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Trichogin GA IV: an antibacterial and protease-resistant peptide‡

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The antibacterial and hemolytic activities of the amphiphilic helical, membrane-active, lipopeptaibol trichogin GA IV and its [Leu[11]-OMe] analogue were compared to those of the partially helical or non-helical 8-meric or 4-meric, *C*-terminal short sequences, respectively. The study on trichogin GA IV was extended to several methicillin-resistant *Staphylococcus aureus* strains. Using a large set of enzymes, we also evaluated the resistance to proteolysis of all of the four peptides. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antibacterial peptide; circular dichroism; lipopeptaibol; proteolytic stability; trichogin

Introduction

'Desperately seeking new antibiotics' is the stimulating title of a *Perspective* contribution appeared in *Science* in September 2008 [1] which reflects the need for new approaches and molecules to combat the rise in bacteria that are resistant to current drugs. Among the properties required for peptide molecules to be exploitable as effective antibiotics, selectivity, hemolytic inactivity, and stability towards proteolytic attacks are of particular relevance. In this connection, here we describe the properties shown by the peptide antibiotic trichogin GA IV, a selected analogue, and two short sequences (the latter aiming at reducing the production costs).

Trichogin GA IV, isolated from the fungus *Trichoderma longibrachiatum* and sequenced by Bodo and coworkers [2,3], is the prototypical member of a class of unusual linear peptides exhibiting membrane-modifying properties. These compounds are referred to as *lipopeptaibols* [4], a term that fully describe their chemical structure. They are *Aib* (α-aminoisobutyric acid)-rich *pept*ides with the *N*-terminus acylated by a *lipo*yl, *n*-Oct (*n*-octanoyl), moiety and an 1,2-aminoalcoh*ol*, Lol (leucinol), at the *C*-terminus. The primary structure of the *N*- and *C*-blocked 10-amino acid peptide trichogin GA IV is as follows:

1 10
n-Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol

We have unambiguously demonstrated by a variety of physicochemical techniques (including X-ray diffraction, CD, and NMR) [5–7] that trichogin GA IV (Tric) and its equipotent undecapeptide [Leu[11]-OMe] (OMe, methoxy) (Tric-OMe) (Table 1) analogue are amphiphilic, right-handed, mixed $3_{10}/\alpha$-helical [8,9] peptides. In these lipopeptides, an N^{α}-blocking fatty acyl moiety of at least six carbon atoms is required for the onset of significant membrane activity [10]. Also, we and others have shown that largely β-turn [11,12] folded, but not helical, forms characterize the *n*-Oct-blocked, short *C*-terminal peptides, while the longest peptides predominantly adopt regular helical structures [13–15]. Membrane activity, although weak, is already seen for main-chain lengths as short as the [Leu[4]-OMe] tetrapeptide (Tric-4) (Table 1), and it progressively increases to the [Leu[8]-OMe] octapeptide (Tric-8) (Table 1) and the undecapeptide.

Materials and Methods

Peptide Synthesis and Characterization

The total chemical syntheses in solution of trichogin GA IV, its Tric-OMe analogue, and the two C-terminal short sequences are reported in refs. [10] and [13]. For the present study the last three peptides have been resynthesized following the same strategy. Their amino acid sequences and mass spectral data [the latter collected on a Perseptive Biosystem (Foster City, MA) Mariner ESI-TOF spectrometer in the positive ionization mode] are reported in Table 1 and the HPLC elution profiles are shown in Figure 1.

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Table 1. Amino acid sequences and molecular masses (M. W.) of the synthetic trichogin GA IV analogues and short sequences studied

Peptide	Amino acid sequence	$[M + H]^+_{calcd}$	$[M + H]^+_{exptl}$[a]
Tric-OMe	*n*-Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe	1094.72	1094.70
Tric-8	*n*-Oct-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe	839.56	839.52
Tric-4	*n*-Oct-Aib-Gly-Ile-Leu-OMe	527.38	527.37

[a] Determined using ESI mass spectrometry (for details see Section on Materials and Methods).

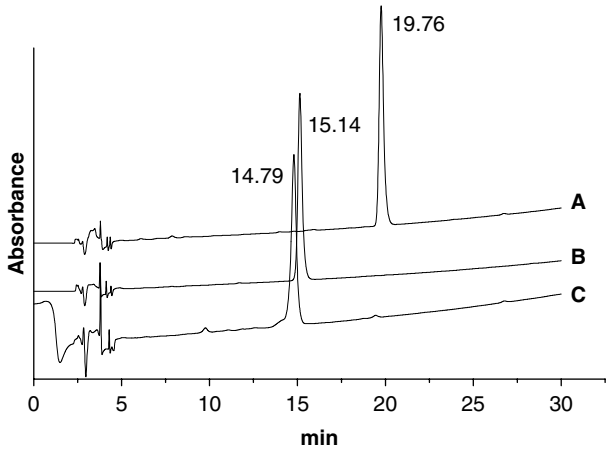

Figure 1. Analytical HPLC elution profiles (retention times) of: (A) Tric-OMe, and (B) the 8-meric (Tric-8) and (C) the 4-meric (Tric-4) short sequences. Experimental conditions: Macherey-Nagel C_4 reverse-phase column; gradient from 40 to 70% B in 30 min A = 0.05% TFA in a 9:1 (v/v) mixture of H_2O/CH_3CN; B = 0.05% TFA in a 1:9 (v/v) mixture of H_2O/CH_3CN]. The absorption was monitored at 220 nm.

Circular Dichroism (CD)

The circular dichroism (CD) spectra were measured on a Jasco (Hachioji City, Japan) model J-710 dichrograph. Cylindrical fused quartz cells of 1.0, 0.2 and 0.1 mm pathlengths (Hellma, Mühlheim, Germany) were used. The values are expressed in terms of $[\theta]_T$, the total molar ellipticity (deg $\times$ cm^2 $\times$ $dmol^{-1}$). Spectrograde 2,2,2-trifluoroethanol (TFE) (Acros, Géel, Belgium) was used as solvent.

Bacterial Strains

Escherichia coli (ATCC 25 922), *Listeria monocytogenes* (ATCC 19 115), and *Staphylococcus aureus* (ATCC 25 923) were obtained from American Type Culture Collection. *Bacillus subtilis* (KCTC 1918), *Staphylococcus epidermidis* (KCTC 3096), and *Pseudomonas aeruginosa* (KCTC 1637) were from the Korean Collection for Type Cultures (KCTC), Korean Research Institute of Bioscience and Biotechnology (KRIBB), Taejon, Korea. To assess the antimicrobial activities of our peptides against antibiotic-resistant bacteria, clinically isolated multidrug-resistant bacterial strains were obtained from the Culture Collection of Antibiotic-Resistant Microbes (CCARM) at Seoul Women's University, Korea. These included five strains of methicillin-resistant *S. aureus* (MRSA) (CCARM 3089, CCARM 3090, CCARM 3108, CCARM 3114, and CCARM 3126).

Antibacterial Activity

To determine the minimal inhibitory concentration (MIC) values for the tested peptides, serial microdilution assays were performed. Cells were grown to log-phase in 10 g/l bactotryptone, 5 g/l yeast extract, and 10 g/l NaCl, pH 7.0, then passed through a 0.22 μm filter and stepwise-diluted in a medium of 1% bactopeptone. Each organism to be tested was suspended at 2×10^6 colony formation units (CFU)/ml in growth medium. A stock solution of each peptide was prepared by dissolving it in the minimum amount of DMSO and diluting with PBS (phosphate buffered saline). The maximum amount of DMSO in each solution was 1.45%. Then, eight solutions, obtained by successive two-fold dilutions, were prepared and tested by mixing 100 μl of each solution with 100 μl of the organism suspension in a microtiter plate well. The test for each peptide was repeated three times at every concentration. The plates were incubated for 18 h at 37 °C. The MIC value was defined as the lowest concentration of peptide that gave no visible growth on the plate. The endpoint was detected by use of a spectrophotometer.

Hemolytic Activity

The hemolytic activity of each peptide was determined using hRBCs (human red blood cells) from healthy donors that were collected on heparin. Fresh hRBCs were washed three times in PBS by centrifugation for 10 min at 800 *g* and resuspension in PBS. The peptide was dissolved in the minimum amount of DMSO, diluted with PBS to the desired concentration (15 μM) and then added to 100 μl of stock hRBCs suspended in PBS (final hRBCs concentration: 8%, v/v). The maximum amount of DMSO in each solution was 1.45%. The samples were incubated with agitation for 1 h at 37 °C and centrifuged at 800 *g* for 10 min. The absorbance of the supernatant was measured at 414 nm. Controls for zero hemolysis (blank) and 100% hemolysis consisted of hRBCs suspended in PBS and 1% Triton X-100, respectively. The percentage hemolysis was calculated using the following equation: % hemolysis = $[(A_{414\ nm}$ with protein solution $- A_{414\ nm}$ in PBS)/($A_{414\ nm}$ with 0.1% Triton X-100 $- A_{414\ nm}$ in PBS)] $\times$ 100. Each measurement was made in triplicate [16]. The hemolytic peptide melittin was synthesized in the Chosun University laboratories.

Antifungal Activity

Microdilution assays were performed to establish the MIC values for the peptides. *Candida albicans* was grown at 28 °C in YPD (2% dextrose, 1% peptone, and 0.5% yeast extract, pH 5.5) for 3 h. Cell densities were counted with a hemocytometer. The fungal cells (2×10^3/well) were seeded on the wells of a flat-bottom 96-well microtiter plate (Greiner, Nurtingen, Germany) containing YPD (100 μl/well). Serial dilutions of the peptide solution were added to each well, and the cell suspension was incubated at 28 °C for 24 h. A 10 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (5 mg/ml) was added to each well, and the plates were incubated at 37 °C for 4 h. The absorbance at 570 nm was measured using an Emax microtiter plate reader (Molecular Devices, California, USA). All assays were performed in triplicate. The assays for antifungal activity against *Fusarium oxysporum*, *Rhizoctonia solani*, *Aspergillus awamori*, *Aspergillus parasiticus*, and

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Astragalus flavus were carried out in 100 × 15 mm Petri dishes containing YPD. After the mycelial colony had developed, sterile blank paper disks (8 mm diameter) were placed 5 mm from the leading edge of the mycelial colony. An aliquot of the peptide test sample in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (20 mM, pH 6.0) was added to each disk, and the plates were incubated at 28 °C for 72 h. Antifungal activity was shown as a clear zone of growth inhibition.

Proteolytic Stability

To evaluate their proteolytic stability, the peptides were dissolved in an enzyme buffer (50 mM Tris·HCl, pH 7.8). The enzymes trypsin, pepsin, chymotrypsin, and endoprotease Glu-c V8 were obtained from Sigma (St.Louis, MO). Also pronase E, a protease from *Streptomyces griseus*, elastase (from porcine pancreas), and subtilisin A (from *Bacillus sp.*) were Sigma products. The enzyme was added to the peptide in the ratio 1 : 250 (w/w) and the mixture was incubated at 37 °C overnight. The samples were analyzed using a C_{18} reverse-phase HPLC column. Appropriate programmed gradients from 40 to 95% B solution for 20 min were applied using eluants A (H_2O/0.1% trifluoroacetic acid, TFA) and B (CH_3CN/0.1% TFA).

Results and Discussion

Circular Dichroism

For our conformational study in the extensively used, secondary structure-supporting solvent TFE we relied heavily on CD spectroscopy. Figure 2 shows the far-UV CD spectra of the undecapeptide Tric-OMe and its two C-terminal short sequences, the tetrapeptide Tric-4 and the octapeptide Tric-8. As expected from its short main-chain length, the curve of Tric-4, with its positive maximum at 203 nm and a cross-over point at 196 nm, is indicative of an unordered conformation [17]. Conversely, the spectrum of Tric-OMe, displaying two negative Cotton effects centered at 203 and 225 nm [10], resembles those of right-handed 3_{10}- and α-helices [17–20]. Moreover, the ratio *R* between the intensities of the 225 nm *versus* 203 band is 0.43, much closer to the value theoretically predicted [18] and experimentally found [19,20] for 3_{10}-helical peptides (0.4) than to that (about 1.0) of α-helical peptides. Not surprisingly, the spectrum (showing negative maxima at 227 and 199 nm) of Tric-8, characterized by a main-chain length intermediate between those of Tric-4 and Tric-OMe, suggests a co-existing population of unordered and helical conformers. The aforementioned CD results in TFE differ only slightly from those obtained for the same peptides in the more rarely used solvent methanol [7,10,13].

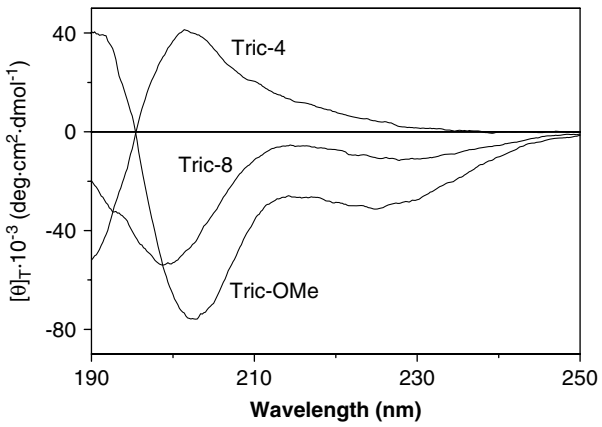

Figure 2. CD spectra (190–250 nm region) of Tric-OMe, and the 8-meric (Tric-8) and 4-meric (Tric-4) short sequences in TFE solution.

Antimicrobial and Hemolytic Activities

Tables 2 and 3 list the activities of trichogin GA IV, its Tric-OMe analogue, and the Tric-4 and Tric-8 C-terminal short sequences against a representative set of Gram-positive and Gram-negative bacteria, including different MRSA strains (part of these results, anticipated in a preliminary form, may be found in ref. 21). Interestingly, trichogin exhibits significant activity only against *S. aureus*. The bacterial cell selectivity of trichogin is mirrored by Tric-OMe. Even more important, trichogin is active also against most of the MRSA strains. The two short sequences do not show any appreciable activity. This latter finding strongly supports the view that the full, terminally-blocked, 10-mer sequence of trichogin GA IV is strictly required for the antibacterial activity. In turn, this result implies that not only the main-chain length itself, but the length-dependent helix stability as well, may play a significant role on the antibacterial activity. Notably, as mentioned above [13], we did find that the inactive 8-mer is nevertheless able to induce significant perturbation on model biomembranes. It is also worth pointing out that we have already shown that the activity of Tric-OMe against the Gram-positive *Streptococcus β-hemolyticus* is comparable to that of bacitracin, an antimicrobial peptide commonly used as standard [22].

To assess the cytotoxicity of the four peptides investigated against mammalian cells, the hemolysis percentage was measured against human erythrocytes after one hour of incubation in PBS at a concentration where trichogin and Tric-OMe exhibit antibacterial activity (15 μM). Low hemolysis (2%) is caused by the two long peptides and 0% by the two inactive short peptides (Tric-4 and Tric-8). The antimicrobial peptide melittin, used as a comparison in our test, exhibited a strong hemolytic activity. In a previous work [10] we found some hemolytic activity for trichogin and Tric-OMe (the threshold effect for peptide concentration is around 16 μM after three hours of incubation in about 1% DMSO solution). Finally, none of the peptides examined shows any measurable antifungal activity against a large spectrum of species (*Candida albicans*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Aspergillus awamori*, *Aspergillus parasiticus*, and *Astragalus flavus*).

Proteolytic Stability

We tested the susceptibilities to proteolytic degradation of the four peptides in the presence of seven proteases: endoproteinase Glu-c, pepsin, chymotrypsin, trypsin, subtilisin A, elastase, and pronase E. Figure 3 shows the HPLC elution profiles of trichogin before and after the proteolytic enzyme treatments. The results clearly indicate that in all experiments performed the natural peptide substrate is effectively shielded from degradation. The behavior of the other three peptides (Tric-OMe, Tric-4, and Tric-8) strictly parallels that of the natural peptide. At this stage of our research, it remains to be unambiguously demonstrated whether the observed stability to proteolytic attack of these hydrophobic peptides would be a result of a specific sequence/3D-structure or of their propensity to self-associate in aqueous solution. Some research groups have already reported significant protection for Aib-containing peptides from

Table 2. Minimal inhibitory concentrations (MIC) (μM) for different bacteria (10^6 CFU/ml)

Peptide	*S. aureus*	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*B. subtilis*	*L. monocytogenes*
Trichogin	7.5–15[a]	>30	30	>30	>30	>30
Tric-OMe	15	>30	30	>30	>30	>30
Tric-8	>40	>40	>40	>40	>40	>40
Tric-4	>60	>60	>60	>60	>60	>60

[a] The MIC value is intermediate between the concentrations 7.5 and 15 μM.

Table 3. Activity of trichogin GA IV against different methicillin-resistant *S. aureus* strains

Parameter	CCRAM 3089	CCRAM 3090	CCRAM 3108	CCRAM 3114	CCRAM 3126
MIC 90 (μM)[a]	12.5	>12.5	12.5	>12.5	12.5
IC 50 (μM)[b]	6.25–12.5	>12.5	6.25–12.5	6.25–12.5	6.25–12.5

[a] Minimal inhibitory concentration (90%).
[b] Inhibitory concentration (50%).

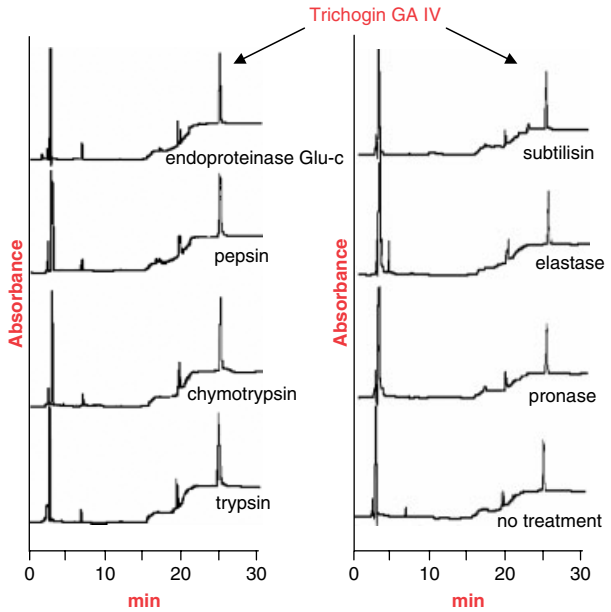

Figure 3. HPLC elution profiles (retention times) of trichogin GA IV before and after proteolytic enzyme treatments (for experimental conditions see Section on Materials and Methods). The absorption was monitored at 220 nm. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

being proteolytically digested [23–28]. However, only in one case [24] the attribution to the Aib-generated helical structure [29–31] in reducing protease recognition was explicitly suggested.

Conclusions

Since their discovery during the twentieth century, antibiotics have substantially reduced the threat posed by infectious diseases that were previously untreatable and fatal. These advancements have been recently jeopardized by the emergence and spread of microbes that are resistant to these drugs. One possible solution to these problems is offered by natural antibacterial peptides. These short peptides (having 10–20 amino acids in their sequence) are often folded in an amphiphilic helical structure and are produced by virtually all organisms as a first line of defense against pathogens. In contrast to traditional antibiotic drugs, these molecules usually act simply by perturbing the bacterial membrane, making it permeable, and thus leading to cell death. For this reason, they represent excellent candidates for the development of novel therapeutic agents. In addition to the need of being bacterial cell-selective and non-cytotoxic, one of the main issues which has limited the widespread clinical application of antibacterial peptides made of protein amino acids is their significant susceptibility to proteolytic degradation.

In this work, we have shown that the naturally-occurring, amphiphilic helical lipopeptaibol trichogin GA IV, and its [Leu^{11}-OMe] analogue as well, exhibit a selective antibacterial activity against *S.aureus* and only a marginal hemolytic effect. Interestingly, trichogin GA IV is active also against several MRSA strains (this property is extremely important as, for example, more people die from the MRSA bacterium than from HIV in the United States [1]). We have also demonstrated that deletion of three and seven *N*-terminal residues, to afford the partially helical and non-helical *C*-terminal 8-mer and 4-mer, respectively, eliminates any antibacterial activity. Finally, evaluation of the effects induced by a variety of proteolytic enzymes on these four peptides has highlighted their remarkable stability to digestion.

Taken together, the present findings strongly support the view that the full 10-mer sequence of trichogin GA IV could represent a promising lead compound for the design and production of novel, selective, non-hemolytic, and protease-resistant antibacterial peptide-based drugs. In this connection, we are currently examining the role of the three Aib residues in supporting the amphiphilic helical secondary structure and in producing the enzymatic stability and bioactivity profiles typical of this lipopeptaibol by replacing each of them in the various positions of the sequence with helicogenic, hydrophobic protein amino acids.

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