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Antibacterial and antifungal properties of α-helical, cationic peptides in the venom of scorpions from southern Africa

Leentje Moerman[1], Suzanne Bosteels[1], Wim Noppe[1], Jean Willems[1], Elke Clynen[2], Liliane Schoofs[2], Karin Thevissen[3], Jan Tytgat[4], Johan Van Eldere[5], Jurg van der Walt[6] and Fons Verdonck[1]

[1]*Interdisciplinary Research Center, Katholieke Universiteit Leuven Campus Kortrijk, Kortrijk;* [2]*Laboratory for Developmental Physiology and Molecular Biology, Katholieke Universiteit Leuven, Leuven;* [3]*F.A. Janssens Laboratory of Genetics, Katholieke Universiteit Leuven, Heverlee;* [4]*Laboratory of Toxicology, Katholieke Universiteit Leuven, Leuven;* [5]*Laboratory for experimental Microbiology, Rega Institute, Katholieke Universiteit Leuven, Leuven, Belgium;* [6]*Department of Physiology, University of Potchefstroom, Potchefstroom, South Africa*

Two novel pore-forming peptides have been isolated from the venom of the South-African scorpion *Opistophtalmus carinatus*. These peptides, designated opistoporin 1 and 2, differ by only one amino acid and belong to a group of α-helical, cationic peptides. For the first time, a comparison of the primary structures of α-helical pore-forming peptides from scorpion venom was undertaken. This analysis revealed that peptides in the range of 40–50 amino acids contain a typical scorpion conserved sequence $S(x)_3KxWxS(x)_5L$. An extensive study of biological activity of synthesized opistoporin 1 and parabutoporin, a pore-forming peptide previously isolated from the venom of the South-African scorpion *Parabuthus schlechteri*, was undertaken to investigate an eventual cell-selective effect of the peptides. Opistoporin 1 and parabutoporin were most active in inhibiting growth of Gram-negative bacteria (1.3–25 μM), while melittin and mastoparan, two well-known cytolytic peptides, were more effective against Gram-positive bacteria in the same concentration range. In addition, the peptides showed synergistic activity with some antibiotics commonly used in therapy. Opistoporin 1 and parabutoporin had hemolytic activity intermediate between the least potent mastoparan and the highly lytic melittin. Furthermore, all peptides inhibited growth of fungi. Experiments with SYTOX green suggested that this effect is related to membrane permeabilization.

Keywords: scorpion venom; cytotoxic peptide; antimicrobial peptide; antifungal agent; amphipathic peptide.

Scorpion venom has been investigated mostly for its neurotoxins acting on different ion channels [1–3]. Recently, α-helical pore-forming peptides have been discovered in scorpion venom (parabutoporin [4], hadrurin [5], IsCTs [6,7] and pandinins [8]). In addition, the cDNA sequence of a peptide from *Buthus martensii* has been described, but biological activity of the peptide has not yet been studied [9]. Pore-forming peptides can be divided into two groups, depending on their primary and secondary structures: (a) linear, mostly α-helical peptides without cysteine residues, and (b) cysteine-rich peptides that form a β-sheet or β-sheet and α-helical structures (for review see [10]). Most of them have amphipathic properties. These peptides are widespread in nature. In animals, their presence has generally been described in body fluids in contact with the external environments, in venom and in hemolymph. Members of the first group have been isolated from the venom of different organisms: bee (melittin [11]), wasp (mastoparan [12]), spider (lycotoxin [13], cupiennin 1 [14], oxyopinin [15]), ant (pilosulin [16], ponericins [17]) and scorpion. Similar peptides are found in the skin secretion of frogs (magainin [18], dermaseptin [19]); for a review of α-helical peptides, see [20]. Peptides containing disulfide bridges are even more ubiquitous in nature. In scorpion venom, representative peptides of this group have been described in *Pandinus imperator* (scorpine [21]). Other disulfide containing peptides were isolated from hemolymph of *Androctonus australis* (androctonin [22]) and *Leiurus quinquestriatus* (scorpion defensin [23]). This group is also largely represented in mammalia. Pore-forming peptides are part of the innate immune system acting as a defense mechanism against invading microorganisms (for review see [24,25]). Despite much literature concerning the antibacterial activities of pore-forming peptides, antifungal activity has been studied for only a few peptides, e.g. dermaseptin [19] and cecropin [26]. Concerning α-helical pore-forming peptides isolated from scorpion venom, antifungal activity has been described only for pandinin 2 [8].

In addition to their defensive role against microorganisms, another function has been described for pore-forming peptides because of their depolarizing effect in excitable cells: lycotoxins, isolated from the venom of the wolf spider *Lycosa carolinensis* act as paralytic agents and may have a

Correspondence to F. Verdonck, Interdisciplinary Research Center, Katholieke Universiteit Leuven Campus Kortrijk, E. Sabbelaan 53, B-8500 Kortrijk, Belgium.
Fax: + 32 56 246997, Tel.: + 32 56 246224,
E-mail: Fons.Verdonck@kulak.ac.be

Abbreviations: CFU, colony-forming unit; Dm-AMP1, antimicrobial peptide isolated from seed of dahlia (*Dahlia merckii*); Myr_2Gro-*P*Cho, 1,2-dimyristoyl-*sn-glycero*-3-phosphocholine; Myr_2Gro-*P*Gro, 1,2-dimyristoyl-*sn-glycero*-3-phospho-*rac*-1 glycerol; LPS, lipopolysaccharide; MIC, minimal inhibitory concentration; PMA, 4β-phorbol 12-myristate 13-acetate.

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function in prey-capture strategy [13]. Pardaxins, pore-forming peptides secreted by the sole fish of the genus Pardachirus, function as shark repellents [27]. The action of pore-forming peptides is not only related to the defense mechanism of higher organisms, but they can also be used by bacteria as a part of their pathogenicity (e.g. aerolysin from the bacterium *Aeromonas hydrophila* [28]).

The interaction between pore-forming peptides and biological membranes has been extensively studied, but is still not fully understood. Different models have been described: pore-forming peptides are thought to destabilize biological membranes via a barrel-stave, a carpet-like or a toroidal mode of action [20].

Besides acting by destabilizing membrane structures and changing ion permeabilities, pore-forming peptides can influence cell functioning by interacting with intracellular signaling. Interaction with G-proteins has been described in different cell types [29]. In granulocytes, intracellular signaling can be influenced by interaction of pore-forming peptides with the NADPH oxidase system [30] and degranulation can be observed. These properties have been studied almost exclusively for mastoparan. Degranulation of human granulocytes has been reported for parabutoporin [4] and IsCT degranulates rat peritoneal mast cells [6]. Although not studied in detail for most amphipathic α-helical peptides, it is most likely that other compounds of this group could have the same activity because the cationic, amphipathic α-helical structure of these peptides is thought to be responsible for these activities [29,30].

Recently, we isolated parabutoporin, a cysteine-free pore-forming peptide of 45 amino acid residues from the venom of the South African scorpion *Parabuthus schlechteri* [4]. Here we describe the isolation of new pore-forming peptides from the venom of *Opistophtalmus carinatus* and compared their activity with the activity of parabutoporin. The peptides were studied for antibacterial, antifungal and hemolytic activities and were compared with the biological activity of melittin (GIGAVLKVLTTGLPALISWIKRK RQQ) and mastoparan (INLKALAALAKKIL). This is the first report of an extensive study on the antifungal activity of α-helical pore-forming peptides isolated from scorpion venom. We also analyzed the primary structures of cysteine-free α-helical peptides that currently have been described in scorpion venom and compared them with sequences of cationic peptides in the venom of other arthropods.

EXPERIMENTAL PROCEDURES

Collection of venom

Venom of *O. carinatus* was collected by electrical stimulation of the telson with a frequency- and voltage-controlled stimulator. Venom drops were transferred in 0.5 mL of deionized water and immediately frozen in liquid nitrogen and stored at −80 °C. For this study, a total volume of about 30 μL venom was used (three animals).

HPLC purification of opistoporin

Lyophilized whole venom was dissolved in 0.1% trifluoroacetic acid and fractionated in a two step reversed-phase HPLC (Alliance Waters) using 0.1% trifluoroacetic acid in water as buffer A and 0.1% trifluoroacetic acid in acetonitrile as buffer B solutions. A linear gradient from 0 to 100% acetonitrile was applied in 25 min at a flow rate of 1 mL·min^{-1}. Fractionation was started on a Prosphere C_4 column (5 μm, 300 Å; Alltech). After determination of the active peak, a subsequent purification was performed on an Xterra RP C18 column (Waters) using a linear gradient from 0 to 60% 0.1% trifluoroacetic acid in acetonitrile in 17.5 min.

Isolation of human granulocytes

Human granulocytes were obtained from the blood of healthy volunteers and purified by Ficoll–Paque centrifugation and hyposmotic lysis of red blood cells as described previously [4].

Procedure for testing of inhibition of superoxide production in human granulocytes

Because inhibition of superoxide production by granulocytes has been reported for mastoparan [31], and because this is a fast and relatively simple screening test, inhibition of superoxide production by the isolated fractions was measured in order to determine the active component. Granulocytes were diluted to a final cell concentration of 2×10^5 mL^{-1} in NaCl/P_i/RPMI. Lyophilized crude venom or purified fractions were dissolved in 1 mL of NaCl/P_i buffer. Thirty microliters of this solution (for controls 30 μL NaCl/P_i solution) were added to 150 μL of granulocyte containing medium and 120 μL RPMI/NaCl/P_i. Control and samples were incubated for 1 h at 37 °C. Thereafter, 50 μL lucigenine (0.5 μg·mL^{-1}) was added and chemiluminescence was measured. A few minutes later, 50 μL PMA (4β-phorbol 12-myristate 13-acetate, 1 μg·mL^{-1}) was added and superoxide production was measured for 10–15 min by a Biolumat 9505. Peak luminescence values were compared and inhibition was calculated as a percentage of superoxide production produced by PMA in control samples (no venom present).

Sequence determination

The primary structure of the peptide was resolved by Edman degradation. For this purpose the sample was dissolved in acetonitrile/water/trifluoroacetic acid (20 : 79.9 : 0.1, v/v/v). Two microliters of the sample were loaded on a glass fiber and subjected to N-terminal amino acid sequencing on a Procise protein sequencer (Applied Biosystems) running in the pulsed liquid mode. Because the complete sequence could not be determined in this way, the peptide was enzymatically digested with 0.5 μg sequencing grade modified trypsin (Promega) for 20 h at 37 °C in 20 μL 0.2 M NH_4HCO_3, pH 8. Subsequently, the mixture was separated by HPLC on a Waters Symmetry C18 column (4.6 × 250 nm). Operating conditions were as follows: 0.1% trifluoroacetic acid in water for 10 min, followed by a linear gradient to 50% acetonitrile (with 0.1% trifluoroacetic acid) for 60 min. The flow rate was 1 mL·min^{-1} and the absorbance was measured simultaneously at 214 and 280 nm.

Mass spectrometry

The molecular mass of the active fraction was determined by nanoflow electrospray (ESI) double quadrupole (Qq) orthogonal acceleration (oa) time of flight (TOF) MS on a Q-TOF system (Micromass, UK). An aliquot of the fraction was dried, redissolved in acetonitrile/water/formic acid (80 : 19.9 : 0.1, v/v/v) and loaded in a gold-coated borosilicate capillary needle (Protana L/Q needle). The multiply-charged ion spectrum was transformed to molecular mass by the Maxent software (Micromass, UK). The masses of the peptides resulting from the tryptic digestion were determined by MALDI-TOF MS on a VG Tofspec (Micromass, UK), equipped with a N2-laser (337 nm) and were compared with those acquired by a theoretical tryptic digestion of the peptide, performed by a computer program (http://www.expasy.ch/cgi-bin/peptide-mass.pl).

Chemical synthesis of parabutoporin and opistoporin

The peptides were prepared by solid-phase synthesis by Ansynth Service B.V. (the Netherlands) using the Fmoc/*tert*-butyl-based methodology with Rink resin as the solid support. The peptides were synthesized manually. The crude peptide was purified by cationic ion exchange chromatography and HPLC on a platinum EPS C18 100 Å 5 μm HPLC column.

Computational analysis of primary and secondary structure

Sequence alignments and percentage identity/similarity in amino acid composition for different peptides were based on Clustal W sequence alignments. Secondary structure predictions were carried out by the secondary structure consensus prediction program. Protein databases were scanned for the conserved amino acids found in pore-forming peptides from scorpion venom by Pattinprot analysis. All programs are available at the NPSA server (http://pbil.ibcp.fr/NPSA).

CD spectroscopy

CD measurements were carried out on a Jasco J-600 A spectropolarimeter using a cuvette of 1 mm pathlength in the far-UV at 25 °C. Base-line normalization was performed at 250 nm. All measurements were performed in 20 mM Tris, pH 7.5 with or without 40% trifluoroethanol, a promotor of the α-helical structure of peptides. Measurements were performed in the presence of Myr_2Gro-*P*Cho or Myr_2Gro-*P*Gro liposomes. The concentration of the peptide was adjusted to 50 μM. The data were expressed as residual ellipticity θ (degrees·cm^2·$dmol^{-1}$).

Preparation of liposomes

Small unilamellar vesicles were prepared by sonication of Myr_2Gro-*P*Cho or Myr_2Gro-*P*Gro dispersions. Dry lipid was dissolved in chloroform. The solvent was then evaporated under a stream of nitrogen. The dry lipid film was resuspended in 5 mM Tes buffer pH 7.0 and then sonicated (peak-to-peak amplitude, 24 μm) for 10 min in an MSE 150-W ultrasonic disintegrator equipped with a 3/8-inch titanium sonication tip.

Antibacterial activity assay

Micro-organisms. *Bacillus subtilis* ATCC 6051, *Bacillus subtilis* IP 5832, *Enterococcus faecalis* ATCC 19433, *Listeria monocytogenes* NCTC 11994, *Micrococcus luteus* ATCC 9341, *Nocardia asteroides* ATCC 3308, *Streptococcus pneumoniae* ATCC 33400 and *Staphylococcus aureus* ATCC 29213 were used in this study as Gram-positive strains. The Gram-negative strains used were *Escherichia coli* ATCC 25922, *Escherichia coli* DH5α, *Haemophilus influenzae* ATCC 19418, *Klebsiella pneumoniae* ATCC 13833, *Salmonella choleraesuis* ATCC 13311, *Serratia marcescens* ATCC 133880 and *Pseudomonas aeruginosa* ATCC 27853.

Determination of minimal inhibitory concentration. The bacteria were grown in Brain Heart Infusion (Oxoid, CM225) at 37 °C and after 4 h, the suspension was diluted in the same medium to a D_{600} of 0.002 ($\pm 5 \times 10^5$ CFU·mL^{-1}). Bacteria were incubated in 96-well microplates in the presence of different concentrations of cationic peptides (twofold serial dilutions) in a final volume of 100 μL. The microplates were incubated at 37 °C with continuous shaking. After 16 h, D_{620} was measured. MIC (minimal inhibitory concentration) is expressed as the lowest concentration that causes 100% inhibition of growth. Results are means of four independent experiments. For growth of *Haemophilus influenzae* 2 μg·mL^{-1} NAD^+, 10 μg·mL^{-1} hemine and 10 μg·mL^{-1} histidine were added to the medium.

Determination of synergism of cationic peptides with conventional antibiotics

Twofold serial dilutions of amoxicillin, levofloxacin, cefuroxime and erythromycin were tested in the presence of a constant amount of peptide equal to one-quarter of the peptide MIC for Gram-negative bacteria and MIC/8 for Gram-positive bacteria. MIC was determined on two independent occasions. Synergism was accepted when the difference of the MIC of the antibiotics in presence and absence of cationic peptides was at least two dilutions. For a more extensive description of the method see [32].

In vitro antifungal activity assay

Micro-organisms. Fungal strains used in this study are *Botrytis cinerea* MUCL 30158, *Fusarium culmorum* MUCL 30162 and *Neurospora crassa* FGSC 2489. Filamentous fungi were grown on six-cereal agar, and conidia were harvested as described previously [33]. *Saccharomyces cerevisiae* strain used was W303–1 A (genotype: *MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*).

Antifungal activity assay. Antifungal activity of the peptides was assayed by microspectrophotometry of liquid cultures grown in microtiter plates as described previously [33]. Briefly, in one well of a 96-well microplate, 20 μL of the protein sample was mixed with 80 μL of half-strength potato dextrose broth (Difco, Detroit, MI, USA), containing

fungal spores at a concentration of 2×10^4 conidia·mL^{-1}. Growth was recorded after 48 h of incubation at 22 °C. The absorbance at 595 nm served as a measure for microbial growth. IC_{50} values (the concentration of the protein required to inhibit 50% of the fungal growth) were calculated from dose–response curves with twofold dilution steps [34]. Antifungal activity against *S. cerevisiae* was determined in an analogous manner (2×10^6 yeast cells per mL, ½ potato dextrose broth). The microplates were incubated at 30 °C without shaking, and the absorbance at 595 nm was recorded after 20 h of incubation.

SYTOX green uptake. Fungal membrane permeabilization was measured by SYTOX green uptake as described previously [35]. Absolute values of fluorescence did not differ more than 50% in independent tests performed under identical conditions.

Hemolytic assay

The hemolytic activity of the peptides was determined using human red blood cells. Fresh human red blood cells with heparin were washed three times (10 min at 200 g) with buffer (0.81% NaCl with 20 mM Hepes pH 7.4) and resuspended in the same buffer. An amount of human red blood cell suspension was added to buffer with the appropriate amount of peptide to reach a final concentration of 10^7–10^8 human red blood cells·mL^{-1} (final volume = 100 μL). The samples were incubated at 37 °C for 30 min. After centrifugation, hemolysis was determined by measuring absorbance at 570 nm of the supernatant. Controls for zero hemolysis and 100% hemolysis consisted of human red blood cells suspended in buffer and distilled water, respectively.

RESULTS

Purification of opistoporin

The venom of the scorpion *O. carinatus* was fractionated by HPLC, as shown in Fig. 1. A first purification gave eight fractions of which only fraction seven inhibited superoxide production by granulocytes (Fig. 1A). Inhibition of superoxide production has been described for mastoparan [30,31], mastoparan-like peptides [30,31] and melittin [36]. This test was used for its simplicity to detect analogous peptides in the venom of scorpions. Fraction 7 was further purified and four subfractions were obtained; fraction B contained the active component (Fig. 1B). After a last purification round, this fraction was separated into two subfractions (Fig. 1C). Inhibition of superoxide production by granulocytes was related to fraction B1. This fraction represents about 5% of the total protein content of the venom, estimated by its relative surface area in the HPLC spectra. The purification of parabutoporin was described previously [4].

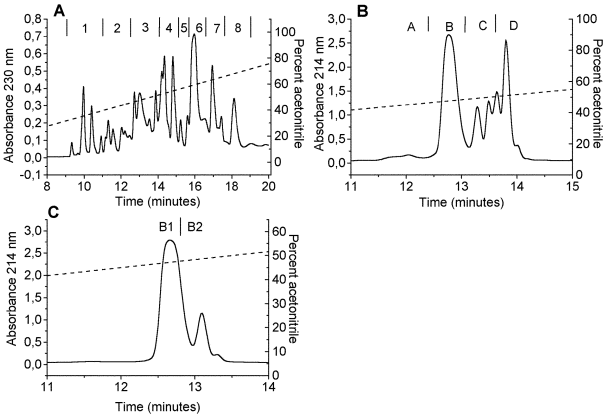

Fig. 1. Purification profile of whole venom components from *Opistophtalmus carinatus*. (A) The whole venom was loaded on a Prosphere C_4 column (5 μm–300 Å Alltech) with a linear gradient from 0 to 100% acetonitrile in 25 min at a flow rate of 1 mL·min^{-1}. The effluent was monitored at 230 nm. The fractions were tested on inhibitory activity on superoxide production by human granulocytes. Fraction 7 contained the active compound. (B) Fraction 7 from the first purification was further separated on an Xterra RP18 column using a linear gradient from 0 to 60% 0.1% trifluoroacetic acid in acetonitrile in 17.5 min. The effluent was monitored at 214 nm. Only fraction B was biologically active. (C) Fraction B from the second purification was again loaded on the Xterra RP18 column using the same linear gradient. Inhibition of superoxide production by human granulocytes was related to peak B1. Dashed lines show the concentration of acetonitrile.

Molecular mass and amino acid sequence of opistoporin

Q-TOF mass spectrometry measurements of the active fraction yielded two series of multiply charged ions, corresponding to two molecular masses, 4836 Da and 4870 Da. The sequence was unambiguously determined by Edman degradation up to amino acid 42. At position 43 a very weak signal corresponding to a proline appeared. Each sequencing cycle yielded a single clear amino acid signal, except for cycle 34 where leucine as well as phenylalanine were detected. Hence, both the mass spectrometric and the amino acid sequencing data indicated the presence of two different peptides with a microheterogeneity on position 34. However, the theoretical masses, calculated according to the 42 amino acid sequences (4652.4 Da and 4686.4 Da) showed a difference of 183.5 Da with the masses observed with Q-TOF mass spectrometry (4836 Da and 4870 Da), indicating the presence of one or two additional amino acids at the carboxyl-terminus. Subsequently, the active fraction was subjected to a tryptic digestion. The mixture of the proteolytic fragments was separated into 10 defined peaks. The masses of these peaks were determined by MALDI-TOF mass spectrometry and compared to those obtained by a theoretical digestion (http://www.expasy.ch/cgi-bin/peptide-mass.pl) of the two sequences. All the masses of the theoretical fragments were found. The fragment with a leucine at position 34 as well as the fragment with phenylalanine at position 34 were present, thereby confirming the presence of two different isoforms. From the observed masses combined with the sequence information, the mass of the C-terminal fragment was deduced (544.3 Da). The fraction containing this mass was subjected to Edman degradation and the sequence was determined as IGATPS. This fragment sequence allowed us to assign the two last residues lacking in the sequence in agreement with the 183.5 Da mass difference between theoretical masses calculated according to the 42 amino acid sequences and

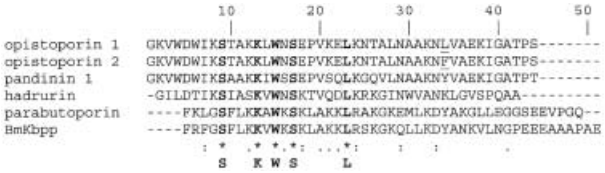

Fig. 2. Comparison of primary structures of cationic pore-forming peptides isolated from scorpion venom based on Clustal W sequence alignment. Sequence alignments of opistoporins, parabutoporin, pandinin 1, hadrurin and BmKBpp showing the conserved amino acid residues in bold. The amino acid difference between opistoporin 1 and 2 is underlined. *, identical amino acids; :, strongly similar amino acids; ., weakly similar amino acids.

those obtained by Q-TOF mass spectrometry. The complete sequence of both peptides is presented in Fig. 2. The peptides were named opistoporin 1 (amino acid 34L) and 2 (amino acid 34F), referring to the scorpion genus from which they were isolated. The average molecular mass values calculated from the sequence data are in complete agreement with molecular mass values measured for both opistoporins.

The purification was started from a mixture of venom from different animals belonging to the same species. To solve the question whether the presence of the two peptides was related to coexpression of both peptides in 1 animal, we analyzed the venom from one single animal. The mass spectrum showed that both peptides can be present in the same venom sample, indicating that one individual scorpion can produce both opistoporins. In some venoms from individual scorpions only one of the two opistoporins, either opistoporin 1 or 2, could be detected.

The peptides contained 12 charged residues (eight lysine, three glutamate and one aspartate), having a charge of +4 at neutral pH. Under the same conditions, the charge of parabutoporin is +7 [4]. These peptides do not contain cysteine residues.

Based on the Clustal W sequence alignment, sequences of different pore-forming peptides isolated from scorpion venom were compared (Fig. 2). The opistoporins have 77.3% identical amino acids and 95.5% (for opistoporin 1) and 97.7% (for opistoporin 2) similar amino acids with pandinin 1 (Fig. 2). The scorpions from which they are isolated, *O. carinatus* (southern Africa) and *P. imperator* (west and central Africa), respectively, both belong to the family of Scorpionidae. The sequences of parabutoporin and BmKbpp contain 61.7% identical amino acids and 89.4% similar amino acids (Fig. 2). Both scorpions (*P. schlechteri*, from southern Africa and *B. martensii*, living in China) belong to the family of Buthidae. An intermediate amount of identical amino acids was observed for hadrurin, isolated from the Mexican scorpion *Hadrurus aztecus* (family Iuridae), with opistoporins (34%) and pandinin 1 (31%). This means that there is a high conservation in amino acid sequence of the peptides in the venom of scorpions that belong to the same family, independent of the continent and region were they live.

Furthermore, we have determined five conserved residues in six of the nine cationic, amphipathic pore-forming peptides isolated from scorpion venom until now. All these peptides contain 41–47 amino acid residues (Fig. 2) and have the sequence $S(x)_3KxWxS(x)_5L$ in their N-terminal half. Pandinin 2 and IsCTs do not have these conserved residues, but these peptides are shorter (24 and 13 amino acid residues, respectively). To our knowledge, this sequence of conserved residues has not been observed in any cationic, α-helical pore-forming peptide known today (based on Pattinprot analysis). Thus these conserved amino acids seem to be a specific signature for cationic pore-forming peptides isolated from scorpion venom.

A larger scale comparison of sequences of parabutoporin and the opistoporins with cysteine-free peptides isolated from venom of other arthropods showed that the highest degree of identical amino acids existed with oxyopinin 1 [15], 25% for parabutoporin and 26% for both opistoporins. Comparison of primary structures with the ponericins [17] showed 22% identical amino acids for parabutoporin and the opistoporins with ponericin G1. In addition, parabutoporin has 24.4% identical amino acids to ponericin L1 and 22.4% to pilosulin [16]. For other cysteine-free peptides isolated from arthropod venom, identities in primary structure were less than 20%. All these homologies are less significant than those observed between peptides isolated from scorpions belonging to the same family. Homologies between cysteine-free peptides from venom of scorpions from different families may be less than homologies between these peptides and cysteine-free peptides recently described in the venom of spiders and insects.

Parabutoporin and opistoporin 1 were synthesized chemically and preliminary studies on antibacterial activity showed that the quality and biological activity of native and synthesized toxins were identical. Due to a shortage of native material, all biological characterizations were carried out using the synthetic peptides.

Secondary structure

Secondary structure predictions have been performed for parabutoporin, and opistoporin 1 and 2 by the secondary structure consensus prediction program. Parabutoporin is predicted to be α-helical from amino acid 3 to amino acid 35. Both opistoporins contain two α-helical domains (residues 3–14 and 20–39) separated by a random coiled region (WNSEP). Such a structure has also been predicted for pandinin 1 [8] and hadrurin [5]. The predictions for parabutoporin and opistoporin 1 were confirmed by means of circular dichroism. The CD spectrum of parabutoporin in 40% trifluoroethanol, a secondary structure promoting solvent, shows two major dips around 208 and 222 nm (Fig. 3A) which is characteristic for the presence of an α-helical structure. The spectrum of parabutoporin in the absence of trifluoroethanol is characteristic of an unordered structure. To mimic the interaction of parabutoporin with cell membranes, CD spectra were recorded in the presence of Myr_2Gro-*P*Cho and Myr_2Gro-*P*Gro small unilamellar vesicles (Fig. 3A). Because the peptides are positively charged at neutral pH, a different interaction of the peptides with negatively charged (Myr_2Gro-*P*Gro) and zwitterionic (Myr_2Gro-*P*Cho) vesicles could be expected. This has recently been described for anoplin [37]. The spectra of parabutoporin in the presence of vesicles resemble those in the presence of 40% trifluoroethanol and indicate that parabutoporin can adopt an α-helical structure in the presence of phospholipids. No great differences in

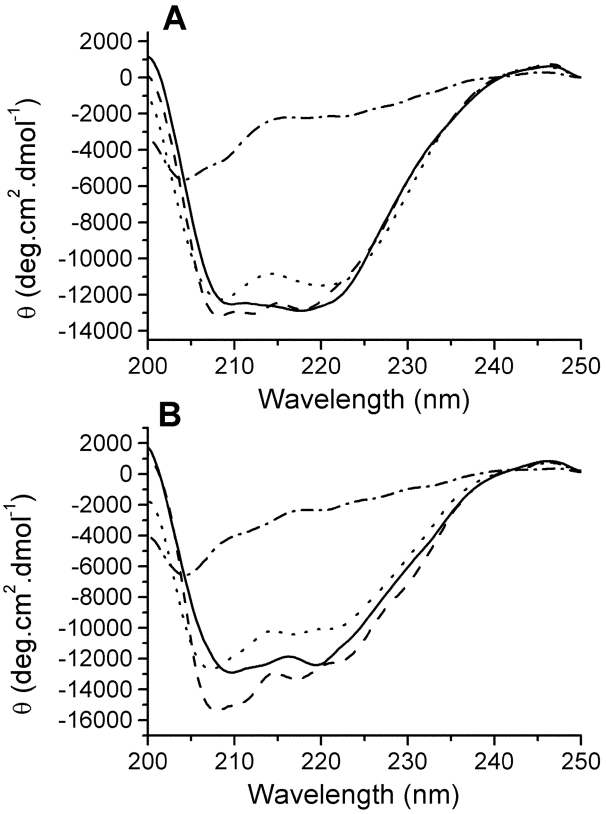

Fig. 3. CD spectra of parabutoporin (A) and opistoporin 1 (B). Spectra were taken at a peptide concentration of 50 μM in absence (dash dot line) or presence of 40% trifluoroethanol (dotted line), 1,2-dimyristoyl-*sn-glycero*-3-phospho-*rac*-1 glycerol (Myr_2Gro-*P*Gro, solid line) or 1,2-dimyristoyl-*sn-glycero*-3-phosphocholine (Myr_2Gro-*P*Cho, dashed line) small unilamellar vesicles.

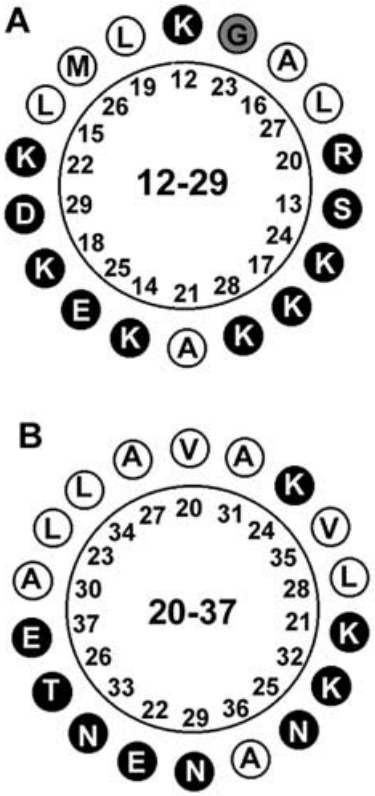

Fig. 4. Helical wheel diagram of parabutoporin (A) and opistoporin 1 (B). For both peptides, a fragment of 18 amino acids that was predicted to be α-helical was shown (parabutoporin amino acids 12–29, opistoporin 1 amino acids 20–37). Hydrophilic residues are given in white letters on a black background, hydrophobic ones are circled and neutral amino acids are given as black letters on a gray background.

secondary structure in the presence of negatively charged or zwitterionic vesicles were observed.

CD spectra of opistoporin 1 indicate also that the peptide is unordered in aqueous solution but can fold into an α-helical structure in a membrane-mimicking environment (Fig. 3B). This phenomenon has also been described for peptides isolated from the venom of other scorpions (IsCTs [6,7], pandinins [8]).

Figure 4 represents helical wheel projections for parabutoporin and opistoporin 1. For both peptides, a fragment of 18 amino acids predicted to be α helical is shown (parabutoporin amino acids 12–29, opistoporin amino acids 20–37). For each peptide, clearly distinct hydrophobic and hydrophilic regions can be distinguished, making both molecules amphipathic.

Antibacterial activity

Because the main function of cationic α-helical peptides has generally been related to their antibacterial activity [20], parabutoporin and opistoporin 1 were tested on Gram-negative and Gram-positive bacteria and their activity was compared with the activity of melittin and mastoparan (Table 1).

Parabutoporin inhibits the growth of all Gram-negative bacteria tested except *S. marcescens* at a concentration of less than 6.5 μM (32.7 $\mu g \cdot mL^{-1}$). The concentration of opistoporin 1 needed to inhibit the growth of the Gram-negative bacteria varied between 1.6 and 50 μM (7.7 and 242 $\mu g \cdot mL^{-1}$), with the peptide being least active against *S. marcescens*. Mastoparan is far less active in inhibiting growth of Gram-negative bacteria and melittin is only active against three of the Gram-negative bacteria at the concentrations tested. However, melittin is the most active in inhibiting growth of Gram-positive bacteria, mastoparan is less active and the two scorpion toxins are the least active (parabutoporin: MIC from 6.3 to > 50 μM, 31.7 to > 251.6 $\mu g \cdot mL^{-1}$, opistoporin 1: MIC 12.5 to > 50 μM, 60.4–242 $\mu g \cdot mL^{-1}$). Thus, these findings show that the peptides isolated from the venom of South-African scorpions are most active in inhibiting growth of Gram-negative bacteria, while melittin and mastoparan are more active against Gram-positive bacteria.

As it has been described that the NH_2-terminal α-helical domain of dermaseptin S (amino acids 1–18) is responsible for antimicrobial activity and is even more antibacterial than dermaseptin (34 amino acids) [19], peptides consisting of amino acids 7–22 of parabutoporin (an α-helical part having the four amino acids LAKK identical to mastoparan) and of the first 28 amino acids of the opistoporins were synthesized and were investigated for antibacterial activity. Almost no activity was seen at concentrations of 50 μM (not shown),

Table 1. Antibacterial activities of parabutoporin, opistoporin, melittin and mastoparan in absence and presence of 5 mM extracellular Mg^{2+} ions. Bacteria were incubated with twofold serial dilutions of peptides and inhibition of growth was measured after 16 h at 37 °C.

	Minimal inhibitory concentration (concentration that inhibits 100% of bacterial growth, μM)							
Mg^{2+} concentration	Parabutoporin 0 mM	Parabutoporin 5 mM	Opistoporin 1 0 mM	Opistoporin 1 5 mM	Melittin 0 mM	Melittin 5 mM	Mastoparan 0 mM	Mastoparan 5 mM
Gram-negative bacteria								
E. coli ATCC 25922	3.1	25	12.5	> 50	25	> 50	25	> 50
E. coli DH5α	3.1	25	6.3	50	50	> 50	12.5	> 50
S. marcescens ATCC 133880	> 50	ND	50	ND	> 50	ND	> 50	ND
P. aeruginosa ATCC 257853	6.3	25	12.5	> 50	> 50	ND	50	ND
K. pneumoniae ATCC 13833	1.6	6.3	6.3	50	50	ND	12.5	25
S. choleraesuis ATCC 13311	3.1	12.5	25	> 50	> 50	ND	50	ND
H. influenzae ATCC 19418	3.1	25	1.6	12.5	> 50	ND	50	ND
Gram-positive bacteria								
B. subtilis ATCC 6051	6.3	6.3	12.5	25	3.1	6.3	6.3	12.5
B. subtilis IP 5832	50	ND	12.5	25	12.5	12.5	12.5	25
L. monocytogenes NCTC 11994	6.3	6.3	12.5	12.5	6.3	3.1	25	25
M. luteus ATCC 9341	25	25	> 50	ND	3.1	3.1	6.3	6.3
E. faecalis ATCC 19433	> 50	ND	12.5	6.3	6.3	3.1	25	25
S. aureus ATCC 292136.3	> 50	ND	> 50	ND	6.3	6.3	12.5	25
S. pneumoniae ATCC 33400	> 50	ND	12.5	12.5	6.3	3.1	12.5	12.5
N. asteroides ATCC 3308	> 50	ND	> 50	ND	6.3	6.3	25	25

> 50, growth is not completely inhibited at 50 μM peptide concentration; ND, not determined.

indicating that these peptides do not contain all the properties required for full antibacterial activity.

Cationic peptides are believed to enter bacteria via the self-promoted uptake [38]. According to this hypothesis interaction of cationic peptides with Gram-negative outer membranes causes structural perturbations and increases outer membrane permeability, which permits the passage of a variety of molecules, including the perturbing peptide itself. It is suggested that the positive charges on the peptide may interact with the negative charges on the LPS of Gram-negative bacteria, enabling the disruption of the outer membrane and facilitating the interaction of the toxin with the inner membrane. In the presence of high Mg^{2+} ions, the MIC of compounds that are taken up via the self-promoted uptake is expected to increase because the Mg^{2+} ions compete for the negatively charged binding places [39]. To investigate whether parabutoporin, opistoporin 1, melittin and mastoparan might be taken up by the self-promoted uptake, we determined the MICs of the peptides in the presence of 5 mM $MgCl_2$. In Table 1, it can be seen that for both parabutoporin and opistoporin 1 the MICs against Gram-negative bacteria were increased by the addition of 5 mM extracellular Mg^{2+}. This suggests that electrostatic interactions between the cationic peptides and the negatively charged binding places on the LPS of Gram-negative bacteria are important for the growth inhibiting effect of the peptides. Experiments with melittin and mastoparan gave the same results. The role of LPS in the interaction was also demonstrated by the lack of effect of extracellular Mg^{2+} on the activity of the peptides against Gram-positive bacteria with the MIC increasing at most 1 dilution (Table 1).

In order to study the mechanism of action of pore-forming peptides, we investigated whether parabutoporin, opistoporin 1, melittin and mastoparan could enhance the antibacterial effects of four antibiotics: amoxicillin, levofloxacin, cefuroxime and erythromycin. Synergism was accepted when the MIC of the antibiotics was decreased at least two dilutions by the addition of cationic peptides.

With the Gram-negative bacterium *Klebsiella pneumoniae*, synergism of parabutoporin, melittin and mastoparan with erythromycin was observed, but not with opistoporin 1, which was less active in this regard (Table 2). On the Gram-positive bacterium *Listeria monocytogenes*, melittin acts synergistically with amoxicillin, cefuroxime and erythromycin. Parabutoporin and opistoporin 1 enhance the antibacterial activity of cefuroxime. Mastoparan shows no synergism with any of the tested antibiotics against *L. monocytogenes*. None of the peptides was synergistic with levofloxacin.

Antifungal properties

Three fungi, namely the saprophytic soil fungus *Neurospora crassa*, the phytopathogenic fungi *Botrytis cinerea* and *Fusarium culmorum*, and the yeast *Saccharomyces cerevisiae* were used as test organisms in the assay. All peptides inhibit 50% of growth of the tested organisms at a concentration of 5 μM at most (see Table 3). Opistoporin 1 is the most active, inhibiting 50% of growth of *N. crassa* and *F. culmorum* at a concentration of 0.8 μM (3.9 $\mu g \cdot mL^{-1}$) and having an IC_{50} of 2 μM (9.7 $\mu g \cdot mL^{-1}$) for growth of the yeast *S. cerevisiae*. *F. culmorum* is the most sensitive organism for all peptides tested.

Mechanism of fungal growth inhibition

To investigate the mechanism of fungal growth inhibition, fungal membrane permeabilization in the presence of the peptides was studied. For this purpose an assay based on the uptake of SYTOX green was used as described by Thevissen

Table 2. Synergism of cationic peptides with conventional antibiotics. Bacterial cells were grown in presence of one-quarter of peptide MIC (for Gram-negative bacteria) or MIC/8 (for Gram-positive bacteria) with twofold serial dilutions of conventional antibiotics for 16 h at 37 °C. Absorbance at 620 nm was a measure of bacterial growth. The second column represents the minimal inhibitory concentration (concentration that inhibits 100% of bacterial growth, μM) in absence of cationic peptides. >256, growth is not completely inhibited at 256 $\mu g \cdot mL^{-1}$ antibiotic concentration.

	Minimal inhibitory concentration (concentration that inhibits 100% of bacterial growth, $\mu g \cdot mL^{-1}$)				
	No cationic peptide	Parabutoporin 0.4 μM	Opistoporin 1 1.6 μM	Melittin 6.3 μM	Mastoparan 3.1 μM
Klebsiella pneumonia ATCC 13833					
Amoxicillin	>256	256	256	256	>256
Cefuroxime	16	8	16	8	8
Levofloxacin	0.12	0.06	0.06	0.12	0.12
Erythromycin	256	64	256	32	64
	No cationic peptide	Parabutoporin 0.8 μM	Opistoporin 1 1.6 μM	Melittin 0.8 μM	Mastoparan 3.1 μM
Listeria monocytogenes NCTC 11994					
Amoxicillin	0.5	0.5	0.5	0.12	0.25
Cefuroxime	130	32	4	4	130
Levofloxacin	2	2	2	1	1
Erythromycin	0.5	0.5	0.5	0.06	0.5

Table 3. Antifungal activity of parabutoporin, opistoporin 1, melittin and mastoparan. Eighty microliters of half-strength potato dextrose broth containing fungal spores at a concentration of 2×10^4 $conidia \cdot mL^{-1}$ was incubated with 20 μL protein sample. Growth was recorded after 48 h of incubation at 22 °C.

	IC_{50} (μM)			
	Parabutoporin	Opistoporin 1	Melittin	Mastoparan
N. crassa	2.5	0.8	0.8	3.1
B. cinerea	3.5	3.1	3.1	3.1
F. culmorum	0.3	0.8	0.8	1.6
S. cerevisiae	2	2	5	5

et al. [35]. SYTOX green is an organic compound that fluoresces upon interaction with nucleic acids and penetrates only cells with leaky plasma membranes [40]. As can be seen in Fig. 5(A), SYTOX green uptake in *N. crassa* rose significantly upon treatment with parabutoporin at concentrations above 1 μM, which correlates well with the concentrations required for growth inhibition. Also for opistoporin 1, a good correlation between inhibition of growth of *N. crassa* and fluorescence of SYTOX green could be observed (Fig. 5B). Similar results were obtained for all combinations of peptides and fungi. This suggests that inhibition of fungal growth is related to membrane permeabilization.

Hemolytic activity

To study the possible preference of bacterial and fungal membranes as targets for these peptides in comparison with mammalian cells, we examined the hemolytic effect of parabutoporin, opistoporin 1, melittin and mastoparan on human red blood cells (Fig. 6). Up to a concentration of 5 μM, parabutoporin induces only a small hemolytic effect on human red blood cells (< 10%). Fifty percent hemolysis is induced by about 38 μM parabutoporin. Opistoporin 1 was less hemolytic, with about 30% hemolysis at a concentration of 100 μM. As can be seen in Fig. 6, parabutoporin and opistoporin 1 are less hemolytic than melittin, but more hemolytic than mastoparan.

DISCUSSION

In this report, we have described the isolation and characterization of amphipathic α-helical peptides from the venom of *Opistophtalmus carinatus*, a scorpion living in southern Africa, and we have made a comparative analysis of the primary structures of all amphipathic α-helical pore-forming peptides isolated from scorpion venom known today. We found that there is a high conservation in amino acid sequence of the peptides in the venom of scorpions belonging to the same family, independent of the continent and region where they live. In this study, the biological activity of parabutoporin and opistoporin 1 is compared with the activity of melittin and mastoparan. Different parameters that can influence the activity of α-helical

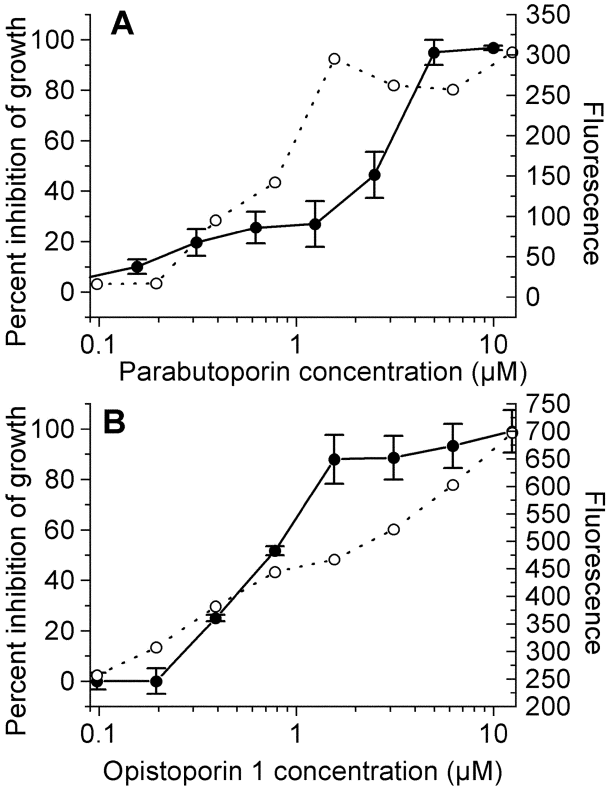

Fig. 5. Antifungal activity of parabutoporin (A) and opistoporin 1 (B) on *Neurospora crassa*. Growth inhibiting effect (closed circle, full line) and membrane permeabilization measured by SYTOX green fluorescence (open circle, dotted line) are shown. Growth inhibiting effect is given as mean ± SE. Values of SYTOX green fluorescence correspond to one representative experiment out of two.

cationic amphipathic peptides have been described (see Table 4 for parameters of parabutoporin, opistoporin 1, melittin and mastoparan): charge, helicity, hydrophobic moment, hydrophobicity and angle subtended by the positively charged residues in a helical wheel diagram [41].

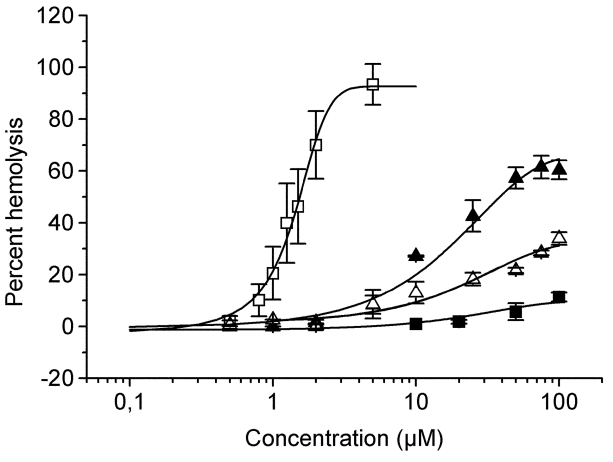

Fig. 6. Hemolytic activity of parabutoporin (closed triangle), opistoporin 1 (open triangle), melittin (open square) and mastoparan (closed square) on human red blood cells in isotonic buffer. Human red blood cells were incubated with different concentrations of peptides for 30 min at 37 °C. Controls for zero and 100% hemolysis were determined by buffer and distilled water, respectively.

As suggested by structure–function studies (for review, see [20,41]), the most cationic peptide (parabutoporin) is the most active on Gram-negative bacteria and the most hydrophobic peptides (melittin and mastoparan) are the most active against Gram-positive bacteria. Hemolytic activity is influenced more by hydrophobic than by electrostatic interactions, but the most hemolytic peptide in our study is melittin, although mastoparan has a higher hydrophobicity. Hydrophobicity also influences the cell selective activity of the pore-forming peptides [42]. The more hydrophobic the peptide, the less cell selective its actions. Parabutoporin is highly hydrophilic and suppresses growth of *E. coli* at concentrations when very few hemolysis occurs.

S. marcescens is relatively resistant to the action of the cationic peptides (Table 1). This has also been observed with other cationic peptides and is supposed to be related to the production of specific proteases [38] and to the presence of fewer negative charges in the cytoplasmic membrane [43,44].

Thus, in general, our findings fit in the theory that amphipathicity, α-helicity and cationicity due to the presence of high amounts of lysine and arginine are the most important factors for activity and are important for the cell selective activity of certain peptides [41,45].

Besides the properties of the peptides, the difference in composition of cell membranes is another determinant for selective activity of pore-forming peptides. The outer leaflet of bacterial membranes contains negatively charged phospholipids while most of the anionic lipids of mammalian membranes are sequestered on the cytoplasmic side of the membranes [10], resulting in more electrostatic interaction between the cationic peptides and bacterial membranes. The presence of cholesterol in eukaryotic membranes is believed to protect eukaryotic cells against the activity of some pore-forming peptides [46]. A third determinant for the activity of a peptide on a certain type of cell is the species, e.g. human red blood cells are much more sensitive to melittin than sheep red blood cells [47]. This difference has been related to the different contents of sphingomyelin (53% of total phospholipids in sheep erythrocytes vs. 25% in human) and phosphatidylcholine (< 2% of total phospholipids in sheep vs. 31% in human) of the two species [48]. A role has been proposed for the major sphingolipid in *S. cerevisiae* membranes [mannose-(inositol-phosphate)$_2$-ceramide] in the interaction with the plant defensin DmAMP1 isolated from *Dahlia merckii*. The sphingolipid can constitute binding sites for DmAMP1 or can be required for anchoring of membrane or cell wall-associated proteins, which interact with DmAMP1 [49]. Other determinants for selective activity of pore-forming peptides are the considerably less inside-negative transmembrane potential of eukaryotic cells compared to prokaryotes [46] and the fact that, unlike bacteria, the respiratory and protein or DNA synthesis machinery are not associated with the cytoplasmic membrane [20].

The growth inhibiting concentration of most effective peptides against bacterial cells has been found to be only slightly below 1 μM [41], making parabutoporin with an MIC of 1.6–6.3 μM (8–31.7 μg·mL^{-1}) a potent peptide against susceptible Gram-negative bacteria. Comparison of the antibacterial activity of different α-helical amphipathic

Table 4. Parameters that influence the activity of cationic peptides (based on the consensus scale of Eisenberg [52]). Parameters were determined for the whole sequence length. H and μ are mean values per residue.

	Amino acids	Charge	α helix	H	μ	θ
Parabutoporin	45	+7	71.1	−0.2347	0.0525	280
Opistoporin 1	44	+4	68.2	−0.1652	0.055	80
Melittin	26	+6	61.5	−0.0858	0.2244	200
Mastoparan	14	+3	64.3	0.0457	0.2206	100

Amino acids, number of amino acids; charge, positive charge at neutral pH; α helix, percentage α-helicity based on secondary structure consensus predictions; H, mean hydrophobicity per residue of the whole peptide sequence; μ, mean hydrophobic moment per residue of the whole peptide sequence; θ, angle subtended by the positively charged residues in a helical wheel projection.

peptides isolated from the venom of scorpions indicates that parabutoporin and opistoporin 1 are especially active against Gram-negative bacteria in comparison to Gram-positive bacteria. Hadrurin [5] is not cell selective, while IsCT [6], IsCT 2 [7] and pandinins [8] are more active in inhibiting growth of Gram-positive bacteria.

As can be seen on the helical wheel diagrams of parabutoporin and opistoporin 1 (Fig. 4), the polar helix surface of parabutoporin is larger than that for opistoporin 1. In addition, the angle subtended by the positively charged residues is more extended for parabutoporin than for opistoporin 1 (Table 4). Together with the higher positive charge of parabutoporin, this could explain the higher antibacterial activity on Gram-negative bacteria for parabutoporin compared to opistoporin 1. Also with magainin analogs, an increase in antibacterial activity against Gram-negative bacteria with increasing angle subtended by the cationic residues was observed [42]. Parabutoporin is predicted to consist of one α-helical segment (amino acids 3–35) while the opistoporins contain two α-helical regions (amino acids 3–14 and 20–39). It is uncertain to which extent this characteristic might effect antibacterial activity. Opistoporin 1 is less active on Gram-negative bacteria than parabutoporin and it has this property in common with pandinin 1, another peptide consisting of two α-helices. Selectivity can not only be related to presence of one or two α-helical fragments because pandinin 1 is more active on Gram-positive bacteria than opistoporin 1. Both peptides differ in only 10 amino acids with five in one single fragment (amino acids 21–27). Although both peptides and both fragments have the same total net charge, opistoporin 1 contains three charged amino acids in this part of the sequence while pandinin 1 contains only one charged amino acid in this fragment. Parameters that might influence antibacterial activity that differ between opistoporin 1 and pandinin 1 are the hydrophobicity (pandinin 1–0.1214, opistoporin 1–0.1652) and the hydrophobic moment, which is nearly double for pandinin 1 (pandinin 1 0.1071, opistoporin 1 0.055). The amino acid substitutions highly responsible for those differences are also situated mainly in the 21–27 fragment of amino acids. A high hydrophobicity and a high hydrophobic moment both have previously been related to a high activity against Gram-positive bacteria [42] in accordance with the reported antibacterial specificity of pandinin 1 [8].

On Gram-negative bacteria, synergism was demonstrated between the pore-forming peptides parabutoporin, melittin and mastoparan and the macrolide erythromycin. This antibiotic inhibits protein synthesis by binding to 50S ribosomal subunits of sensitive microorganisms and it has to pass both inner and outer membranes to be active. Synergy of cationic peptides with erythromycin has been explained as a destabilization of the outer membrane so that erythromycin can pass the outer membrane more easily [50]. Opistoporin 1 is less active in this regard. On Gram-positive bacteria, melittin acts synergistically with amoxicillin, cefuroxime and erythromycin. Parabutoporin and opistoporin 1 enhance the antibacterial activity of cefuroxime. This antibiotic is a β-lactam compound that inhibits the synthesis of peptidoglycan. Because of this inhibition, cationic peptides can probably pass through the altered peptidoglycan layer more easily. This mechanism has been suggested to explain the synergistic effect of cecropin B and benzylpenicillin on *S. epidermidis* [51]. The proteins inhibited by β-lactam antibiotics are located on the outer side of the inner membrane of bacteria, β-lactams do not have to pass the inner membrane to be active. Amoxicillin inhibits the growth of bacteria via the same mechanism, but apparently only melittin can pass through this truncated peptidoglycan layer. In our study, none of the peptides showed synergistic activity with the quinolone levofloxacin. Neither could cationic model peptides demonstrate synergism with this antibiotic [50].

The action of membrane destabilizing peptides in venom of scorpions seems to be part of an antibacterial and antifungal defense system. However they probably also contribute to neuronal hyperexcitability and induction of pain during scorpion envenomation by their depolarizing action on nociceptive nerve endings. Depolarization of rat dorsal root ganglion cells has been described for parabutoporin [4]. This mechanism may contribute to the immobilization of the envenomated prey.

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