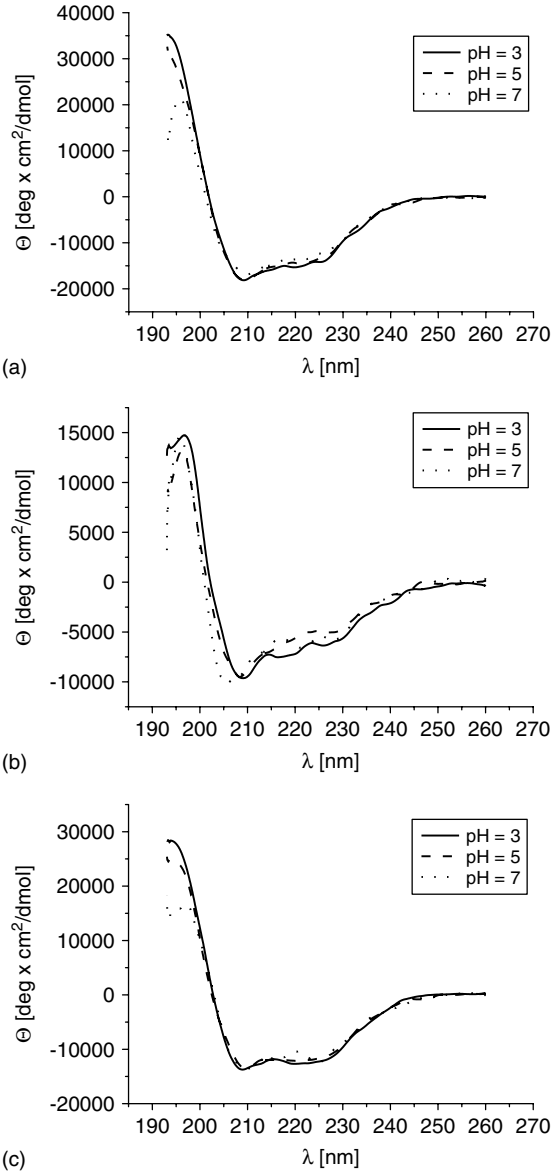

Figure 3 CD spectra of 0.1 mM: (a) TA, (b) retro-TA and (c) (6-1)(7-13)-TA in phosphate buffers of pH 3 (solid), 5 (dash) and 7 (dot), in the presence of SDS micelles.

determining the antibacterial activity of TA analogues. Hujakka *et al.* described a dimeric TA analogue, which gained cytocidal activity also for gram-negative bacteria [14]. Wade *et al.* have studied 18 analogues of temporin A and concluded that the helicity of the peptide can also be one of the critical parameters for its antibacterial character [15]. This would be in agreement with our results. According to the CD spectroscopy results, all peptides investigated by us form mainly random coils at different pH in phosphate buffer solutions. In water with relatively small TFE concentration (30%), TA and (6–1)(7–13)-TA adopt mainly α-helical structures. In the presence of SDS micelles, both peptides form mainly α-helices at different pH values. Thus, it can be concluded that TA and (6–1)(7–13)-TA adopt α-helical structures in hydrophobic environments, which is in good agreement with the postulated membrane interaction. The results of CD experiments for TA are similar to those reported in the literature [6,8]. For instance, retro-TA assumes mostly unordered conformation under hydrophilic or hydrophobic conditions. This can explain the lower antimicrobial potential of this analogue. However, a change in the peptide sequence resulting in a slightly lower activity of retro-TA is compensated by its nonproblematic synthesis.

Thus, retro-TA can even be a more attractive molecule than the original one. We recommended this slightly less-active but easily and inexpensively synthesizable molecule as an alternative for the expensive, naturally

Table 1 Antimicrobial activity of TA and its retro-analogues

Organism	Antimicrobial activity (μg/ml)					
	TA		Retro-TA		(6–1)(7–13)-TA	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive bacteria						
Bacillus subtilis	8	8	16	16	8	8
Enterococcus hirae	32	32	128	256	64	64
Rhodococcus equi	2	4	8	8	8	8
Staphylococcus epidermidis	4	4	16	16	8	16
Staphylococcus aureus	8	16	32	32	8	8
Staphylococcus saprophyticus	8	8	16	16	16	16
Gram-negative bacteria						
Escherichia coli	512	512	1024	1024	512	512
Proteus vulgaris	512	1024	>1024	>1024	512	1024
Pseudomonas aeruginosa	512	512	>1024	>1024	512	1024
Serratia marcescens	>1024	>1024	>1024	>1024	>1024	>1024
Stenotrophomonas maltophila	1024	>1024	>1024	>1024	1024	>1024
Fungi						
Candida albicans	32	32	64	64	32	32
Aspergillus niger	64	256	128	512	64	256

occurring TA. On the other hand, retro-TA can be considered as a starting material for further structure–function studies, for example, looking for single amino–acid substitutions, resulting in a higher antimicrobial potency.

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