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Lasioglossins: Three Novel Antimicrobial Peptides from the Venom of the Eusocial Bee *Lasioglossum laticeps* (Hymenoptera: Halictidae)

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Three novel structurally related pentadecapeptides, named lasioglossins, were isolated from the venom of the eusocial bee *Lasioglossum laticeps*. Their primary sequences were established as H-Val-Asn-Trp-Lys-Lys-Val-Leu-Gly-Lys-Ile-Ile-Lys-Val-Ala-Lys-NH_2 (LL-I), H-Val-Asn-Trp-Lys-Lys-Ile-Leu-Gly-Lys-Ile-Ile-Lys-Val-Ala-Lys-NH_2 (LL-II) and H-Val-Asn-Trp-Lys-Lys-Ile-Leu-Gly-Lys-Ile-Ile-Lys-Val-Val-Lys-NH_2 (LL-III). These lasioglossins exhibited potent antimicrobial activity against both Gram-positive and Gram-negative bacteria, low haemolytic and mast cell degranulation activity, and a potency to kill various cancer cells in vitro. The lasioglossin CD spectra were measured in the presence of trifluoroethanol and sodium dodecyl sulfate solution and indicated a high degree of α-helical conformation. NMR spectroscopy, which was carried out in trifluoroethanol/water confirmed a curved α-helical conformation with a concave hydrophobic and convex hydrophilic side. To understand the role of this bend on biological activity, we studied lasioglossin analogues in which the Gly in the centre of the molecule was replaced by other amino acid residues (Ala, Lys, Pro). The importance of the N-terminal part of the molecule to the antimicrobial activity was revealed through truncation of five residues from both the N and C termini of the LL-III peptide. C-terminal deamidation of LL-III resulted in a drop in antimicrobial activity, but esterification of the C terminus had no effect. Molecular modelling of LL-III and the observed NOE contacts indicated the possible formation of a bifurcated H-bond between hydrogen from the Lys15 CONH peptide bond and one H of the C-terminal $CONH_2$ to the Ile11 oxygen atom. Such interactions cannot form with C-terminal esterification.

Introduction

The growing resistance of pathogenic bacteria to conventional antibiotics has resulted in a search for new classes of antimicrobial agents with different modes of action than traditional antibiotics. Antimicrobial peptides (AMPs) have been identified in almost all forms of life and represent the first line of defence against infection.[1–4] AMPs belong to a new family of antibiotics that have stimulated research and clinical interest as new therapeutic options.[5–8] The significant advantage of AMPs resides in their mechanism of action, which is markedly different from that of conventional antibiotics. Whereas AMPs often exhibit activities comparable to those of conventional antibiotics, the physical nature of their action implies a faster killing process.[3,9] Although the precise mechanism of AMP action is still not exactly known, it is generally accepted that these positively charged peptides target the anionic bacterial cell envelope, accumulate in the cell wall, interact with the membrane surface and integrate into the lipid bilayer and disrupt its structure, which leads to leakage of cytoplasmic components and cell death.[3,10–12]

Among more than 900 AMPs[13] isolated from a wide range of organisms, the linear cationic α-helical peptides represent the most studied group.[11] These are small peptides, usually 10–45 amino acid residues long and rich in hydrophobic and basic amino acid residues, and can adopt an α-helical amphipathic secondary structure in the cell membrane environment or in the presence of such membrane-mimicking substances as trifluoroethanol (TFE) or sodium dodecyl sulfate (SDS). This category of peptides, together with low-molecular-mass compounds, enzymes, neurotoxins and other peptides, are often found in the venom of stinging insects such as the hymenopterans.[14] For example, mastoparans,[15] anoplin,[16] eumenitin[17] and decoralin,[18] isolated from the venom of wasps, and ponericins,[19] isolated from Ponerinae ants, are all cytolytic peptides possessing potent antimicrobial activity against a broad range of bacteria. Such peptides are good candidates as potential templates for the development of new and effective antibiotic peptides. The major drawback in using these peptides

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as antibiotics, however, is their ability to lyse eukaryotic cells as well; this is commonly expressed as haemolytic activity or toxicity to red blood cells.

Recently, we identified a new antimicrobial peptide in the venom of the cleptoparasitic bee *Melecta albifrons* named melectin.[20] This 18-residue α-helical amphipathic peptide, representing the major peptide component of the venom, exhibits strong antimicrobial activity against both Gram-positive and Gram-negative bacteria and has low haemolytic activity. We report on the structural characterisation and biological activity of three novel pentadecapeptides isolated from the venom of the wild eusocial bee *L. laticeps* that we have named lasioglossins. This eusocial bee occurs in all parts of Europe except for the northernmost areas.[21] They nest in clay soil, but are also frequently found in areas of anthropogenic activity, for example, between the stones of a cobbled street or in wall clefts. Solitary, overwintering gynes establish their nests in the early spring and become queens when their worker daughters emerge during May.[22]

Lasioglossins and melectin, which have strong antimicrobial and low haemolytic activity, belong to a new class of antimicrobial peptides found in the venom of wild bees. Our recent efforts in the search for new antimicrobial agents from natural sources indicate that the venom of wild bees is a rich source of this type of pharmacologically interesting compounds.

Results

Purification and sequence determination

RP-HPLC purification of the venom extract obtained from eight venom reservoirs gave a profile with relatively few intense peaks (Figure 1). The MALDI-TOF MS of the components eluted in the peaks at $t_R = 18.0$, 28.2, 30.6, 31.6 and 33.9 min showed clear protonated molecular ion MS peaks $[M+H]^+$ at *m/z* 2005.9, 2892.9, 1723.4, 1737.3 and 1765.3, respectively, accompanied with their corresponding sodium and potassium adducts. The ESI-QTOF MS tandem mass spectra of the peptides eluted at $t_R = 30.6$, 31.6 and 33.9 min showed almost the complete b-ion and y-ion series, thereby allowing the prediction of peptide sequences. These predicted sequences agreed with the sequences obtained by Edman degradation, which provided the entire sequences in 20 cycles. These sequences were: Val-Asn-Trp-Lys-Lys-Val-Leu-Gly-Lys-Ile-Ile-Lys-Val-Ala-Lys ($t_R = 30.6$), Val-Asn-Trp-Lys-Lys-Ile-Leu-Gly-Lys-Ile-Ile-Lys-Val-Ala-Lys ($t_R = 31.6$), and Val-Asn-Trp-Lys-Lys-Ile-Leu-Gly-Lys-Ile-Ile-Lys-Val-Val-Lys ($t_R = 33.9$). Internally calibrated electrospray mass spectra showing doubly, triply and quadruply charged molecules were deconvoluted by using in-house software, resulting in accurate masses of 1722.1 ± 0.1, 1736.1 ± 0.1 and 1764.1 ± 0.1, respectively. This signifies that these three peptides are C-terminally amidated.

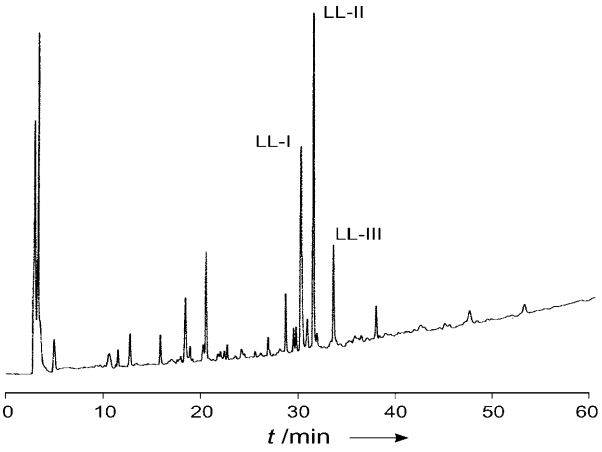

Figure 1. RP-HPLC profile of *Lasioglossum laticeps* venom extract at 222 nm. An elution gradient of solvents from 5–70% MeCN/H_2O/0.1% TFA was applied for 60 min at a flow rate of 1 mL min^{-1}.

Comparison of the lasioglossin sequences with those in the SwissProt and GenBank™/EMBL Data Banks (Blast program in Swiss-Prot) indicated that although they have some sequence homology with mastoparans of the vespidae family, their sequences are not integrated in the antimicrobial peptide database.[13] The three lasioglossins (LL-I, LL-II and LL-III), therefore, can be considered as new antimicrobial peptides. Determination of the primary structure of the peptides eluted in both the peaks at $t_R = 18.0$ and 28.2 min by tandem mass spectrometry and by Edman degradation did not result in unambiguous sequences.

Peptide synthesis

LL-I, LL-II and LL-III and their analogues (Table 1) were prepared by using standard peptide coupling chemistry. Bromophenol blue indicator was used to nondestructively monitor the conversion of free amino groups during coupling.[23] This procedure showed that coupling during the first few cycles was

Table 1. Amino acid sequences and MS data of the synthetic lasioglossins (LL-I, LL-II, LL-III) and their analogues.

Acronym	Peptide sequence	Monoisotopic molecular mass [Da]	
		calcd	found
LL-I	VNWKKVLGKIIKVAK-NH_2	1722.14	1721.8
LL-I/1	**NV**WKKVLGKIIKVAK-NH_2	1722.14	1721.9
LL-I/2	VNWKKVL**A**KIIKVAK-NH_2	1736.16	1736.8
LL-I/3	VNWKKVL**K**KIIKVAK-NH_2	1793.21	1793.8
LL-I/4	VNWKKVL**P**KIIKVAK-NH_2	1762.17	1761.9
LL-II	VNWKKILGKIIKVAK-NH_2	1736.16	1736.0
LL-II/1	**NV**WKKILGKIIKVAK-NH_2	1736.16	1736.0
LL-II/2	VNWKKIL**A**KIIKVAK-NH_2	1750.17	1750.8
LL-II/3	VNWKKIL**K**KIIKVAK-NH_2	1807.23	1807.8
LL-II/4	VNWKKIL**P**KIIKVAK-NH_2	1776.19	1776.3
LL-III	VNWKKILGKIIKVVK-NH_2	1764.19	1763.9
LL-III/1	**NV**WKKILGKIIKVVK-NH_2	1764.19	1764.2
LL-III/2	VNWKKIL**A**KIIKVVK-NH_2	1778.20	1778.9
LL-III/3	VNWKKIL**K**KIIKVVK-NH_2	1835.26	1835.9
LL-III/4	VNWKKIL**P**KIIKVVK-NH_2	1804.22	1804.7
LL-III/5	VNWKKILGKIIKVVK-**OH**	1765.17	1765.5
LL-III/6	VNWKKILGKIIKVVK-**OMe**	1779.19	1779.3
LL-III/7	VNWKKILGKI-NH_2	1196.77	1196.7
LL-III/8	ILGKIIKVVK-NH_2	1108.81	1108.7
LL-III/9	VNWKKILG-NH_2	955.60	955.6
LL-III/10	GKIIKVVK-NH_2	882.64	882.5

completed within several minutes. Later, as indicated by a sluggish colour change, the coupling time had to be extended to several hours. Crude peptides were further purified by preparative RP-HPLC to provide analytical HPLC purity ranging from 96 to 99%. The retention times of the purified peptides are given in Table 2 and the results of MS analyses confirming their identities are provided in Table 1.

Table 2. Physical properties of lasioglossins (LL-I, LL-II, LL-III) and their analogues.

Peptide	H[a]	μ_H[b]	t_R [min]	α-Helical fraction (f_h)				
				TFE [%]			SDS [mM]	
				0	10	40	0.16	16
LL-I	−0.117	0.340	30.73	0.16	0.23	0.47	0.15	0.59
LL-I/1	−0.117	0.411	30.29	–	–	–	–	–
LL-I/2	−0.111	0.335	32.98	–	–	–	–	–
LL-I/3	−0.201	0.407	30.05	–	–	–	–	–
LL-I/4	−0.132	0.351	26.39	–	–	–	–	–
LL-II	−0.104	0.346	32.01	0.15	0.19	0.45	0.14	0.54
LL-II/1	−0.104	0.420	32.18	–	–	–	–	–
LL-II/2	−0.098	0.341	33.78	–	–	–	–	–
LL-II/3	−0.188	0.414	31.23	–	–	–	–	–
LL-II/4	−0.119	0.358	27.58	–	–	–	–	–
LL-III	−0.085	0.364	33.98	0.14	0.20	0.53	0.20	0.63
LL-III/1	−0.085	0.436	34.61	0.16	0.19	0.51	0.16	0.66
LL-III/2	−0.079	0.360	36.03	0.16	0.22	0.57	0.19	0.62
LL-III/3	−0.169	0.431	31.69	0.17	0.22	0.46	0.19	0.63
LL-III/4	−0.100	0.376	29.28	0.14	0.18	0.40	0.18	0.45
LL-III/5	−0.085	0.364	33.29	–	–	–	–	–
LL-III/6	−0.085	0.364	32.88	0.19	0.21	0.42	0.18	0.47
LL-III/7	−0.088	0.314	26.73	0.14	0.17	0.31	0.12	0.30
LL-III/8	0.066	0.426	21.80	0.20	0.20	0.35	0.19	0.36
LL-III/9	−0.064	0.231	24.36	0.11	0.16	0.24	0.13	0.19
LL-III/10	−0.075	0.437	14.71	0.20	0.22	0.26	0.20	0.40

[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.[53] [b] The mean hydrophobic moment (μ_H), used as a quantitative measure of amphipathicity, was calculated according to the formula given in ref. [36].

Biological activity

The LL-I, LL-II and LL-III peptides showed comparably high antimicrobial potency against the two Gram-positive and two Gram-negative bacteria tested (Table 3). The Gram-negative bacterium *P. aeruginosa* appeared to be the most resistant. LL-III was more effective against *S. aureus* than LL-I and LL-II. All three peptides had no detectable haemolytic activity up to a concentration of 200 μM (Table 3).

A simple interchange of Val1 with Asn2 gave the LL-I/1, LL-II/1 and LL-III/1 analogues and resulted in "ideal" amphipathic helices according to the wheel projection (Figure 2) of Edmundson,[24] but this did not improve their antimicrobial qualities. Conversely, antimicrobial activity of the three analogues against *S. aureus* and *P. aeruginosa* were slightly reduced compared to the natural peptides. Substitution of the Gly residue at position 8 by an α-helix-stabilising Ala (analogues LL-I/2, LL-II/2 and LL-III/2) either did not change or only slightly enhanced their antimicrobial activities against *S. aureus*.

Table 3. Antimicrobial and haemolytic activity of lasioglossins (LL-I, LL-II, LL-III) and their analogues.

Peptide	Antimicrobial activity MIC [μM]				Haemolytic activity[a]
	B.s.	*S.a.*	*E.c.*	*P.a.*	LC_{50} [μM]
LL-I[b]	0.8	14.3	1.7	15.8	>200
LL-I/1	1.3	71.6	2.5	22.7	>200
LL-I/2	0.6	7.2	0.7	26.9	>200
LL-I/3	0.7	15.6	0.7	19.0	>200
LL-I/4	0.9	>100	5.0	100	>200
LL-II[b]	0.7	9.0	1.4	14.4	>200
LL-II/1	0.7	19.7	1.1	23.3	>200
LL-II/2	0.7	4.3	0.7	22.5	66.5
LL-II/3	0.7	15.6	0.7	26.1	88.2
LL-II/4	0.7	80	2.5	100	>200
LL-III[b]	0.7	3.9	1.4	18.7	>220
LL-III/1	0.7	9.0	1.4	23.1	>200
LL-III/2	0.6	2.2	1.4	27.1	55.1
LL-III/3	0.7	9.6	0.7	26.1	72.2
LL-III/4	0.8	32.5	1.2	100	>200
LL-III/5	1.2	40	3.5	40	>200
LL-III/6	0.5	2.1	1.0	22.5	79.3
LL-III/7	4.7	>100	23.3	75	>200
LL-III/8	40	>100	>40	>100	>200
LL-III/9	>200	>100	>100	>100	>200
LL-III/10	100	>100	>100	>100	>200

[a] Concentration of the peptide causing lysis of 50% of red bood cells. [b] Mast cell degranulation activity represented 25–35% of the activity of mastoparan.

Lasioglossins with a net charge of +6 fit into the large group of native AMPs having a net charge ranging from +4 to +6, which might represent the optimal charge for antimicrobial activity.[11] It would appear that a further increase in their net positive charge does not substantially promote their antimicrobial activity. The replacement of Gly by cationic and α-helix-stabilising Lys residue (analogues LL-I/3, LL-II/3 and LL-III/3) resulted in slightly enhanced activity against *E. coli* only, the activity against *B. subtilis*, *S. aureus* and *P. aeruginosa* was unmodified or slightly reduced. Unfortunately, both Gly8 substitutions increased the undesirable haemolytic activity in the case of the LL-II and LL-III analogues. On the other hand, the substitution of Gly with the helix-breaker Pro residue (analogues LL-I/4, LL-II/4 and LL-III/4) resulted in a desirable decrease in haemolytic activity but was accompanied by a substantially lower antimicrobial effect against *S. aureus* and *P. aeruginosa*.

The C-terminally deamidated analogue (LL-III/5) showed an approximately twofold decline in antimicrobial activity and no haemolytic activity. Esterification of the C terminus (LL-III/6) resulted in an increase in haemolytic activity without affecting its potent antimicrobial activity. The N-terminal decapeptide fragment of lasioglossin III (LL-III/7) possessed significantly decreased antimicrobial activity (~70×, >25×, ~17× and ~4× for *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*, respectively) in comparison to LL-III. The C-terminal decapeptide fragment (LL-III/8) was almost inactive (slight activity only against *B. subtilis*). Further truncation of these two fragments by two amino acid residues led to completely inactive compounds (LL-III/9 and LL-III/10).

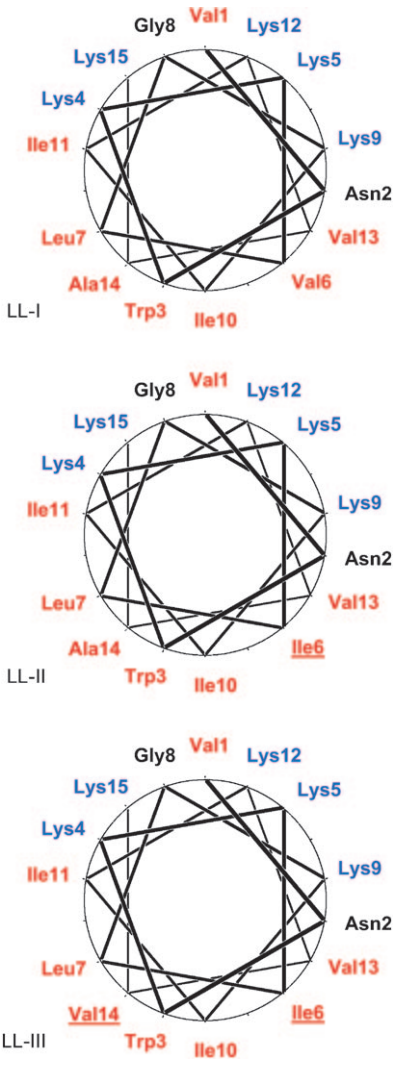

Figure 2. Wheel diagram of lasioglossins. Sector of hydrophobic amino acids is shown in red. The hydrophilic amino acids sector (shown in black and blue) is dominated by five Lys residues (shown in blue). Differences in the sequences are underlined.

Because the lasioglossin sequences show some homology to mastoparans that were isolated from wasps of the Vespidae family, they also display some mast cell degranulation activity. Their activity is about 25–35% that of mastoparan.[25]

Cytotoxicity

Lasioglossins I, II and III were tested for cytotoxicity against the PC-12 cancer cell line (Figure 3). LL-III, the compound most toxic against these cancer cells and that also had the best antimicrobial activity profile, was also tested for cytotoxicity against several other malignant cell lines (Table 4). This peptide showed a high potency against leukaemia cells (IC_{50} of approximately 5 μM). LL-III was two to three-times less potent against cells derived from solid tumours (PC12, HeLa S3 and SW480). The lowest toxicity was observed against the rat normal epithelial cells (IEC-6, 19 μM). The results of HeLa cell viability,

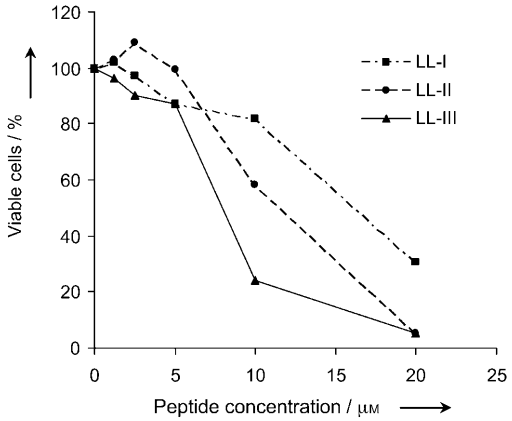

Figure 3. Viability of PC-12 cells incubated in the presence of lasioglossins.

Table 4. Anticancer activity of LL-III against some malignant cell lines.

Cel line[a]	L1210	CCRF-CEM T	HL-60	HeLa S3	PC12	SW480
IC_{50} [μM]	4.7[b]	5.0[b]	4.5[b]	8.4[b] 5.0[c]	10.0[c]	15.0[c]

[a] L1210, Mouse lymphocytic leukaemia; CCRF-CEM T, human lymphoblastic leukaemia; HL-60, human promyelocytic leukaemia; HeLa S3, human cervix carcinoma; PC12, pheochromocytoma of the rat adrenal medulla; SW480, human colon adenocarcinoma. [b] XTT test. [c] MTT test.

determined by using both XTT and MTT methods, were comparable (Table 4).

CD analysis and structural features

Far-UV spectra for the lasioglossins were acquired through CD spectroscopy in both the absence and presence of the helix-promoting solvent TFE and in the anisotropic environment of the SDS micelles. In water and in the presence of small amounts of TFE or SDS, lasioglossins exhibit spectra (Figure 4) characteristic of an unstructured peptide with a broad minimum at 200 nm containing approximately 15% of α-helix (Table 2). The CD spectra underwent a considerable change after increasing the concentration of TFE in 10% increments, demonstrating a predominantly α-helical structure as indicated by the appearance of double minimum bands at 207 and 221 nm. The spectra of LL-III (Figure 4A) at 30, 40 and 50% TFE showed the highest ellipticity at these minima, indicating a maximum α-helical content (53%) at 30% and 40% TFE (Table 2). A similar α-helical structure formation was observed for LL-III in the anisotropic environment of the SDS micelles (Figure 4B), reaching a maximum α-helical fraction (63%) at 16 mM concentration of SDS.

The CD spectra of the structurally related LL-I and LL-II peptides follow practically the same profile as the spectra of LL-III (not shown). As expected, the substitution of Pro for Gly8 (LL-III/4) considerably reduced the propensity for α-helix formation in the analogues. This peptide reached its maximal α-helical fraction of 40% and 45% at higher concentrations of TFE or

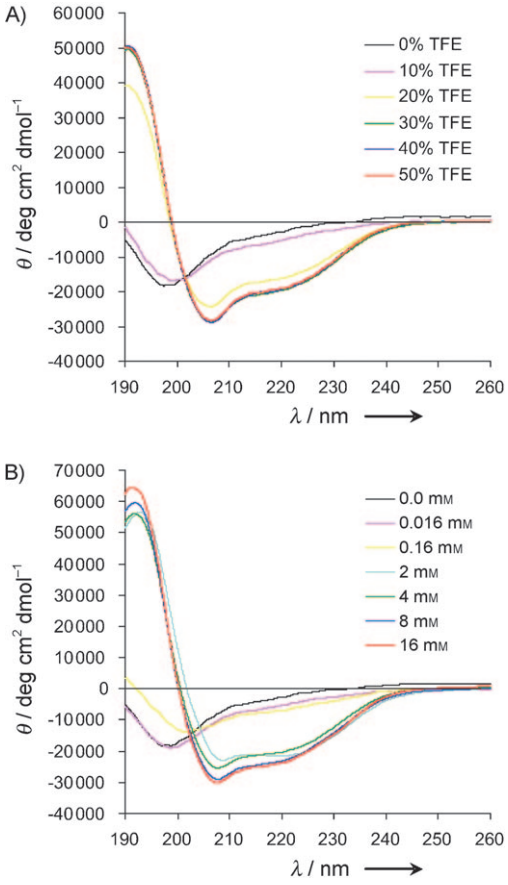

Figure 4. UV-CD spectra of LL-III in water, in the presence of various concentrations of A) TFE (*v/v*) and B) SDS (mM). The peptide concentration was 0.25 mg mL^{-1}.

SDS, respectively (Table 2). The replacement of Gly8 in LL-III by the α-helix-promoting residues Ala (LL-III/2) or Lys (LL-III/3) and the interchange of Val1 and Asn2 to produce the perfectly amphipathic analogue LL-III/1 did not influence the propensity of these analogues to form an α-helix, as indicated by their CD spectra (not shown).

The N-terminal (LL-III/7) and C-terminal (LL-III/8) fragments of lasioglossin III, which consisted of 10 amino acid residues, adopted an α-helical secondary structure in the presence of TFE or SDS to lesser extent only. Moreover, LL-III/8 showed a large proportion of β-sheet content at 2 mM SDS. Further truncation of these fragments by two amino acid residues resulted in a reduced propensity to form an α-helix in the case of the N-terminal octapeptide fragment (LL-III/9) but not in the case of the C-terminal octapeptide (LL-III/10), whose ability to form a helical structure was retained.

NMR spectroscopy

Resonance assignment of proton signals in all the peptides studied was achieved through a standard sequential resonance assignment strategy[26] by using 2D COSY, 2D TOCSY and 2D NOESY spectra. Spectra collected at different temperatures allowed an unambiguous resonance assignment in the case of overlap of amide proton signals. The survey of NMR spectroscopic data for the peptides studied is presented in the Supporting Information. The NMR spectra confirmed both the structure and purity of the peptides.

Secondary structure

Inter-residual NOEs, coupling constants $^3J_{NH,\alpha H}$, chemical shift index values (CSI) and temperature coefficients of NH proton chemical shifts ($\Delta\delta_{NH}/\Delta T$) were used as indicators of secondary structure.

NMR spectra of lasioglossins in water

The proton NMR spectroscopic data for the eight chosen peptides (in water at 27 °C) are summarised in Tables S1–8 in the Supporting Information. The NMR spectra all exhibit close similarity except those for residues in positions 6, 8, 14 and/or 15. Their NOESY spectra contain only intraresidual and sequential NOEs between neighbouring residues. Coupling constants $^3J_{NH,\alpha H}$ appear in the 6–8.5 Hz range and chemical shifts of Hα protons are very close to the random coil values[27] ($\Delta\delta_{\alpha H}$ is either slightly positive or negative but, in general, lower than 0.10 ppm). The large $\Delta\delta_{NH}/\Delta T$ values that were observed (in the range of −6 to −9 ppb, except for Leu4 with values of −2 to −2.5 ppb) are typical for random coil peptides.[28] All these observations are characteristic for flexible peptides adopting multiple conformations. The spectra that were obtained at lower temperature (5 °C) show a small decrease in $^3J_{NH,\alpha H}$ (typically by 0.2–0.5 Hz) that might indicate some propensity to form a helical conformation.

NMR spectra of lasioglossins in 30% TFE solution

TFE is known to induce helix formation in those parts of the peptide that have a clear helical propensity. The complete NMR data of the eight peptides (in 30% TFE solution at 27 °C) are presented in Tables S9–16 in the Supporting Information. Similar amino acid sequences led again to similarity of data, except for those around positions with different residues.

The NOESY spectra indicate, in addition to the intraresidual and neighbouring sequential NOEs, NOEs typical of helical conformation (see Figure 5 for LL-III). Most of the $^3J_{NH,\alpha H}$ couplings are lower than 6 Hz and characteristic for α-helices. Signals of Hα protons are significantly shifted upfield. The $\Delta\delta_{NH}/\Delta T$ values of most residues are much lower than for those in water solution (typically < -4 ppb). Amide NH protons of Leu7, Ile10, Ile11, Val14 and Lys15 displayed higher values (> -4 ppb), indicating easier access of solvent. A continuous series of negative CSI (> -0.1 ppm) from residues 3 to 14 indicated helical conformation over the whole peptide chain (except for the first three residues). Secondary structure indicators for the eight 15-residue peptides studied are summarised in Figures S1–8.

A closer look at the NMR data indicates periodical changes in some NMR parameters along the peptide chain, that is, chemical shifts of NH protons (δ_{NH}), and temperature coeffi-

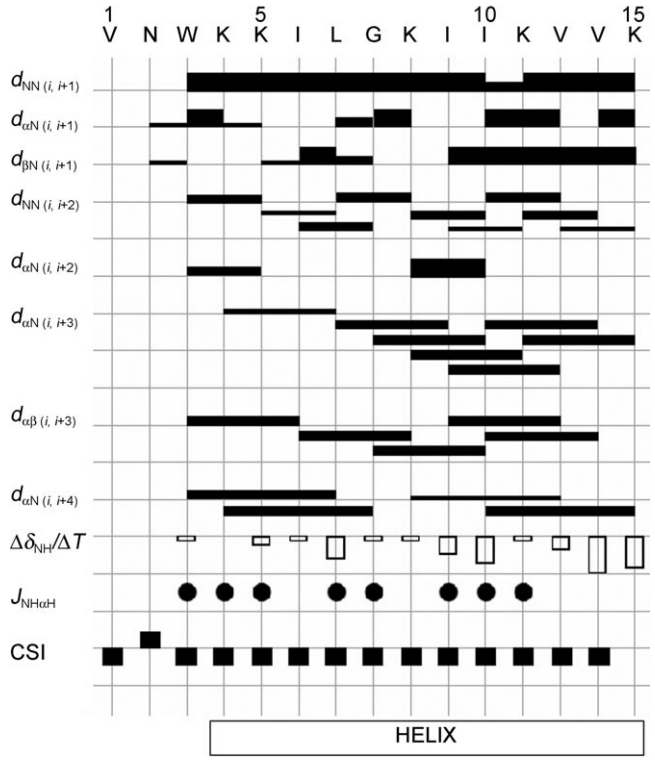

Figure 5. Secondary structure determination of LL-III in 30% TFE. The intensity of NOE cross-peaking is indicated by the thickness of the lines. The open boxes indicate relative sizes of temperature coefficients $\Delta\delta_{NH}/\Delta T$. Filled circles are shown when $J_{NH,\alpha H} = < 6$ Hz. The filled squares above and below the horizontal line represent CSI values of +1 and −1, respectively.

cients of NH proton chemical shifts ($\Delta\delta_{NH}/\Delta T$). Such periodical changes appear in the whole series of peptides, as shown in Figures 6 and 7, and have also been described in the literature[29–31] for amphipathic helical peptides with curvature of

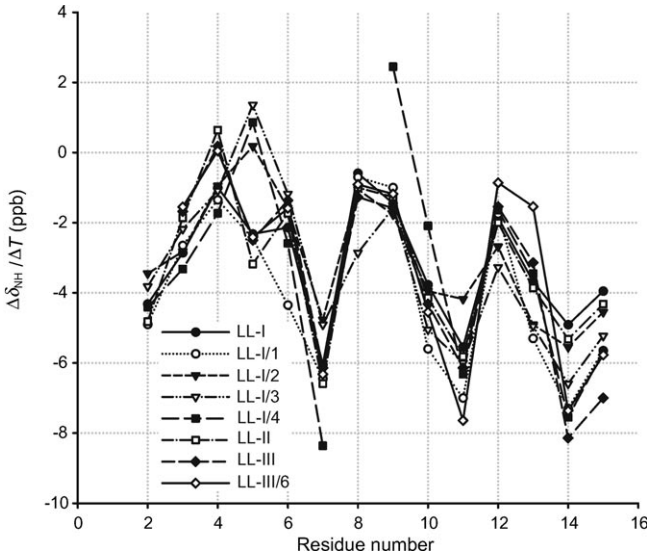

Figure 7. Plot of the temperature coefficients of amide protons in 30% TFE for individual residues in the series of LL peptides.

helix due to a shortening of hydrogen bonds on the hydrophobic side and a lengthening of hydrogen bonds on the hydrophilic side.[29,32] The hydrogen-bonding lengths *d* (between the amide proton and nearby oxygen atom in Å) can be calculated from the difference in the chemical shifts of amide NH protons in the helix and in the random coil $\Delta\delta_{NH}$ according to the equation $\Delta\delta_{NH} = 19.2\,d^{-3} - 2.3$.[33,34] We calculated the hydrogen-bonding lengths for our peptides by using NH chemical shifts in 30% TFE (helix) and in water (where random coil conformation had been proven; see above). The calculated H-bond lengths in our peptides again show some periodic variation, as illustrated in Figure 8. Summarised data indicate that the average H-bond length (distance O···H in the C=O···H–N

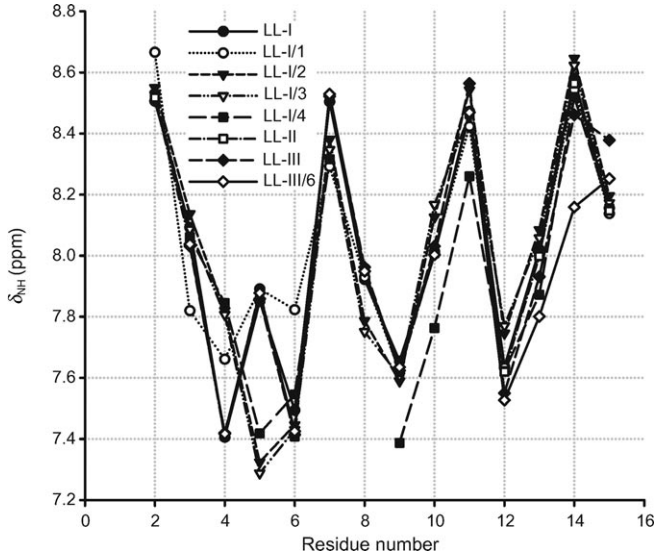

Figure 6. Plots of chemical shifts of amide protons (δ_{NH}) in 30% TFE for individual residues in the series of LL peptides

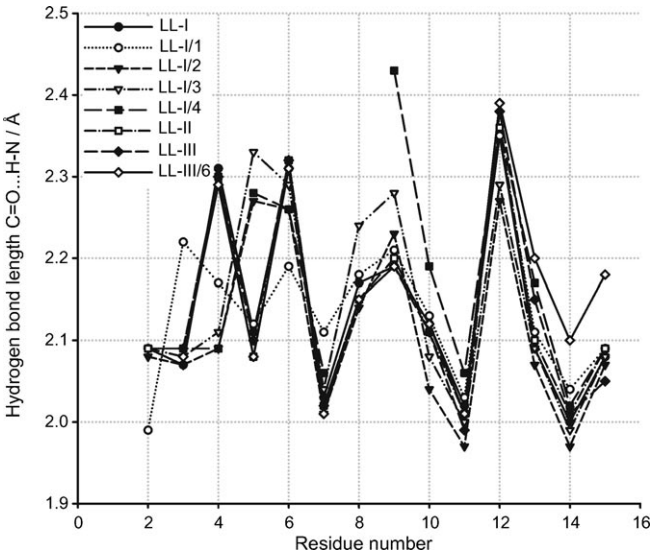

Figure 8. Calculated hydrogen bond lengths (H···O distance) for individual residues in the series of LL peptides.

fragments) of the residues on the hydrophilic side of the helices in all our peptides is always longer (2.16–2.22 Å) than the average H-bond length on the hydrophobic side (2.08–2.12 Å). We can conclude, therefore, that the preferred conformation of our peptides in 30% TFE is a curved helix with a concave hydrophobic and a convex hydrophilic side. Detailed calculations of the 3D structures of some peptides selected from our series will be the subject of a future study.

Differences between chemical shifts of Hα protons in 30% TFE and in water ($\Delta\delta_{\alpha H}$) for our peptides are shown in Figure 9. For most of our peptides, all of these values are more

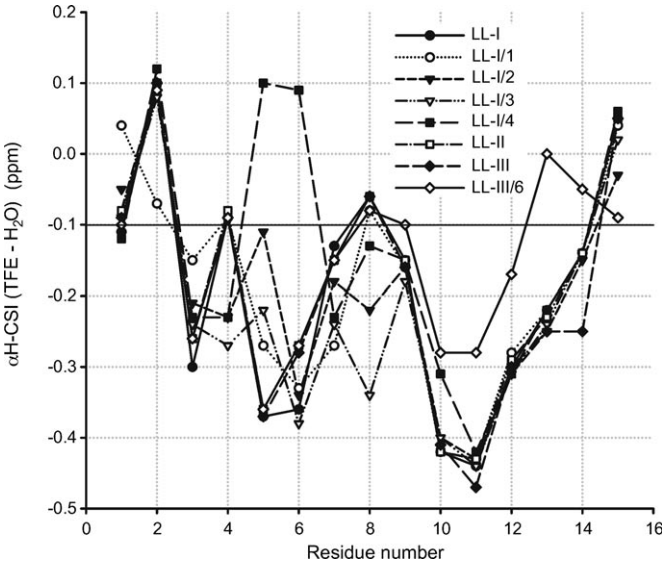

Figure 9. Plot of differences between chemical shifts of αH protons ($\Delta\delta_{\alpha H}$) in 30% TFE (helix) and in water (random coil) for individual residues in the series of LL peptides.

negative than −0.1 ppm; this indicates a helical conformation for all but the first two and last residues. The LL-I/4 peptide with Pro8 shows a markedly different $\Delta\delta_{\alpha H}$ of about +0.1 ppm for residues 6 and 5, clearly due to perturbation of the regular helix form by proline.

Discussion

The three newly isolated peptides from the venom of the eusocial bee *L. laticeps*, which we have named lasioglossins (LL-I, LL-II and LL-III), show no significant homology to other known antimicrobial peptides categorised in the peptide database (http://aps.unmc.edu/AP/main.php). They display comparable antimicrobial activity against each of four bacteria (*B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*) in the μM range and show low mast cell degranulation activity. The VNWK sequence motif of lasioglossins is homologous to the INWK sequence motif of some mastoparans that were isolated from different species of social wasps.[15,35]

CD spectroscopic measurements of the lasioglossins and LL-III analogues in the presence of TFE or SDS confirmed the presence of a significant amount of α-helical structure, and therefore, we assume that these sequences can adopt an amphipathic α-helical conformation as shown in Figure 2. In this Edmundson wheel projection, all the hydrophilic amino acid residues are situated on one side of the α-helix, whereas the hydrophobic amino acid residues, except Val12, are on the opposite side.

According to the literature, the ability of AMPs to form a well-defined amphipathic α-helix strongly correlates with their antimicrobial activity.[36] The quantitative measure of peptide amphipathicity is the hydrophobic moment μ_H, which is calculated as the vector sum of individual amino acid hydrophobicities normalised to an ideal helix.[36,37] We compared the hydrophobic moments (Table 2) of our peptides with MIC values to see whether this parameter correlates with antimicrobial activity. Among the three natural lasioglossins, the consecutive increase in their activity against *S. aureus* (Table 3) is possibly reflected in their hydrophobic moments, which increase in series, that is, LL-I < LL-II < LL-III. Surprisingly, the perfectly amphipathic analogues with highest μ_H values of the studied series (LL-I/1, LL-II/1, and LL-III/1) and theoretically with the best propensity to adopt α-helical structure due to Asn as the best helix-capping residue at the N terminus,[38] possessed lower antimicrobial activity than the natural peptides. Also, no effect of this modification on hemolytic activity was detected within the experimental range of peptide concentration. We have previously observed an analogous phenomenon when testing perfectly amphipathic analogues that have N-terminal Asn residues; these analogues were derived from other AMPs that we isolated from the venom of Polistinae wasps.[15]

The presence of a flexible glycine-induced hinge region in the middle of the peptide chain of α-helical AMPs seems to be responsible for different sensitivity of bacterial and eukaryotic target cells.[39] We showed that replacement of Gly in the central part of the lasioglossin molecule by α-helix-promoting residues (Ala and Lys) increased the haemolytic activity of LL-II and LL-III. This probably confirms the general fact that increasing the helicity of α-helical AMPs results in a marked enhancement in their haemolytic activities.[40,41] An increase in the net positive charge of the lasioglossins through the incorporation of cationic Lys residue to position 8 resulted in reduced antimicrobial activity against *S. aureus*, the analogous effect was already observed by us in the case of another AMP.[20] This might reflect induced resistance of *S. aureus* against AMPs via the *aps* AMP sensor/regulator system, which includes the D-alanylation of teichoic acid in the cell wall, the incorporation of lysyl-phosphatidylglycerol in the bacterial membrane, and putative AMP transport systems that cause the repulsion of strongly cationic LL analogues.[42] An additional level of protection might also depend on the proteolytic activity secreted by *S. aureus* against the basic residues that are present in LL analogues.[43]

We also tried to correlate the retention times of our peptides that were measured on the C-18 HPLC column with their calculated hydrophobicities and hydrophobic moments in order to observe any relationships between these parameters and biological activity (Tables 2 and 3). In this way we wished to observe whether the interactions of helical peptides with lipidic C-18 groups of the stationary phase during RP-HPLC

were similar to the process that governs the interactions of AMPs with biological membranes.[44] Although RP-HPLC separates peptides mainly through hydrophobic interactions, the presence of amphipathic structures in peptides induced within lipid environments influences their retention times.[44,45] The hydrophobicity and hydrophobic moment values for the LL-I, LL-II and LL-III natural lasioglossins correlate very well with retention time (Table 2) and antimicrobial activity against *S. aureus* (Table 3).

The substitution of Ala (LL-I/2, LL-II/2 and LL-III/2) or Lys (LL-I/3, LL-II/3 and LL-III/3) for Gly8 in the lasioglossins influenced the hydrophobicity of the analogues in a way that perfectly reflects their elution times. As shown in Table 2, however, analogues with a Pro substitution for Gly8 (LL-I/4, LL-II/4 and LL-III/4) eluted markedly earlier than natural peptides; this suggests that the disruption of lasioglossin α-helical structure imposed by Pro somehow perturbs the hydrophobic interactions of those analogues with a C-18 stationary phase, causing them to permeate faster through the column. Note that this HPLC behaviour also reflects lower antimicrobial activity of Pro8-containing analogues against *S. aureus* and *P. aeruginosa*.

The α-helical amphipathic N-terminal fragment LL-III/7 showed measurable antimicrobial activity (Table 3) compared to the almost biologically inactive C-terminal fragment LL-III/8 of the same length, despite the fact that the calculated value of its hydrophobic moment is lower than that of LL-III/8. On the other hand, LL-III/7 eluted later than LL-III/8, despite its lower calculated H and μ_H values. This might be an indication of unfolding in the helical structure of LL-III/8 and the loss of the nonpolar face of its amphipathic helix, which is a preferred binding domain during RP-HPLC.

The LL-III/6 analogue with a hydrophobic C-terminal methyl ester group unexpectedly eluted one minute earlier than the less hydrophobic peptide amide LL-III. The difference in retention time between the esterified analogue and the original peptide is indicative of the peptide's conformational change during interaction with the stationary phase of the column. We undertook molecular modelling of both compounds in an effort to explain the unexpected HPLC behaviour of LL-III/6 (Lys15 COOMe) and LL-III (Lys15 $CONH_2$). The energy-minimised structure of LL-III indicated that one of the $CONH_2$ α-amide protons of Lys15 might be participating in a bifurcated H-bond (together with the Lys15 peptide bond NH) to the Ile11 oxygen atom of the backbone carbonyl group as shown in Figure 10. The presence of such a bond is supported by the large difference in $\Delta\delta_{NH}/\Delta T$ values of Lys15 $CONH_2$ protons (−1.86 ppb for CONHa at δ 7.158 against −8.73 ppb for CONHb at δ 6.932) and by the NOE contacts of CONHa to some of the protons of Val14, Val13 and Lys12. The involvement of the Lys15 $CONH_2$ group in the hydrogen-bonding network could reduce polarity and contribute to the structural rigidity of LL-III. On the other hand, the absence of such an interaction in L-III/6 with the Lys15 methyl ester group might cause structural perturbation of the C-terminal part of its helix, affecting its ability to interact with the stationary phase. A lowering of the helical content in the C-terminal part of LL-III/6 is supported by the observation that the $\Delta\delta_{\alpha H}$ of its last three residues are between −0.03 to −0.05 ppm, that is, less than the −0.1 ppm that is required by CSI for helix formation (Figure 9). Further, the CD spectroscopy experiments indicate a lower helicity for the C-terminally esterified peptide, both in the presence of TFE and in the presence of SDS micelles.

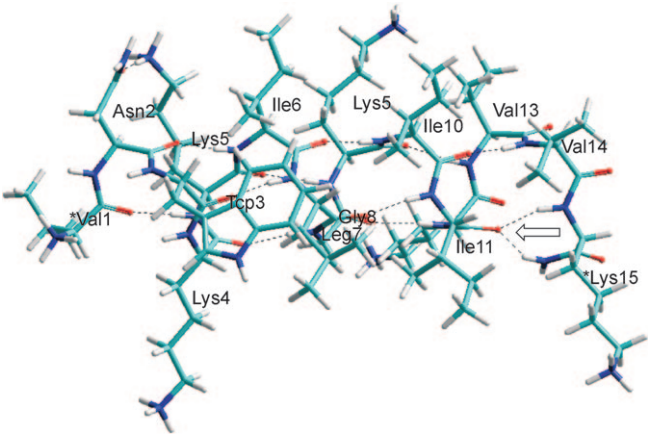

Figure 10. Molecular model of the helical conformation of LL-III. Dotted lines indicate hydrogen bonds. The bifurcated H-bond of one hydrogen of Lys15-$CONH_2$ and the hydrogen of Lys15 peptide bond to the Ile11 oxygen atom of the carbonyl group is indicated with an arrow.

Conclusions

Lasioglossins, the novel peptides first identified in the venom of wild eusocial bee, have high antimicrobial and low haemolytic activity and represent an attractive subgroup of antimicrobial peptides that have potential to be further modified into therapeutics for treating certain infections.

Experimental Section

Materials: Fmoc-protected L-amino acids, Rink Amide MBHA resin and 2-chlorotrityl chloride resin were purchased from IRIS Biotech GmbH, Marktredwitz, Germany. Tetracycline, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidine, LB broth, LB agar, MTT reagent, XTT reagent and insulin were bought from Sigma–Aldrich. Media for cell culturing were purchased from Biotech, Prague. 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; and *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which were obtained as multiresistant clinical isolates. The following cancer cell lines were all from ATCC (Manassas, VA, USA): rat pheochromocytoma PC12, human colon adenocarcinoma SW480 (CCL-228), mouse lymphocytic leukaemia L1210 (CCL-219), CCRF-CEM T lymphoblastoid (human acute lymphoblastic leukaemia, CCL-119), human promyelocytic leukaemia HL-60 (CCL-240), human cervix carcinoma HeLa S3 and normal rat epithelial cells (IEC-6, CRL-1592).

Sample preparation and peptide purification: Bee specimens were collected in the urban area of the town of Hořice in the northeast of the Czech Republic during May 2007. The bees were captured while approaching or leaving their nests, which were

built into the clefts of a sandstone pedestal in a family house, and were then kept frozen at −20 °C for several days. The venom reservoirs of five individuals were removed by dissection, and their contents were extracted with a mixture of MeCN and H_2O (1:1) that contained 0.1% TFA (25 μL). The extract was centrifuged, and the supernatant was fractionated by using RP-HPLC. Chromatography was carried out on a Thermo Separation Product instrument with a Vydac C-18 column (250×4.6 mm; 5 μm) set at a 1.0 mL min^{-1} flow rate and by using a solvent gradient ranging from 5 to 70% MeCN/H_2O/0.1% TFA over 60 min. After collecting the major fractions that were detected by UV absorption at 222 nm (Figure 1), the solvent was evaporated in a Speed-Vac, and the material was analysed by mass spectrometry and subjected to Edman degradation.

Mass spectrometry: Mass spectra for the peptides were acquired on a Reflex IV MALDI-TOF mass spectrometer (Bruker) equipped with a UV 337 nm nitrogen laser operating 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) was mixed with the matrix (1 μL), applied to a spot on a MALDI plate and allowed to dry at room temperature. Tandem mass spectra were recorded by using a Micromass Q-Tof microTM mass spectrometer (Waters) equipped with an electrospray ion source. A mixture of MeCN and H_2O (1:1) containing 0.1% acetic acid was continuously delivered to the ion source at a 20 μL min^{-1} flow rate. Samples dissolved in the mobile phase were introduced by 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 by 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) by using the manufacturer's pulse-liquid Edman degradation chemistry cycles.

Peptide synthesis: The lasioglossins and their analogues were prepared by using the solid-phase method in 5 mL polypropylene syringes with a Teflon filter in the bottom. Synthesis was done by using the N^{α}-Fmoc chemistry protocol on a Rink Amide MBHA resin (100 mg) with 0.7 mmol g^{-1} substitution. Protected amino acids were coupled in fourfold excess with DMF as the 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/H_2O/TIS (95:2.5:2.5) for 3.5 h and precipitated with *tert*-butyl methyl ether. The LL-III/5 and LL-III/6 compounds were similarly synthesised on 2-chlorotrityl chloride resin. LL-III/5 was deprotected and cleaved from the resin as described above. LL-III/6 was cleaved from the resin in its protected form by a mixture of CH_2Cl_2/trifluoroethanol/acetic acid (7:2:1) for 1 h. The solvent was concentrated under vacuum, and the residue was triturated with a mixture of *tert*-butyl methyl ether/*n*-hexane. The protected peptide was suspended in MeOH and esterified with a solution of diazomethane in Et_2O. The solvent was evaporated, the residue was triturated with *n*-hexane, and the peptide was deprotected by using the standard procedure. The crude peptides were purified by preparative RP-HPLC by using a Vydac C-18 column (250×10 mm) at a 3.0 mL min^{-1} flow rate and by using the same instrument and solvent gradient as described above.

Determination of antimicrobial activity: A quick qualitative estimate of antimicrobial properties was undertaken by using the drop-diffusion test on Petri dishes by the double-layer technique. Quantitative minimum inhibitory concentrations (MICs) were established by observing bacterial growth in multiwell plates.[46,47] Mid-exponential phase bacteria were added to individual wells containing solutions of the peptides at different concentrations in LB broth (final volume 0.2 mL, final peptide concentration in the range of 0.5 to 100 μM). The plates were incubated at 37 °C for 20 h while being continuously shaken in a Bioscreen C instrument (Oy Growth Curves AB Ltd., Helsinki, Finland). The absorbance was measured at 540 nm every 15 min and each peptide was tested at least 3 times in duplicate. Routinely, 1.2×10^3–7.5×10^3 CFU of bacteria per well were used for activity determination. Tetracycline in a concentration range of 0.5–50 μM was tested as a standard.

Determination of haemolytic activity:[48] The peptides were incubated with rat red blood cells for 1 h at 37 °C in a physiological solution at a final volume of 0.2 mL (final erythrocyte concentration 5% (*v*/*v*) and final peptide concentration 1–200 μM). The samples were then centrifuged for 5 min at 250 g, and the absorbance of the supernatant was determined at 540 nm. Supernatants of red blood cells suspended in physiological solution and 0.2% Triton X100 in physiological solution served as controls for zero haemolysis (blank) and 100% haemolysis, respectively. Each peptide was tested in duplicate in at least two independent experiments.

Mast cell degranulation test:[35,49] Mast cells were obtained by peritoneal washing of adult Wistar rats. Degranulation potency was determined by measuring the activity of β-D-glucosaminidase colocalised 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 substrate solution (*p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidine) and incubated for a further 6 h at 37 °C. The reaction was stopped by the addition of 0.2 M Tris buffer, pH 9 (150 μL), and the absorbance was determined at 405 nm. Mast cells that were incubated in a physiological solution and 0.2% Triton X100 served as controls for zero degranulation and 100% degranulation, respectively. Synthetic Mastoparan was used as a standard.

Cell viability (toxicity) was tested by using two systems:

MTT assay: Cells (PC12, SW480 and IEC-6) were cultured under standard conditions in RPMI or DMEM medium supplemented by inactivated HS or FBS, glutamine (2 mM) and penicillin and streptomycin (100 U mL^{-1}). In the case of IEC-6 cells, insulin was added. The cells were seeded at 5000 cells/0.2 mL of the medium onto 96-well plates and cultured for 24 to 48 h. The medium was then exchanged for fresh medium with or without different concentrations of the peptides. After 90 min incubation, 12.5 μL of the MTT reagent (5 mg mL^{-1} in physiological solution) was added to the cells, and these were incubated for a further 30–60 min. The supernatant was then aspirated, and the blue formazan crystals were dissolved in DMSO (200 μL). The absorbance was determined on a Tecan spectrophotometer (Mannendorf, Switzerland) at 540 nm. Zero viability and 100% viability were determined by absorbance of DMSO in wells without cells and with cells incubated without peptides, respectively. The IC_{50} was determined as the concentration of peptides that caused a 50% decrease in cell viability (Figure 3). In preliminary experiments, to establish the best conditions for routine testing of toxicity, the cells were incubated with the peptides for 30 min, 60 min, 2 h, 6 h, 24 h and 48 h and the IC_{50} value was found to be comparable within the experimental error. From practical reasons, to guarantee the same conditions for all peptides tested, we have chosen the incubation time 90 min.

XTT assay: L1210 cells, CCRF-CEM cells and HL-60 cells were cultivated in RPMI 1640 medium supplemented with foetal calf serum

for 24 h by using 24 or 96-well tissue culture plates before adding the peptides. The endpoint of cell growth was 72 h after drug addition. The sessile HeLa S3 cells were treated similarly except that they were cultivated for 48 h before drug addition. Cell viability was quantified by using a XTT-PMS[50] standard spectrophotometric assay (Roche Molecular Biochemicals). The inhibitory potency of the compounds tested was expressed as an IC_{50} value.

CD spectra measurement: The secondary structures of the lasioglossins and their analogues were determined on a Jasco 815 spectropolarimeter (Tokyo, Japan) at room temperature by using a 0.1 cm path length with CD signal monitoring from 190 to 300 nm. All peptides were measured in H_2O, in a TFE/H_2O mixture (10, 20, 30, 40 and 50% (*v*/*v*) of TFE), and in the presence of SDS at concentrations of 0.16 mM to 16 mM (below and above the critical micelle concentration). A concentration of 0.25 mg mL^{-1} was used for all the peptides studied. For each experiment, data were averaged over four scans, taken with a 2 s time constant and with the blank subtracted. The final spectra (Figure 4) are expressed as molar ellipticity per residue. Assuming a two-state model, the observed mean residue elipticity at 222 nm was converted into an α-helix fraction (f_H) by using a methodology proposed in the literature.[51,52]

NMR spectroscopy: Eight peptides (LL-I, LL-I/1, LL-I/2, LL-I/3, LL-I/4, LL-II, LL-III and LL-III/6) were selected for NMR spectroscopic measurement. Each peptide (3–6 mg) was dissolved in a H_2O/D_2O mixture (9:1). A trace amount of dioxane was added as an internal reference and the solvent was acidify with HCl to pH 3–4. After a complete set of NMR spectroscopy experiments, CF_3CD_2OH (TFE) was added to produce a 30% TFE solution. NMR spectroscopy experiments were carried out by using the Bruker AVANCE 600 spectrometer with a cryo-probe at four different temperatures 5, 16, 27, and 40 °C. The 1D spectra and a set of homonuclear 2D spectra, COSY, TOCSY and NOESY, were collected for each sample under the given conditions. Possible misinterpretation of the NOE due to spin-diffusion was eliminated by checking for the presence of the corresponding ROE peaks in ROESY spectra. A spin-lock time of 90 ms was used for TOCSY, and a mixing time of 300 ms was used for NOESY spectra. A typical 2D NMR data set consisted of 2048 complex points in the t_2 dimension with 512 complex t_1 increments and a spectral width of 6600 Hz in both dimensions.

Glossary

LB, Luria-Bertani; *E.c.*, *Escherichia coli*; *B.s.*, *Bacillus subtilis; S.a.*, *Staphylococcus aureus*; *P.a.*, *Pseudomonas aeruginosa;* DIPC, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; MS, mass spectrometry; RP-HPLC, reversed-phase, high-performance liquid chromatography; TFA, trifluoroacetic acid; TIS, triisopropylsilan.

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Keywords: antimicrobial activity · helical structures · NMR spectroscopy · peptides · wild-bee venom

[1] A. Parisien, B. Allain, J. Zhang, R. Mandeville, C. Q. Lan, *J. Appl. Microbiol.* **2008**, *104*, 1–13.
[2] R. E. W. Hancock, H.-G. Sahl, *Nat. Biotechnol.* **2006**, *24*, 1551–1557.
[3] O. Toke, *Biopolymers* **2005**, *80*, 717–735.
[4] M. Zasloff, *Nature* **2002**, *415*, 389–395.
[5] M. Zaiou, *J. Mol. Med.* **2007**, *85*, 317–329.
[6] A. Giuliani, G. Pirri, S. F. Nicoletto, *Cent. Eur. J. Biol.* **2007**, *2*, 1–33.
[7] Y. J. Gordon, E. G. Romanowski, A. M. McDermott, *Curr. Eye Res.* **2005**, *30*, 505–515.
[8] E. Andrès, J. L. Dimarcq, *J. Int. Med. Res.* **2004**, *255*, 519–520.
[9] H. G. Boman, *J. Int. Med. Res.* **2003**, *254*, 197–215.
[10] M. R. Yeaman, N. Y. Yount, *Pharm. Rev.* **2003**, *55*, 27–55.
[11] A. Tossi, L. Sandri, A. Giangaspero, *Biopolymers* **2000**, *55*, 4–30.
[12] Z. Oren, Y. Shai, *Biopolymers* **1998**, *47*, 451–463.
[13] Z. Wang, G. Wang, *Nucleic Acids Res.* **2009**, *37*, D933-D937.
[14] L. Kuhn-Nentwig, *Cell. Mol. Life Sci.* **2003**, *60*, 2651–2668.
[15] V. Čeřovský, J. Slaninová, V. Fučík, H. Hulačová, L. Borovičková, R. Ježek, L. Bednárová, *Peptides* **2008**, *29*, 992–1003.
[16] K. Konno, M. Hisada, R. Fontana, C. C. B. Lorenzi, H. Naoki, Y. Itagaki, A. Miwa, N. Kawai, Y. Nakata, T. Yasuhara, J. R. Neto, W. F. de Azevedo Jr., M. S. Palma, T. Nakajima, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **2001**, *1550*, 70–80.
[17] K. Konno, M. Hisada, H. Naoki, Y. Itagaki, R. Fontana, M. Rangel, J. S. Oliveira, M. P. dos Santos Cabrera, J. R. Neto, I. Hide, Y. Nakata, T. Yasuhara, T. Nakajima, *Peptides* **2006**, *27*, 2624–2631.
[18] K. Konno, M. Rangel, J. S. Oliveira, M. P. dos Santos Cabrera, R. Fontana, I. Y. Hirata, I. Hide, Y. Nakata, K. Mori, M. Kawano, H. Fuchino, S. Sekita, J. R. Neto, *Peptides* **2007**, *28*, 2320–2327.
[19] J. Orivel, V. Redeker, J.-P. Le Caer, F. Krier, A.-M. Revol-Junelles, A. Longeon, A. Chaffotte, A. Dejean, J. Rossier, *J. Biol. Chem.* **2001**, *276*, 17823–17829.
[20] V. Čeřovský, O. Hovorka, J. Cvačka, Z. Voburka, L. Bednárová, L. Borovičková, J. Slaninová, V. Fučík, *ChemBioChem* **2008**, *9*, 2815–2821.
[21] A. W. Ebmer, *Teil III, Naturkundliches Jahrbuch der Stadt Linz* **1971**, 17, 63–156.
[22] L. Packer, *Insectes Soc.* **1983**, *30*, 367–375.
[23] V. Krchňák, J. Wágner, P. Šafář, M. Lebl, *Collect. Czech. Chem. Commun.* **1988**, *84*, 2542–2548.
[24] M. Schiffer, A. B. Edmundson, *Biophys. J.* **1967**, *7*, 121–135.
[25] Y. Hirai, T. Yasuhara, H. Yoshida, T. Nakajima, M. Fujino, C. Kitada, *Chem. Pharm. Bull.* **1979**, *27*, 1942–1944.
[26] M. Billeter, W. Braun, K. Wüthrich, *J. Mol. Biol.* **1982**, *155*, 321–346.
[27] D. S. Wishart, A. M. Nip, *Biochem. Cell Biol.* **1998**, *76*, 153–163.
[28] G. Merutka, H. J. Dyson, P. E. J. Wright, *J. Biomol. NMR* **1995**, *5*, 14–24.
[29] N. E. Zhou, B. Zhu, B. D. Sykes, R. S. Hodges, *J. Am. Chem. Soc.* **1992**, *114*, 4320–4326.
[30] I. D. Kuntz, P. A. Kosen, E. C. Craig, *J. Am. Chem. Soc.* **1991**, *113*, 1406–1408.
[31] D. S. Wishart, B. D. Sykes, *Methods Enzymol.* **1994**, *239*, 363–392.
[32] J.-Y. Suh, K.-H. Lee, S.-W. Chi, S.-Y. Hong, B.-W. Choi, H.-M. Moon, B.-S. Choi, *FEBS Lett.* **1996**, *392*, 309–312.
[33] G. Wagner, A. Pardi, K. J. Wüthrich, *J. Am. Chem. Soc.* **1983**, *105*, 5948–5949.
[34] A. Pardi, G. Wagner, K. Wüthrich, *Eur. J. Biochem.* **1983**, *137*, 445–454.
[35] M. A. Mendes, B. M. de Souza, M. S. Palma, *Toxicon* **2005**, *45*, 101–106.
[36] N. Pathak, R. Salas-Auvert, G. Ruche, M.-H. Janna, D. McCarthy, R. G. Harrison, *Proteins Struct. Funct. Genet.* **1995**, *22*, 182–186.
[37] T. Wieprecht, M. Dathe, M. Krause, M. Beyermann, W. L. Maloy, D. L. MacDonnald, M. Bienert, *FEBS Lett.* **1997**, *417*, 135–140.
[38] A. Chakrabartty, A. J. Doig, R. L. Baldwin, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11332–11336.
[39] Y. R. Bommineni, H. Dai, Y.-X. Gong, J. L. Soulages, S. C. Fernando, U. DeSilva, O. Prakash, G. Zhang, *FEBS J.* **2007**, *274*, 418–428.
[40] I. Cornut, K. Büttner, J.-L. Dasseux, J. Dufourcq, *FEBS Lett.* **1994**, *349*, 29–33.

[41] J. M. Conlon, N. Al-Ghaferi, B. Abraham, J. Leprince, *Methods* **2007**, *42*, 349–357.
[42] M. Li, D. J. Cha, Y. Lai, A. E. Villaruz, D. E. Sturdevant, M. Otto, *Mol. Microbiol.* **2007**, *66*, 1136–1147.
[43] M. Sieprawska-Lupa, P. Mydel, K. Krawczyk, K. Wojcik, M. Puklo, B. Lupa, P. Suder, J. Silbering, M. Reed, J. Pohl, W. Shafer, F. McAleese, T. Foster, J. Travis, J. Potempa, *Antimicrob. Agents Chemother.* **2004**, *48*, 4673–4679.
[44] K. Büttner, S. E. Blondelle, J. M. Ostresh, R. A. Houghten, *Biopolymers* **1992**, *32*, 575–583.
[45] Y. Chen, M. T. Guarnieri, A. I. Vasil, M. L. Vasil, C. T. Mant, R. S. Hodges, *Antimicrob. Agents Chemother.* **2007**, *51*, 1398–1406.
[46] Z. Oren, Y. Shai, *Biochemistry* **1997**, *36*, 1826–1835.
[47] O. Lequin, A. Ladram, L. Chabbert, F. Bruston, O. Convert, D. Vanhoye, G. Chassaing, P. Nicolas, M. Amiche, *Biochemistry* **2006**, *45*, 468–480.
[48] B. M. Souza, M. A. Mendes, L. D. Santos, M. R. Marques, L. M. M. César, R. N. A. Almeida, F. C. Pagnocca, K. Konno, M. S. Palma, *Peptides* **2005**, *26*, 2157–2164.
[49] B. D. Gomperts, P. E. R. Tatham, *Methods Enzymol.* **1992**, *219*, 178–189.
[50] D. A. Scudiero, R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff, M. R. Boyd, *Cancer Res.* **1988**, *48*, 4827–4833.
[51] B.-M. Backlund, G. Wikander, T. Peeters, A. Gräslund, *Biochim. Biophys. Acta, Biomembr.* **1994**, *1190*, 337–344.
[52] C. A. Rohl, R. L. Baldwin, *Methods Enzymol.* **1998**, *295*, 1–26.
[53] D. Eisenberg, E. Schwarz, M. Komaromy, R. Wall, *J. Mol. Biol.* **1984**, *179*, 125–142.

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