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Melectin: A Novel Antimicrobial Peptide from the Venom of the Cleptoparasitic Bee *Melecta albifrons*

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A novel antimicrobial peptide designated melectin was isolated from the venom of the cleptoparasitic bee Melecta albifrons. *Its primary sequence was established as H-Gly-Phe-Leu-Ser-Ile-Leu-Lys-Lys-Val-Leu-Pro-Lys-Val-Met-Ala-His-Met-Lys-NH_2 by Edman degradation and ESI-QTOF mass spectrometry. Synthetic melectin exhibited antimicrobial activity against both Gram-positive and -negative bacteria and it degranulated rat peritoneal mast cells, but its hemolytic activity was low. The CD spectra of melectin measured in the presence of trifluoroethanol and sodium dodecyl sulfate showed a high content α-helices, which indicates that melectin can adopt an amphipathic α-helical secondary structure in an anisotropic environment such as the bacterial cell membrane. To envisage the role of the proline residue located in the middle of the peptide chain on biological activity and secondary structure, we prepared several melectin analogues in which the Pro11 residue was either replaced by other amino acid residues or was omitted. The results of biological testing suggest that a Pro kink in the α-helical structure of melectin plays an important role in selectivity for bacterial cells. In addition, a series of N- and C-terminal-shortened analogues was synthesized to examine which region of the peptide is related to antimicrobial activity.*

Introduction

Antimicrobial peptides (AMPs) are evolutionarily conserved components of the host's innate immunity system that form the first line of defense against infections. They have been identified in almost all classes of life.[1,2] Although the precise mechanism of the broad spectrum of antimicrobial activity of these peptides is not yet fully understood, they appear to act through a specific, but not receptor-mediated, formation of transmembrane pores or ion channels on the cellular membrane. This causes leakage of essential metabolites and results in the disruption of microbial cell structure and leads to cell death.[3–5] In contrast to conventional antibiotics, they do not appear to induce microbial resistance and require only a short time to induce killing.[6]

Among AMPs, those from insects constitute a remarkable group. Since insects are uniquely adapted to a variety of natural environments that are often considered rather unhealthy by human standards, they have developed an amazing resistance to bacterial infection. Upon encountering bacteria, a complex genetic cascade is triggered that ultimately results in the synthesis of a battery of AMPs and their release into the haemolymph.[7] Over the course of their evolution, stinging insects such as hymenopterans have developed venom that is stored in venom reservoirs in addition to the AMPs released into the haemolymph. It contains antimicrobial and cytolytic peptides, together with a complex mixture of enzymes, neurotoxins, low molecular mass compounds and other peptides.[8] Despite different compositions, the main function of venom is to subdue prey and defend against predators.

The venom peptides of hymenoptera that are best characterized include the mastoparans,[9,10] chemotactic peptides[9,11] and kinins[12] isolated from hornets and wasps; hemolytic melittin[8,13,14] and neurotoxic apamine[13,15] from honeybees; cytolytic bombolitins[16] from bumble bees and ponericins[17] isolated from Ponerinae ants. Among these peptides, mastoparans[18,19] and ponericins[17] exhibit potent antimicrobial activity against broad range of bacteria.

Recently, we have investigated the peptide composition of the venoms isolated from social wasps of Polistinae subfamily collected in the Dominican Republic.[19] We found that the venoms contain peptides of the mastoparan group that posses strong antimicrobial activity against Gram-positive and -negative bacteria.[20] In the present work we describe the structural characterization and biological activities of a novel peptide named melectin (MEP), which we isolated from the venom of cleptoparasitic bee *Melecta albifrons*. Cleptoparasitic bees are commonly called "cuckoo bees" because their behavior is similar to that of cuckoo birds. The females do not construct their own nests, but enter nests of pollen collecting bee species where they lay their eggs in cells constructed by the host bees. When the cuckoo bee larva hatches, it consumes the host larva's pollen supply, and kills and eats the host larva.

MEP, the major component of the *Melecta albifrons* venom, is composed of 18 amino acid residues. It exhibits both antimicrobial and mast cell degranulating activity, but low hemolytic activity. This is the first antimicrobial component found in soli-

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tary bee venom. Unlike AMPs found previously in hymenoptera venom, which were identified as cationic peptides with amphipathic α-helical conformation, MEP possesses one Pro residue in the middle of the peptide chain; this makes MEP structurally unique. As a part of this study, several peptide analogues were synthesized in order to characterize the role of this Pro residue on the biological activity of MEP.

Results

Purification and sequence determination

RP-HPLC purification of the venom extract obtained from four venom reservoirs gave simple profile (Figure 1) with a few intense peaks. The MALDI-TOF MS of the component eluted in

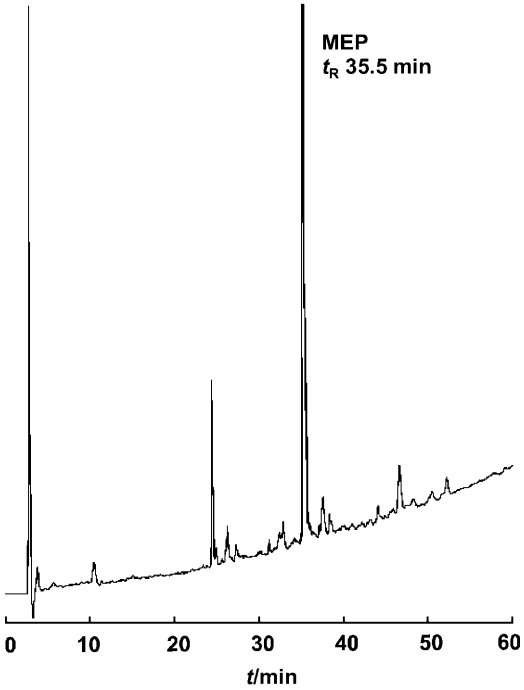

Figure 1. RP-HPLC profile of *Melecta albifrons* venom extract at 222 nm. An elution gradient of solvents from 5%–70% acetonitrile/water/0.1% TFA was applied for 60 min at a 1 mL min^{-1} flow rate.

the most intense peak at t_R 35.5 min showed a protonated molecular ion $[M+H]^+$ at *m/z* 2038.9, and sodium adduct $[M+Na]^+$ at *m/z* 2060.9. The tandem mass spectrum of the triply charged peptide ion (*m/z* 680.4) showed the complete y-type ion series, and most of the b-ions (not shown). The peptide sequence predicted from the MS/MS spectrum using BioLynx software (Waters) agreed with the sequence obtained by Edman degradation. It gave the entire sequence in 20 cycles, as follows: Gly-Phe-Leu-Ser-Ile-Leu-Lys-Lys-Val-Leu-Pro-Lys-Val-Met-Ala-His-Met-Lys. The accurate mass, 2038.24 Da, which was determined by ESI-QTOF MS with internal calibration, indicates that the C-terminus of the peptide is amidated. A comparison of the peptide with sequences in the SwissProt and GenBank™/EMBL Data Banks (Blast program in Swiss-Prot) showed that it does not have any significant sequence homology with other known peptides. Thus, MEP can be considered as a new antimicrobial peptide.

Peptide synthesis

MEP and its analogues (Table 1) were prepared by standard DIPC/HOBt chemistry. Bromphenol blue indicator was used to nondestructively monitor the conversion of free amino groups during coupling.[21] This procedure showed that coupling during the first few cycles was completed within several minutes. Later on, as indicated by the sluggish color change, the coupling time had to be extended to several hours. Crude peptides were further purified by preparative RP-HPLC providing analytical HPLC purity in the 95-98% range. The retention times of the purified peptides are given in Table 3, and the results of the MS analyses that confirmed their identities are in Table 1.

Table 1. Amino acid sequences and MS data of synthetic melectin (MEP) and its analogues.

Acronym	Peptide sequence	Monoisotopic molecular mass [Da]	
		calcd	found $[M+H]^+$
MEP	GFLSILKKVL**P**KVMAHMK-NH_2	2038.23	2039.2
MEP-1	GFLSILKKVL**K**KVMAHMK-NH_2	2069.27	2070.4
MEP-2	GFLSILKKVL–KVMAHMK-NH_2	1941.18	1941.9
MEP-3	GFLSILKKVL**A**KVMAHMK-NH_2	2012.22	2013.3
MEP-4	GFLSILKKVL**G**KVMAHMK-NH_2	1998.28	1999.2
MEP-5	KVMAHMK-NH_2	842.46	843.5
MEP-6	**P**KVMAHMK-NH_2	939.51	940.5
MEP-7	L**P**KVMAHMK-NH_2	1052.60	1053.3
MEP-8	VL**P**KVMAHMK-NH_2	1151.67	1152.4
MEP-9	GFLSILKKVL**P**-NH_2	1212.80	1213.7
MEP-10	GFLSILKKVL-NH_2	1115.74	1116.6

Table 2. Antimicrobial and hemolytic activity of MEP and its analogues.

Peptide	Antimicrobial activity MIC [μM]				Hemolytic activity[a]
	B.s.	*S.a.*	*E.c.*	*P.a.*	LC_{50} [μM]
MEP[b]	0.8	6.8	2.0	18.5	>100
MEP-1	1.4	13.8	1.8	25.3	27.3
MEP-2	0.9	5.0	1.8	27.5	22.9
MEP-3	1.3	4.7	1.6	18.3	29.7
MEP-4	1.3	4.3	1.8	14.4	41.4
MEP-5	>100	n.t.[c]	>100	n.t.	>100
MEP-6	>100	n.t.	>100	n.t.	>100
MEP-7	>100	≫100	>100	≫100	>100
MEP-8	>100	≫100	>100	≫100	>100
MEP-9	13.0	>100	78.0	>100	>100
MEP-10	2.9	10.9	9.1	59.0	>100

[a] Concentration of the peptide causing lysis of 50% of red blood cells. [b] Mast cell degranulation activity EC_{50} = 19.4 μM. [c] Not tested

Table 3. Physical properties of melectin (MEP) and its analogues.

Peptide	H[a]	μ_H[b]	t_R [min]	α-Helical fraction (f_h) TFE [%]			SDS [mM]	
				0	10	40	0.16	16
MEP[a]	−0.011	0.274	36.48	0.12	0.16	0.41	0.41	0.45
MEP-1	−0.068	0.326	41.88	0.11	0.20	0.57	0.29	0.61
MEP-2	−0.007	0.265	40.83	0.13	0.20	0.51	0.28	0.60
MEP-3	0.007	0.258	43.14	0.13	0.59	0.52	0.60	0.64
MEP-4	0.002	0.262	42.47	0.12	0.21	0.59	0.28	0.85
MEP-5	−0.184	0.093	10.27	0.07	0.08	0.11	0.08	0.14
MEP-6	−0.170	0.214	11.93	–	–	–	–	–
MEP-7	−0.092	0.123	17.93	–	–	–	–	–
MEP-8	−0.029	0.130	18.40	0.10	0.10	0.20	0.09	0.29
MEP-9	0.100	0.331	33.87	–	–	–	–	–
MEP-10	0.117	0.325	36.89	0.12	0.17	0.43	0.24	0.48

[a] The mean hydrophobicity (H) of each peptide was calculated as the average of hydrophobicities of each amino acid in the peptide chain using the hydrophobicity Eisenberg consensus scale.[25] [b] The mean hydrophobic moment (μ_H), used as a quantitative measure of amphipathicity, was calculated according to the formula given in ref. [23].

Biological activities

As shown in Table 2, MEP and its analogues with Pro residue in position 11 substituted by other amino acid residues (MEP-1, MEP-3 and MEP-4), showed comparably high antimicrobial potency against both Gram-positive and -negative bacteria, although Gram-negative bacteria appeared to be slightly less susceptible. Figure 2 shows typical bacteria growth curves for different MEP concentrations. The excision of Pro from the peptide chain (MEP-2) had no effect on antimicrobial activity. However, all modifications resulted in an undesirable increase of hemolytic activity of the analogues. The N-terminal fragments of the sequence (MEP-9, MEP-10) possessed antimicrobial activity, in contrast to the C-terminal fragments (MEP-5, MEP-6, MEP-7, MEP-8); the latter were inactive against all bacteria tested.

Interestingly, the N-terminal fragment with Pro residue on its C-terminus (MEP-9) exhibited a substantially lower antimicrobial activity compared to the N-terminal fragment lacking a C-terminal Pro (MEP-10). Although the primary structure of MEP shows no homology to any known mastoparan, surprisingly, this peptide induced degranulation of mast cells (EC_{50} = 19.4 μM).

CD analyses and structural features

The secondary structure of MEP was estimated by CD spectroscopy both in the absence and presence of the helix promoting solvent trifluoroethanol, and the anisotropic environment of SDS micelles. The CD spectra (Figure 3A) obtained in water and in the presence of 10% TFE are characteristic of an unordered structure, and contain at most 11.8% and 15.8% of α-helix, respectively. When the concentration of TFE was increased in 10% increments, the peptide became readily structured, with a relatively high (41%) α-helical content at 40% TFE (Table 3), as indicated by the appearance of two minimum bands at 207 and 221 nm in the CD spectra (Figure 3A). Similar

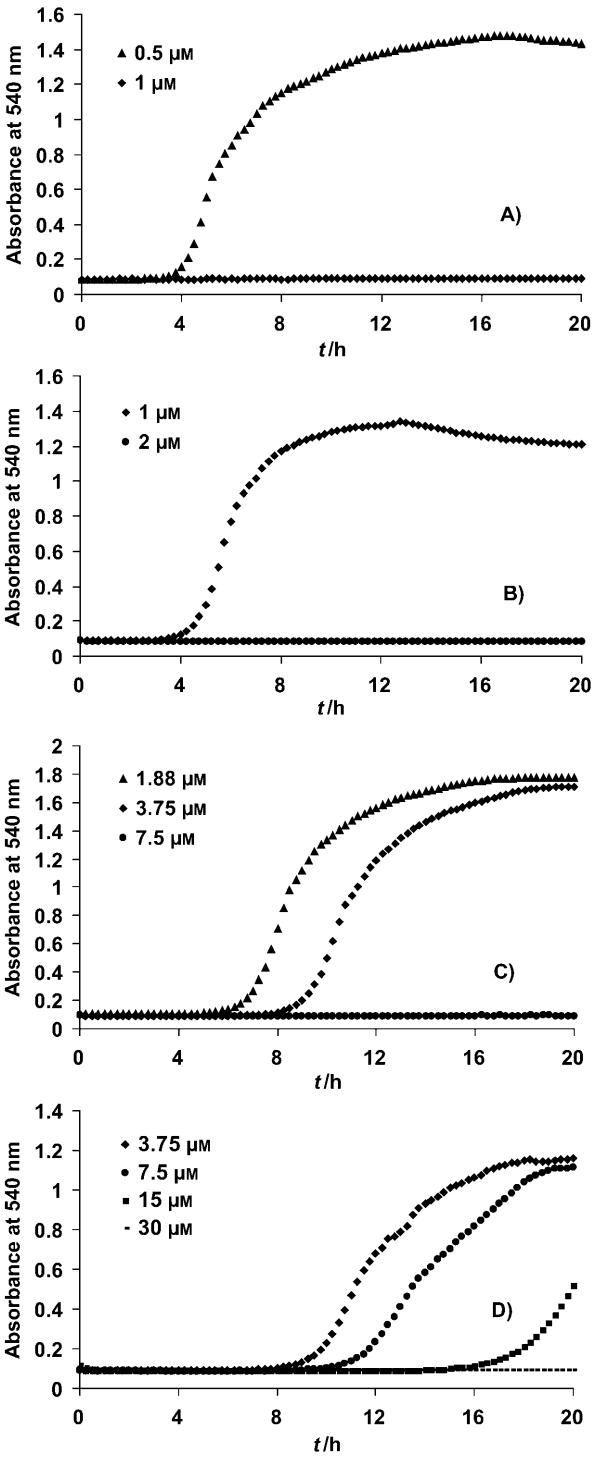

Figure 2. Growth curves of bacteria *B. subtilis* (A), *E. coli* (B), *S. aureus* (C) and *P. aeruginosa* (D) in the presence of different concentrations [μM] of MEP. As can be seen from the figure, 1 μM, 2 μM, 7.5 μM and 30 μM MEP completely inhibited the growth of *B.s.*, *E.c.*, *S.a.* and *P.a.*, respectively, for 20 h. ▲, ◆, ●, ■, – refer to different concentrations of MEP [μM], as indicated in the figure legends.

formation of a MEP α-helical structure was observed in the anisotropic environment of the SDS micelles. A maximum (44.8%) α-helical fraction was reached at a SDS concentration of 16 mM. As expected, replacing Pro11 by Ala (MEP-3) or Lys

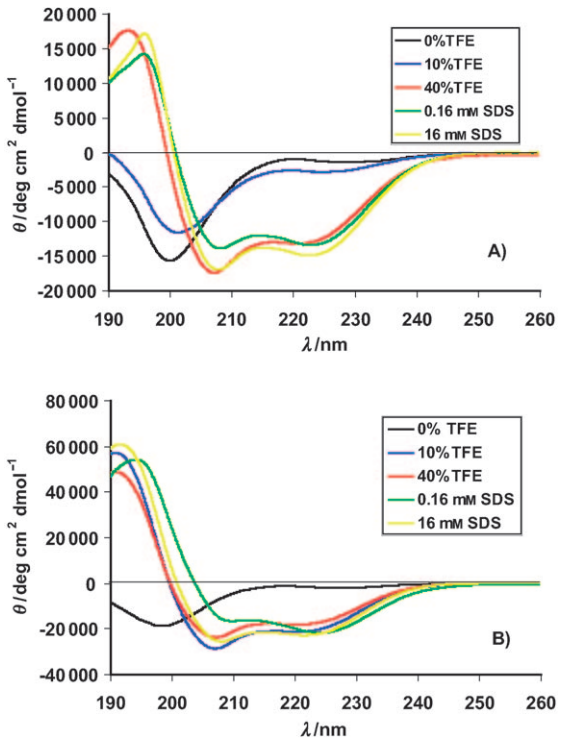

Figure 3. UV–CD spectra of MEP (A) and MEP-3 (B) in water, in the presence of TFE (10 and 40% of TFE/water mixture, *v/v*) and SDS (0.16 mM and 16 mM). Peptide concentration was 0.25 mg mL^{-1} for both samples studied.

(MEP-1) increased significantly the α-helicity of the analogues, as shown in Figure 3B for MEP-3. These peptides became structured at lower concentrations of TFE or SDS (Table 3). Surprisingly, also the MEP-4 analogue, in which Pro was replaced by Gly, showed a strong propensity to form an α-helix. In this case, the maximum value of helicity (85%), was reached at 16 mM SDS concentration. Also, the N-terminal fragment of MEP, which consists of only ten amino acid residues (MEP-10), readily adopted a helical secondary structure, almost to the same extent as MEP, opposed to the C-terminal fragment (MEP-5, seven residues). The secondary structure of MEP-5 was a random coil, even at high concentrations of TFE or SDS (Table 3). Extension of this fragment by three amino acid residues (MEP-8, 10-residues) resulted in a slightly higher propensity to form an α-helix (Table 3).

Since CD spectroscopic measurements of MEP and analogues in the presence of TFE or SDS confirmed the presence of a significant amount of α-helical structure, we assume that this sequence can adopt an amphipathic α-helical conformation as shown in Figure 4. In this Edmundson wheel projection,[22] all of the hydrophilic amino acid residues except His16 are situated on one side of the α-helix, whereas all of the hydrophobic amino acid residues except Ala15 are on the opposite side.

According to the literature, the ability of AMPs to form a well-defined amphipathic α-helix correlates strongly with their antimicrobial activity.[23,24] The quantitative measure of peptide amphipathicity is the hydrophobic moment, μ_H, calculated as the vector sum of individual amino acid hydrophobicities, and

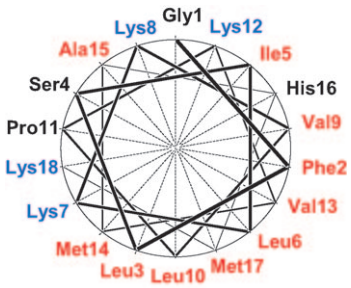

Figure 4. Wheel diagram of MEP. Sector of hydrophobic amino acids is shown in red. The hydrophilic amino acids sector (shown in black) is dominated by four Lys residues (shown in blue).

normalized to an ideal helix.[23] We compared the hydrophobic moments (Table 3) of the studied peptides with MIC values in order to see whether or not this parameter correlates with antimicrobial activity. For MEP and its analogues of the same length (MEP-1–MEP-4), the hydrophobic moment values generally are not reflected in the slight differences between MIC values. For the peptide fragments (MEP-5–MEP-10), the remarkable differences between the MIC values of MEP-10 and MEP-9 versus MEP-5–MEP-8 are reflected in their hydrophobic moments.

Discussion

We have isolated a new AMP from the venom of the solitary cleptoparasitic bee, *Melecta albifrons*. This novel AMP comprises its major peptide component, and we have named it malectin (MEP). Unlike solitary wasps that lay their eggs on the bodies of paralyzed prey, the cuckoo bee females lay their eggs in the food storages of host bee nest cells. In the solitary wasp case, some of the AMPs found in the venom may play a role in the prevention of potential infections due to prey ingestion.[26] We speculate that the primary role of MEP in the venom may be in protecting the solitary bee against enemies, with the antimicrobial role more or less secondary.

Peptides with antimicrobial properties isolated from the venoms of different wasp families are able to adopt an amphipathic α-helical conformation in membrane-mimicking environments, a prerequisite for their biological activities.[20,27] MEP, with a sequence rich in hydrophobic and basic amino acid residues, also belongs to the category of cationic amphipathic α-helical peptides. However, it does not display any sequence homology with such wasp venom peptides, for example, of the mastoparan class.[20] On the other hand, when the MEP sequence is compared to the sequences of other α-helical AMPs obtained from natural sources, some obvious positional conservation in terms of residue types can be observed.[4] In particular, Gly1, Ser4, Lys8, the presence of aromatic residue near the N-terminus, and C-terminal amidation, all are common to these types of AMPs. Plotting the sequence of MEP onto a α-helical wheel projection (Figure 4) reveals a well-defined hydrophobic sector with large aliphatic residues, and a hydrophilic sector dominated by Lys residues. Generally, the distribution of amino acid residues in the MEP sequence fits very well to the

statistical analysis diagram of the residue distribution of α-helical AMPs from natural sources, as proposed by Tossi in his review.[4] According to his scheme, the presence of Pro within the hydrophilic sector is not typical for the analyzed series of α-helical AMPs.

Since Pro is an imino acid, its X-Pro peptide group readily exists in the *trans*, as well as the *cis* form, unlike other peptide groups, which predominantly adopt the *trans* form. In short peptides and unfolded proteins, the relative ratio of *trans* to *cis* isomers is approximately three to one.[28] In native proteins, prolines generally adopt a single isomeric state, which is dictated by other interactions within the protein. As evident from the MEP CD spectra measured in a membrane-mimicking environment (that is, in the presence of TFE or SDS micelles) this peptide adopts a α-helical structure. Thus, within this α-helix, the Leu10–Pro11 peptide group must exist in the *trans* form. As proline lacks the hydrogen on its amide, the possibility of making a hydrogen bond to the preceding turn is lost, and because its ring structure restricts its backbone dihedral angle ϕ, a kink is introduced to the helical structure.[29] This conformational element, imposed by proline on the MEP peptide chain, appears to govern its biological functions. As shown in Table 2, the replacement of Pro11 by Lys, Ala or Gly or its elimination, resulted in a negligible change in antimicrobial activity but a remarkable increase in hemolytic activity. These modifications increased significantly the α-helicity of the analogues as shown by CD spectra measurement (Figure 3, Table 3). The fact that increasing the helicity of AMPs results in a significant increase in their hemolytic activitiy has already been observed.[30,31] Our results indicate that the kink imposed by the Pro residue at the central position of MEP plays an important role in its differentiation between prokaryotic and eukaryotic cells. A similar study of another α-helical amphipathic octadecapeptide, P18, in which Pro9 was substituted by α-helix stabilizing residues, also confirmed the significance of the Pro kink for antimicrobial activity without undesirable hemolytic properties.[32] For example, the Leu9 analogue of the P18 peptide is highly hemolytic. On the other hand, substitution of Ala for Pro14 in the 24 amino acid residue AMP gaegurin resulted in lower antimicrobial activities, but no increase in hemolytic activity.[33] Those examples indicate that the insertion of the Pro residue into the central part of α-helical amphipathic AMPs, which causes slight bending of the helix, might lead to the design of analogues with improved properties.

The α-helical and amphipathic N-terminal fragments (MEP-9, MEP-10), showed a noticeable antimicrobial activity (Table 2) as compared to inactive C-terminal fragments of similar length (MEP-5–MEP-8). These C-terminal fragments, however, have a disordered secondary structure in the presence of TFE or SDS and lower hydrophobic moments (Table 3). We may therefore conclude that it is the N-terminal region of MEP that is involved in the interaction of the peptide with the bacterial membrane.

It has been shown that the interactions of helical peptides with lipidic C-18 stationary phase groups during RP-HPLC are similar to the process that governs the interactions of AMPs with biological membranes.[34] Although RP-HPLC separates peptides mainly by hydrophobic interactions, the induced peptide secondary structures also influence their retention times.[34] The substitution of Lys for Pro in MEP-1 reduced the hydrophobicity of the peptide in a way that theoretically should shorten its retention time measured on the C-18 RP-HLC column. However, as shown in Table 3, this peptide eluted much later than MEP, suggesting, that the bend in the α-helical structure of MEP imposed by Pro somehow perturbs the hydrophobic interactions of the MEP with the C-18 stationary phase, making it more "movable" within the column. The increase in retention times of MEP-2, MEP-3, and MEP-4 compared to the retention time of MEP (Table 3) may be due to higher α-helicity of these analogues rather than to their mean hydrophobicity values (Table 3). The effect of hydrophobicity on the increase of peptide retention times correlates well within the series MEP-1 (Lys), MEP-4 (Gly) and MEP-3 (Ala).

Conclusions

MEP, a novel peptide with a Pro kink helical structure that was firstly identified in solitary bee venom possesses high antimicrobial and low hemolytic activity and may serve as a potential new template for the development of effective antibiotic peptides.

Experimental Section

Materials: Fmoc-protected L-amino acids were purchased from IRIS Biotech GmbH (Marktredwitz, Germany), Rink Amide MBHA resin was obtained from Merck-Novabiochem (Darmstadt, Germany). Tetracycline, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidine, LB broth and LB agar were from Sigma–Aldrich. All other reagents, peptide synthesis solvents, and HPLC-grade acetonitrile were of the highest purity available from commercial sources. As test organisms we used: *Bacillus subtilis* 168, kindly provided by Prof. Yoshikawa (Princeton University, Princeton, NJ), *Escherichia coli* B, from the Czech Collection of Microorganisms (Brno, Czech Republic), and *Staphylococcus aureus* and *Pseudomonas aeruginosa* were obtained as multi-resistant clinical isolates.

Sample preparation and peptide purification: Bee specimens were collected in the urban area of northwest Prague, Czech Republic, during May 2007 and kept frozen at $-20\,^{\circ}C$ for several days. The venom reservoirs of four individuals were removed by dissection and their contents were extracted with a mixture of acetonitrile-water (1:1) containing 0.1% TFA (25 μL). The extract was centrifuged, and the supernatant was fractionated by RP-HPLC. Chromatography was carried out on the Thermo Separation Product instrument with a Vydac C-18 column (250×4.6 mm; 5 μm) at a 1.0 mL min^{-1} flow rate, using a solvent gradient ranging from 5–70% acetonitrile/water/0.1% TFA over 60 min. The major fractions detected by the UV absorption at 222 nm were collected, the solvent was evaporated in a speed-vac, and the material analyzed by mass spectrometry and subjected to Edman degradation.

Mass spectrometry: Mass spectra of the peptides were acquired on a Reflex IV MALDI-TOF mass spectrometer (Bruker) equipped with a UV 337 nm nitrogen laser operated in the reflectron mode. The matrix was a 10 mg mL^{-1} concentration of α-cyano-4-hydroxycinnamic acid in acetone. Each sample (1 μL) mixed with the matrix (1 μL) was applied onto a spot on a MALDI plate and

allowed to dry at room temperature. Tandem mass spectra were recorded using a Micromass Q-Tof micro™ mass spectrometer (Waters) equipped with an electrospray ion source. A mixture of acetonitrile and water (1:1) containing 0.1% acetic acid was delivered continuously to the ion source at a 20 μL min^{-1} flow rate. Samples dissolved in the mobile phase were introduced using a 2 μL loop. The capillary voltage, cone voltage, desolvation temperature and source temperature were 3.5 kV, 20 V, 150 °C and 90 °C, respectively. MS/MS spectra were obtained using CID at 28 eV collision energy.

Peptide sequencing by Edman degradation: The N-terminal amino acid sequence was determined on the Procise–Protein Sequencing System (PE Applied Biosystems, 491 Protein Sequencer, Foster City, USA) using manufacturer's pulse-liquid Edman degradation chemistry cycles.

Peptide synthesis: MEP and its analogues were synthesized manually by using a solid-phase method in 5 mL polypropylene syringes with a bottom Teflon filter. Synthesis was done by using a N^{α}-Fmoc chemistry protocol[35] on a Rink Amide MBHA resin (100 mg) with 0.7 mmol g^{-1} substitution. Protected amino acids were coupled in fourfold excess in DMF as solvent and DIPC (7 equiv)/HOBt (5 equiv) as coupling reagents. The peptides were deprotected and cleaved from the resin with a mixture of TFA/1,2-ethanedithiol/H_2O/thioanisol/triisopropylsilane (TIS) (90:2.5:2.5:3:2) for 3.5 h and precipitated with *tert*-butyl methyl ether. Crude peptides were purified by preparative RP-HPLC using a Vydac C-18 column (250× 10 mm) at a 3.0 mL min^{-1} flow rate on the same instrument with a solvent gradient as described above.

Antimicrobial activity determination: A quick qualitative estimate of the antimicrobial properties was done by taking advantage of the double-layer technique originally developed for bacteriophage titration by microbial geneticists. We poured melted "soft" agar (2 mL), prepared from LB broth with 0.5% agar and bacteria (about 10^7 colony forming units (CFU)) over the surface of Petri dishes (90 mm in diameter) containing LB agar (20 mL). Fresh bacterial cultures were always prepared in the LB broth and added when the melted soft agar cooled down to about 45 °C. Antimicrobial peptides (0.001–10 mg mL^{-1}) diluted in water were dropped (2 μL) on the surface of the solidified upper layer, and the plates were incubated at 37 °C. Cleared zones of inhibition appeared within a few hours and remained clear for days. The potency was estimated by the diameter and clarity of the zones formed. Quantitatively, the minimal inhibitory concentrations (MICs) were established by observing bacteria growth in multi-well plates.[11,36,37] Bacteria in mid-exponential phase were added to individual wells containing solutions of different concentrations of tested peptides (final volume 0.2 mL, final peptide concentration ranged from 0.5 to 100 μM) in the LB broth. These were incubated at 37 °C for 20 h, while being shaken continuously in a Bioscreen C instrument (Helsinki, Finland). The absorbance was measured at 540 nm every 15 min, and each peptide was tested at least 3 times in duplicates. Routinely $1.2x10^3$–$7.5x10^3$ CFU of bacteria per well were used for the activity determination. Tetracycline (0.5–50 μM) was tested as a standard.

Determination of the hemolytic activity:[18] Peptides were incubated with rat red blood cells for 1 h at 37 °C (final volume 0.2 mL) in a physiological solution (final erythrocyte concentration 5% *v/v*, and final peptide concentration 1–100 μM). The samples were then centrifuged for 5 min at 250 g, and absorbance of the supernatant was determined at 540 nm. Controls for zero hemolysis (blank) and 100% hemolysis consisted of supernatants of red blood cells suspended in physiological solution and 0.2% Triton X-100 in physiological solution, respectively. Each peptide was tested at least in two independent experiments in duplicate.

Mast cell degranulation test:[10,38] Mast cells were obtained by peritoneal washing of adult Wistar rats. The degranulation potency was determined by measuring the activity of β-D-glucosaminidase that colocalized with histamine in the mast cells. The mast cells were incubated in the presence of the peptide (1–100 μM, total volume 0.2 mL) for 15 min at 37 °C, and then centrifuged at 250 g for 10 min. Aliquots of the supernatant (50 μL) were added to the solution of the substrate (p-nitrophenyl-*N*-acetyl-β-D-glucosaminidine), and incubated further for 6 h at 37 °C. The reaction was stopped by the addition of Tris buffer (0.2 M buffer, pH 9, 150 μL), and the absorbance was determined at 405 nm. Controls for zero degranulation and 100% degranulation were obtained from mast cells incubated in a physiological solution and 0.2% Triton X-100, respectively.

CD spectra measurement: Far-UV CD spectra were recorded at room temperature on a Jasco 815 spectropolarimeter (Tokyo, Japan). All peptides were measured in water, in a TFE/water mixture (10, 20, 30, 40 and 50%, *v/v*), and in the presence of SDS at a concentration below 0.16 mM and above the critical micelle concentration (CMC) (16 mM). For all of the studied peptides, the concentration was 0.25 mg mL^{-1}. The optical path length was 0.1 cm, and CD signal was monitored from 190 nm to 300 nm. For each experiment, the data were averaged over four scans, taken with a 2 s time constant and with blank subtracted. The final spectra are expressed as molar ellipticity per residue. Assuming the two-state model, the observed mean residue ellipticity at 222 nm was converted into an α-helix fraction (f_H) using the method proposed in literature.[39,40]

Glossary: LB, Luria-Bertani; MIC, minimal inhibitory concentration; *E.c.*, *E. coli*; *B.s.*, *B. subtilis; S.a.*, *S. aureus*; *P.a.*, *P. aeruginosa;* DIPC, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TIS, triisopropylsilane; SDS, sodium dodecyl sulfate.

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