Comparison of synthesis and antibacterial activity of temporin A

Irene Harjunpää[a], Pentti Kuusela[b,c], Marek T. Smoluch[d], Jerzy Silberring[d], Hilkka Lankinen[a], David Wade[e,*]

[a] *Haartman Institute, Peptide and Protein Laboratory, Department of Virology, P.O. Box 21, FIN-00014 Helsinki University, Helsinki, Finland*
[b] *Haartman Institute, Department of Bacteriology and Immunology, FIN-00014 Helsinki University, Helsinki, Finland*
[c] *HUCH Diagnostics, Department of Bacteriology and Immunology, P.O. Box 402, FIN-00029 University Central Hospital, Helsinki, Finland*
[d] *Faculty of Chemistry and Regional Laboratory, Jagiellonian University, Ingardena 3, PL-30-060 Krakow, Poland*
[e] *Kuwait University, Faculty of Medicine, Department of Biochemistry, P.O. Box 24923, 13110 Safat, Kuwait*

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Abstract Temporin A is a small, basic, highly hydrophobic, antibacterial peptide found in the skin of the European red frog, *Rana temporaria*. It was synthesized twice by the FastMoc solid phase method using amino acids protected at the N^{α}-position with either 9-fluorenylmethoxycarbonyl or 2-(4-nitrophenylsulfonyl)ethoxycarbonyl. The syntheses of temporin A demonstrates the difference between 2-(4-nitrophenylsulfonyl)ethoxycarbonyl and 9-fluorenylmethoxycarbonyl amino acids. The purified peptide showed also antibacterial activity against clinically important Gram-positive bacteria. It was found to have a moderately good activity against both methicillin resistant and sensitive strains of *Staphylococcus aureus*, but a weaker activity against vancomycin resistant strains of *Enterococcus faecium*.

Key words: Temporin A; Synthetic peptide; 2-(4-nitrophenylsulfonyl)ethoxycarbonyl/9-fluorenylmethoxycarbonyl amino acids; Antibacterial activity; *Staphylococcus aureus*; *Enterococcus faecium*

1. Introduction

The temporins (temporins A–G, H, K, L) are a group of 10 small (13 amino acids), basic (net charge at pH 7 = +1–+2), hydrophobic peptide amides that have been isolated from the skin of the European red frog, *Rana temporaria* [1]. They are among the smallest antibacterial peptides that have been isolated from animal sources. They are active against both Gram-positive and -negative bacteria and are found to be non-toxic to human red blood cells.

Temporin A (FLPLIGRVLSGIL-NH_2) has been synthesized previously by the solid phase 9-fluorenylmethoxycarbonyl (Fmoc) methodology ([1], Donatella Barra, personal communication). Compared with two similar antibacterial peptides isolated from insect sources, melittin (a 26 amino acid peptide amide [2]) and cecropin A (a 37 amino acid peptide amide [3]), temporin A was 10-fold less potent than melittin against *Staphylococcus aureus* Cowan I (S.a.CI), a common laboratory test strain of *S. aureus* (lethal concentration (LC) = 2.3 versus 0.2 μM), but greater than 100-fold more potent than cecropin A against the same bacterium (LC = 2.3 versus > 200 μM).

The purposes of this investigation were (1) to synthesize temporin A using amino acids protected at the N^{α}-group with Nsc, a relatively new protecting group for the solid phase peptide synthesis [4], with a standard automated synthesis program, (2) to compare the synthesis with the one done using Fmoc-protected amino acids, (3) to confirm the identity of the products of each synthesis by standard analytical techniques and (4) to assay the peptide for activity against clinically important strains of Gram-positive bacteria.

2. Materials and methods

2.1. Peptide synthesis, purification and characterization

Temporin A was synthesized twice on an Applied Biosystems model 433A Peptide Synthesizer with the manufacturer's pre-defined FastMoc chemistry options which use a 10-fold molar excess of amino acids over resin loading, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/*N*-hydroxybenzotriazole/*N*,*N*-diisopropylethylamine activation, deprotection with 20% piperidine and *N*-methylpyrrolidone as the solvent. Syntheses were done in a 0.05 mmol scale with 9 min coupling times and under feedback controlled repeats of 2 min deprotection reactions. Each cycle of amino acid addition included the capping reaction in which unreacted primary amines were acetylated with acetic anhydride. The syntheses differed in two ways: (1) the type of N^{α}-protection was either Fmoc or 2-(4-nitrophenylsulfonyl)ethoxycarbonyl (Nsc) and (2) the length of the time for deprotection of the N^{α}-group was modified from 2 min/reaction with Fmoc to 5 min with Nsc in the first reaction and 3 min in each repeat. The deprotection times programmed for each cycle of the Nsc synthesis were increased because the Nsc group is known to require more time for removal than the Fmoc group [4]. Fmoc amino acids were purchased either from Novabiochem (San Diego, California, USA) or Senn Chemicals AG, (Dielsdorf, Switzerland). Nsc amino acids were a generous gift from Dr Hack-Joo Kim (Research Institute, Hyundai Pharm., Seoul, South Korea). The side chain protecting groups were the same for both the Nsc and Fmoc amino acids, t-butyl ether for serine and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl for arginine. The solid phase used was Rink amide resin (Novabiochem, substitution = 0.56 mmol/g). After synthesis was completed, the peptide was de-protected and cleaved from the resin by treatment with trifluoroacetic acid (TFA)/thioanisole/ethanedithiol/water (90/5/2.5/2.5) at room temperature for 2 h. The peptide was then precipitated from the cleavage solution by adding ice-cold diethyl ether into the mixture, followed by centrifugation. The precipitate was washed twice with 40 ml of ether, dissolved in water, frozen and lyophilized.

The peptide synthesized with Nsc amino acids was then analyzed by reversed phase high performance liquid chromatography (RP-HPLC) on a 0.46×25 cm, 5 μm particle size, Vydac 218TP54 C18 column, eluting with a 50 min gradient of 0–100% acetonitrile (CH_3CN)-0.1% TFA/H_2O-0.1% TFA with a flow rate of 1 ml/min and monitoring the eluate at 210 nm. Three major peaks were detected. In order to obtain enough material for bacteriological assays, a 12.5 mg amount of crude peptide was subjected to preparative RP-HPLC (Fig. 1A), using conditions similar to those mentioned above, except that the column was a Vydac 1×25 cm, 10 μm particle size, 218TP101522 C18 and the flow rate was 5 ml/min. Three main fractions were collected, CH_3CN was evaporated by a stream of N_2 and the aqueous peptide solutions were frozen and lyophilized. The three resulting purified fraction powders were each analyzed by electrospray ionization mass spectrometry (ESI-MS) as described previously [5].

*Corresponding author. Fax: (965) (533) 8908.
E-mail: david@hsc.kuniv.edu.kw

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For the peptide synthesized with Fmoc amino acids, the HPLC procedures were similar. Analytical RP-HPLC gave a tailing major peak which had the same retention time as temporin A in the Nsc synthesis. Purification of 13 mg of crude peptide was done with a 10 min gradient of 0–21% CH_3CN/H_2O-0.1% TFA, followed by a 40 min gradient of 21–70% CH_3CN/H_2O-0.1% TFA and 10 min of isocratic elution at 70% CH_3CN-H_2O-0.1% TFA. Fractions corresponding to the main peak (Fig. 1B) were pooled, lyophilized and subjected to ESI-MS.

Amino acid analysis was carried out using a Pharmacia-LKB 4151 Alpha Plus analyzer after hydrolysis for 24 h at 110°C in evacuated tubes with 6 M hydrochloric acid containing 0.5% (w/v) phenol.

2.2. Bacterial strains and media

Three bacterial strains of *S. aureus* and two strains of *Enterococcus faecium* were used in the studies. Methicillin sensitive *S. aureus* strain K52 and methicillin resistant strain K35 as well as the vancomycin resistant *E. faecium* strains 18578 and 18172, which carried the *vanA* and *vanB* genes, respectively, were clinical isolates originating from the Department of Bacteriology and Immunology, HUCH Diagnostics, Helsinki University Central Hospital. The third staphylococcal strain was the laboratory strain, Newman. For the experiments, bacterial strains were maintained on chocolate agar plates.

2.3. Determination of minimal inhibitory concentration (MIC) and bacterial killing

MIC values were determined by using a microdilution technique. Bacterial suspensions of each strain, corresponding to a McFarland value of 1.0, were diluted 1:1000 in Mueller-Hinton broth containing Ca^{2+} and an aliquot (25 μl) was mixed with an aliquot (25 μl) of 2-fold dilutions of temporin A in de-ionized H_2O. The MIC was defined as the lowest concentration of temporin A allowing no visible growth after 20 h of incubation at 37°C.

To determine the bacterial killing, 10 μl of a suitable dilution made from microdilution wells was spread on Mueller-Hinton agar plates and colony forming units (CFUs) were counted after an overnight incubation at 37°C. Dilutions giving 20–500 bacterial colonies per plate were used for colony counting.

3. Results

3.1. Peptide synthesis, purification and characterization

During automated Fmoc synthesis, the N^{α}-deprotection steps of each cycle of amino acid addition are feed-back monitored by measuring the amount of a conductive piperidine-carbamate salt that is formed. The FastMoc protocol specifies that a deprotection step is repeated until there is less than 4% difference in conductivities between successive couplings. The conductivity of the Nsc synthesis was recorded as in the Fmoc synthesis. A comparison of the Fmoc and Nsc synthesis showed that for the Nsc synthesis each deprotection reaction was repeated three times (14 min in total) except for serine which was repeated twice (11 min in total), whereas for the Fmoc synthesis each deprotection reaction was repeated only twice (6 min in total), indicating, as expected, a slower removal of Nsc.

Fig. 1 shows preparative RP-HPLC chromatograms of the crude product obtained from cleavage of the peptides from resin. As indicated by the ESI-MS (figure insets) for the purified peptide fractions, temporin A was found in the Nsc synthesis preparation in the central major peak (Fig. 1A) and in Fmoc synthesis preparation in the major peak (Fig. 1B). Each preparation had a molecular ion corresponding to the expected mass to charge (m/z) ratio of the single charged species, 1397, and also a predominant ion corresponding to the double charged species, m/z 699. Analytical RP-HPLC of the purified temporin As from both syntheses gave chromatograms with single peaks, indicating pure peptide products

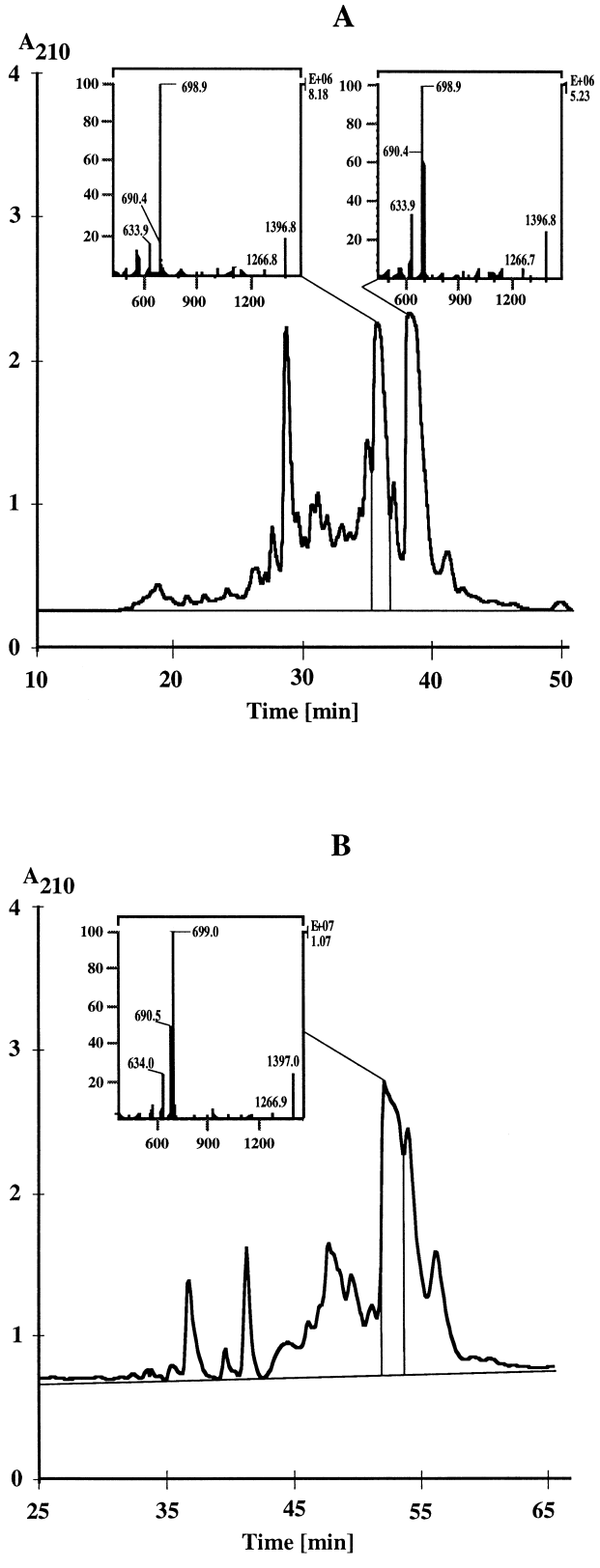

Fig. 1. Chromatograms from preparative RP-HPLCs of the crude peptide products from Nsc (A) and Fmoc (B) syntheses of temporin A shown as absorbance U at 210 nm post injection. In (A), the peak of purified temporin A is the central major peak and in (B), it is the major peak. ESI-MS results of all temporin A containing fractions are shown as insets, with the m/z ratio as the horizontal axis and abundance as the vertical axis.

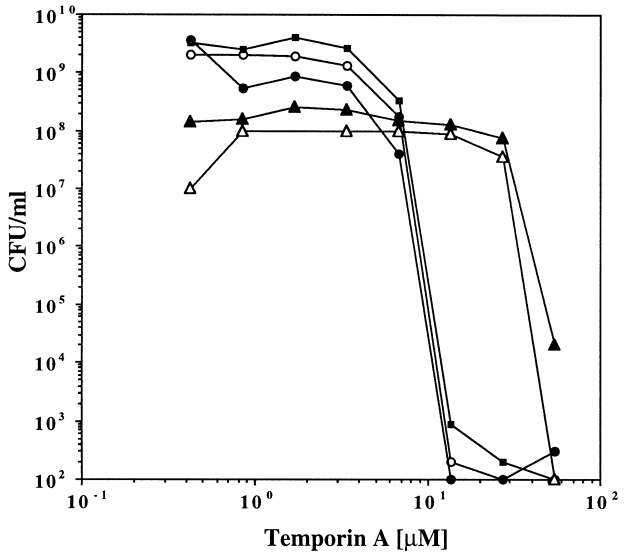

Fig. 2. The bacteriocidal effect of temporin A against *S. aureus* strain Newman (open circle) and strains K35 (closed circle) and K52 (closed square) and against *E. faecium* strains 18578 (open triangle) and 18172 (closed triangle).

(data not shown). Separation of temporin A into two major fractions was observed in Nsc but not in Fmoc synthesis. This may reflect a tendency of temporin A to aggregate either with itself or with the observed synthetic side-product(s).

Amino acid analysis confirmed that the purified temporin A had the correct amino acid composition and the result was also used to establish the concentrations of peptide solutions in subsequent bacteriological assays.

3.2. Bacteriological assays

Temporin A inhibited the growth of both *S. aureus* and *E. faecium*. MICs for *S. aureus* strain Newman and both *S. aureus* strains K35 and K52 were 6.8 μM and 13.7 μM, respectively (Table 1). The corresponding values for *E. faecium* strains 18578 and 18172 were 27.3 μM and 54.6 μM, respectively (Table 1).

Temporin A was also bacteriocidal to *S. aureus* and *E. faecium*. CFUs of *S. aureus* strains were reduced more than 90% at a temporin A concentration of 6.8 μM and further 5–6 orders of magnitude at temporin A concentrations of 13.7 μM (Fig. 2). The peptide was bacteriocidal to *E. faecium* only at the highest concentrations used (54.6 μM), where the bacterial numbers were reduced five orders of magnitude for *E. faecium* strain 18578 and less than four orders of magnitude for the strain 18172 (Fig. 2).

Table 1
Capability of temporin A to inhibit the growth of Gram-positive bacteria

Bacterium	Strain	MIC[a]	
		μg/ml	μM
S. aureus:	Newman	9.5	6.8
	K35	19.5	13.7
	K52	19.5	13.7
E. faecium	18578	38.1	27.3
	18172	76.3	54.6

[a]Minimal inhibitory concentration.

4. Discussion

There is one report in the literature about temporin peptides [1]. It contains the first description of the naturally occurring temporins and compares the biological properties of the naturally isolated forms, A, B, D and H, to the synthetic versions of the peptides. It was shown that temporins A and B have antimicrobial activities and that these activities were the same for both naturally occurring and synthetic versions. The goals of this investigation were to attempt the FastMoc synthesis of temporin A using amino acids protected at the N^{α}-group with Nsc and to compare the synthetic product with that obtained using Fmoc-protected amino acids. The additional purpose was to test the antimicrobial effects of the purified peptide against clinically important methicillin sensitive and resistant *S. aureus* strains, as well as against vancomycin resistant *E. faecium* strains.

The Nsc group has been proposed as an appropriate temporary protecting group for the α-amine in amino acids during solid phase synthesis [6]. It has an increased stability in neutral and weakly basic dimethylformamide solutions as compared to Fmoc. It apparently has the same mechanism of cleavage by organic bases as does the Fmoc group, although at rates of cleavage that are 3–8-fold slower than for Fmoc. Comparisons of interferon α2 (130-137), acyl carrier protein (65-74) and ACTH(1-24), that were synthesized in the solid phase by using both Nsc and Fmoc amino acids and identical side chain protecting groups, revealed that the crude products of Nsc and Fmoc syntheses yielded essentially the same HPLC profiles [6]. The results of the syntheses of temporin A using Nsc or Fmoc amino acids were in line with previous syntheses. Temporin A is a hydrophobic peptide compared to the three other peptides mentioned above and very hydrophobic peptides often present synthetic difficulties like deletion and termination peptides [7]. In the Nsc synthesis, the relative amount of deletion peptides (50%) eluting prior to temporin A was estimated to be 6% more than in Fmoc synthesis (44%) (Fig. 1). The observed synthesis difference is most probably due to incomplete N^{α}-deprotections in case of Nsc, a result which for long or difficult peptides proposes low yields in FastMoc protocols using Nsc amino acids. Lengthening of the reaction time up to 3–8-fold appears necessary, but extends deprotection cycles (20–50 min in a 0.1 mmol scale). Therefore, use of a higher concentration of piperidine or complementation of 20% piperidine with the stronger base 1,8-diazabicyclo(5,4,0)undec-7-ene-1,1,3,3-tetramethyl guanidine are feasible changes for Nsc synthesis [6].

In regard to the bacteriological experiments with temporin A derived from the Nsc synthesis, the peptide had a moderately good activity against the three *S. aureus* strains but a weak activity against the *E. faecium* strains. CFUs of staphylococcal strains were reduced more than 90% at a temporin A concentration of 6.8 μM. Whereas a similar effect against *E. faecium* strains was seen at a temporin A concentration higher than 27.3 μM. The values are somewhat higher than the lethal concentration of 2.3 μM reported for the S.a.CI laboratory strain [1]. It is difficult to compare the values because different methods were used to determine the bacteriocidal concentration (also Cowan I was not included in the present study). Interestingly, the bacteriocidal effect of temporin A appeared similar against both the methicillin resistant and sensitive strains, which suggests that methicillin sensitivity

is not related to the mechanism of action of this peptide. Methicillin is a β-lactam that inhibits the bacterial peptidoglycan synthesis, whereas the mechanism of action of temporin A is thought to involve the formation of ion channels or pores in the bacterial plasma membrane. There appeared to be a slight difference in the potency of the peptide against the two vancomycin resistant enterococci. In a separate experiment (data not shown), a vancomycin sensitive *E. faecium* strain was found to be as sensitive to temporin A as the vancomycin resistant strains, suggesting that vancomycin sensitivity, like methicillin sensitivity, is also unrelated to the temporin A sensitivity. Differences in susceptibilities to vancomycin may be related to differences between bacterial strains or differences in their cell walls. In summary, these antibacterial results are promising and it should be possible to improve the antibiotic activities of temporin A by synthetic manipulations of its primary structure, as has been accomplished for many similar peptides [8].

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