

Novel antimicrobial peptides from the venom of the eusocial bee *Halictus sexcinctus* (Hymenoptera: Halictidae) and their analogs

Lenka Monincová · Miloš Buděšínský · Jiřina Slaninová · Oldřich Hovorka ·
Josef Cvačka · Zdeněk Voburka · Vladimír Fučík · Lenka Borovičková ·
Lucie Bednářová · Jakub Straka · Václav Čerovský

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Abstract Two novel antimicrobial peptides, named halictines, were isolated from the venom of the eusocial bee *Halictus sexcinctus*. Their primary sequences were established by ESI-QTOF mass spectrometry, Edman degradation and enzymatic digestion as Gly-Met-Trp-Ser-Lys-Ile-Leu-Gly-His-Leu-Ile-Arg-NH₂ (HAL-1), and Gly-Lys-Trp-Met-Ser-Leu-Leu-Lys-His-Ile-Leu-Lys-NH₂ (HAL-2). Both peptides exhibited potent antimicrobial activity against Gram-positive and Gram-negative bacteria but also noticeable hemolytic activity. The CD spectra of HAL-1 and HAL-2 measured in the presence of trifluoroethanol or SDS showed ability to form an amphipathic α -helical secondary structure in an anisotropic environment such as bacterial cell membrane. NMR spectra of HAL-1 and HAL-2 measured in trifluoroethanol/water confirmed formation of helical conformation in both peptides with a

slightly higher helical propensity in HAL-1. Altogether, we prepared 51 of HAL-1 and HAL-2 analogs to study the effect of such structural parameters as cationicity, hydrophobicity, α -helicity, amphipathicity, and truncation on antimicrobial and hemolytic activities. The potentially most promising analogs in both series are those with increased net positive charge, in which the suitable amino acid residues were replaced by Lys. This improvement basically relates to the increase of antimicrobial activity against pathogenic *Pseudomonas aeruginosa* and to the mitigation of hemolytic activity.

Keywords Antimicrobial peptides · Analogs · Wild-bee venom · Hemolytic activity · NMR spectroscopy · CD spectroscopy

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L. Monincová · M. Buděšínský · J. Slaninová · O. Hovorka ·
J. Cvačka · Z. Voburka · V. Fučík · L. Borovičková ·
L. Bednářová · V. Čerovský (✉)
Institute of Organic Chemistry and Biochemistry,
Academy of Sciences of the Czech Republic,
Flemingovo 2, 16610 Prague 6, Czech Republic
e-mail: cerovsky@uochb.cas.cz

L. Monincová
Department of Biochemistry, Faculty of Science,
Charles University in Prague, Hlavova 8,
12840 Prague 2, Czech Republic

J. Straka
Department of Zoology, Faculty of Science,
Charles University in Prague, Viničná 7,
12844 Prague 2, Czech Republic

Introduction

The emergence of bacterial strains resistant to the most conventional antibiotics has resulted in an intensive search for alternative antimicrobial agents with fundamentally different modes of action (Parisien et al. 2008). Antimicrobial peptides (AMPs), which are part of the innate defense system of practically all living organisms, ranging from prokaryotes to humans, are among the most promising lead compounds for developing medicines in the fight against resistant pathogens (Gordon et al. 2005; Zaiou 2007; Giuliani et al. 2007). Since the discovery of cecropin, the first AMP isolated from the hemolymph of the *Cecropia* moth (Hultmark et al. 1980), almost 1,000 AMPs have so far been listed in the Antimicrobial Peptide Database (Wang et al. 2009). These peptides, upon interaction with biological membrane or a membrane-mimicking environment, are able to fold into highly amphipathic

conformations with hydrophobic and hydrophilic moieties segregated into distinct patches on the molecular surface. Due to the frequent occurrence of lysine and arginine residues in their amino acid sequences, most AMPs are cationic molecules. This allows them preferentially to interact with the anionic phospholipids of bacterial membranes, which is followed by integration of the peptides into the lipid bilayer and disruption of the membrane structure that leads to leakage of cytoplasmic components and cell death (Oren and Shai 1998; Tossi et al. 2000; Yeaman and Yount 2003; Toke 2005). Some studies have revealed that the killing process may proceed with relatively little membrane disruption but occur rather through interactions with putative key intracellular targets (Yeaman and Yount 2003). In addition to their direct action against Gram-positive and Gram-negative bacteria, some AMPs are active against fungi (Ajesh and Sreejith 2009) or protozoa (Rivas et al. 2009), or they may lyse cancer cells (Dennison et al. 2006). The most studied group of cationic AMPs is comprised of the linear α -helical peptides (Tossi et al. 2000; Dennison et al. 2005). These are usually peptides composed of 10–45 amino acid residues. They are rich in hydrophobic residues and can adopt an amphipathic α -helical secondary structure within the cell membrane environment or in the presence of membrane-mimicking substances such as sodium dodecyl sulfate (SDS) or some organic solvents like trifluoroethanol (TFE).

Numerous studies have shown that peptides of this category are frequently found in the venom of arthropods such as scorpions, spiders, and stinging hymenopterans (Kuhn-Nentwig 2003). Among those peptides, the group of mastoparans (Čerovský et al. 2008b) isolated from the venom of *Vespidae* wasps and other short peptides found in the venom of solitary wasps, such as anoplin (Konno et al. 2001), eumenitin (Konno et al. 2006) and decoralin (Konno et al. 2007), have been extensively studied with regard to their antimicrobial potency. The main drawback in using these peptides as antibiotics, however, consists in their ability also to lyse eukaryotic cells, which is commonly expressed as hemolytic activity or toxicity to red blood cells.

Recently, we have found that the venom of several wild bees offers another promising source of α -helical amphipathic peptides which show potent antimicrobial and low or moderate hemolytic activity. Those are melectin (Čerovský et al. 2008a), lasioglossins (Čerovský et al. 2009), and macropin (Monincová et al. 2009) isolated from the venom of the cleptoparasitic bee *Melecta albifrons*, primitively eusocial bee *Lasioglossum laticeps* and the solitary bee *Macropis fulvipes*, respectively.

In this work, we describe the structural characterization and biological activity of two novel dodecapeptides named halictines (HAL-1 and HAL-2) which we isolated from the venom of the wild eusocial bee *Halictus sexcinctus*. In

addition, we prepared a large series of HAL-1 and HAL-2 analogs to evaluate the effect of their chemical modification on antimicrobial and hemolytic activities, as well as in an effort to obtain compounds with high antimicrobial activity and minimum hemolytic activity. Halictines, which are composed of 12 amino acid residues, are among the shortest linear cationic α -helical AMPs found in nature. This makes them advantageous in terms of their potential for chemical modification and possible applications.

Halictus sexcinctus is a bee of the family Halictidae with several narrow, white felt bands on its abdomen. It is 14–16 mm in size. Females collect pollen for their brood from various host plant species with wide-open flowers, usually of the family Asteraceae. They nest in sandy or loess soil individually or in small aggregations. This bee occurs in all parts of Europe as far north as southern Sweden. Because the social organization of this species is extremely variable, *H. sexcinctus* has come to be a very useful model for studying the evolution of eusocial behavior.

Materials and methods

Materials

Fmoc-protected L-amino acids and Rink Amide MBHA resin were purchased from IRIS Biotech GmbH, Marktredwitz, Germany. Tetracycline, LB broth, LB agar, and TLCK-treated α -chymotrypsin 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* (*B. s.*) 168, kindly provided by Prof. Yoshikawa (Princeton University, Princeton, NJ, USA); *Escherichia coli* (*E. c.*) B from the Czech Collection of Microorganisms, Brno; and *Staphylococcus aureus* (*S. a.*) and *Pseudomonas aeruginosa* (*P. a.*) obtained as multi-resistant clinical isolates.

Sample preparation and peptide purification

Bee specimens of *H. sexcinctus* were collected in the vicinity of the village of Veska in Eastern Bohemia (Czech Republic) during June 2008 and were then kept frozen at -20°C . The venom reservoirs of six individuals were removed under the microscope by dissection and their contents were extracted with a mixture of 25 μl of acetonitrile/water (1:1) containing 0.1% trifluoroacetic acid. The extract was centrifuged, and the supernatant was fractionated by RP-HPLC. Chromatography was carried out on an Agilent Technologies 1200 Series module with a Vydac

C-18, 250 × 4.6 mm; 5 µm, column (Grace Vydac, Hesperia, CA, USA) at a 1 mL/min flow rate using a solvent gradient ranging from 5 to 70% acetonitrile/water/0.1% TFA over 60 min (Fig. 1). The elution was monitored by absorption at 220, 254, and 280 nm utilizing a diode-array detector. The instrument was controlled and UV spectra evaluated using ChemStation Software. The selected fractions (peaks detected at 220 nm) were collected, the solvent evaporated in a Speed-Vac, and the material tested for antimicrobial activity against *M. luteus* and *E. coli* by drop diffusion assay. The active fraction were further analyzed by mass spectrometry and subjected to Edman degradation and enzymatic digestion.

Mass spectrometry

Mass spectra of the peptides were acquired on a Micromass Q-ToF micro mass spectrometer (Waters) equipped with an electrospray ion source. A mixture of acetonitrile/water 1:1 with 0.1% formic acid was continuously delivered to the ion source at a 20 µl/min flow rate. Samples dissolved in 20 µl of the mobile phase were introduced using a 2-µl loop. The measurement was performed in the positive mode, and the capillary voltage, cone voltage, desolvation temperature, and ion source temperature were 3.5 kV, 20 V, 150°C, and 90°C, respectively. The exact masses were measured using an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. The mobile phase composition and flow rate were the same as in the case of the Q-ToF micro. Spray voltage, capillary voltage, tube lens voltage, and capillary temperature were 5.0 kV, 10 V, 110 V and 300°C, respectively. The mass spectra were internally calibrated using protonated phthalic anhydride as the lock mass.

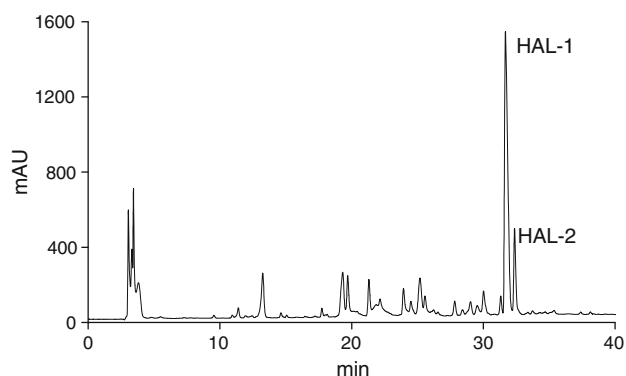


Fig. 1 RP-HPLC profile of *Halictus sexcinctus* venom extract at 220 nm. An elution gradient of solvents from 5 to 70% acetonitrile/water/0.1% TFA was applied for 60 min at a 1 ml/min flow rate

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, CA, USA) using the manufacturer's pulse-liquid Edman degradation chemistry cycles.

Peptide synthesis

The halictines and their analogs were synthesized manually according to the standard N^α-Fmoc protocol on a Rink Amide MBHA resin (100 mg with 0.7 mmol/g substitution) in 5 ml polypropylene syringes with a Teflon filter in the bottom. Protected amino acids (4 eq) were coupled using *N,N'*-diisopropylcarbodiimide (DIPC, 7 eq) and 1-hydroxybenzotriazole (HOBt, 5 eq) as coupling reagents in *N,N*-dimethylformamide (DMF) as a solvent. Free amino group conversion during coupling was monitored by a bromophenol blue indicator. Due to the steric coupling hindrance between Ile and *N*-methylleucine (MeL) in positions 6 and 7 of HAL-1/28 analog, Fmoc-Ile-OH (4 eq) had to be recoupled using HATU (3.9 eq)/*N*-ethyl-diisopropylamine (8 eq). Deprotections of the α-amino group were performed with 20% piperidine in DMF. The peptides were fully deprotected and cleaved from the resin with a mixture of TFA/thioanisole/H₂O/1,2-ethanedithiol/triisopropylsilane (90:3:2.5:2.5:2) for 3.5 h and then precipitated with *tert*-butyl methyl ether. Crude peptides were purified by preparative RP-HPLC on a Thermo Separation Product instrument using a Vydac C-18 column (10 × 250 mm; 5 µm) at a 3.0 ml/min flow rate using the solvent gradient as described above. The fractions were detected by UV absorption at 280 or 222 nm. The main dominant fraction containing the required peptide product was lyophilized.

Determination of antimicrobial activity

A quick qualitative estimate of antimicrobial properties was undertaken using the drop-diffusion test on Petri dishes by the double-layer technique (Čerovský et al. 2008a). Quantitative minimum inhibitory concentrations (MICs) were established by observing bacterial growth in multi-well plates (Oren and Shai 1997; Lequin et al. 2006). 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 0.5–100 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 three times in duplicate. Routinely, 1.2×10^3 to

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 hemolytic activity

The peptides were incubated with rat red blood cells (or human red blood cells of healthy donors) 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 $250\times g$, and 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 hemolysis (blank) and 100% hemolysis, respectively (Souza et al. 2005). Each peptide was tested in duplicates in at least two independent experiments.

CD spectra measurement

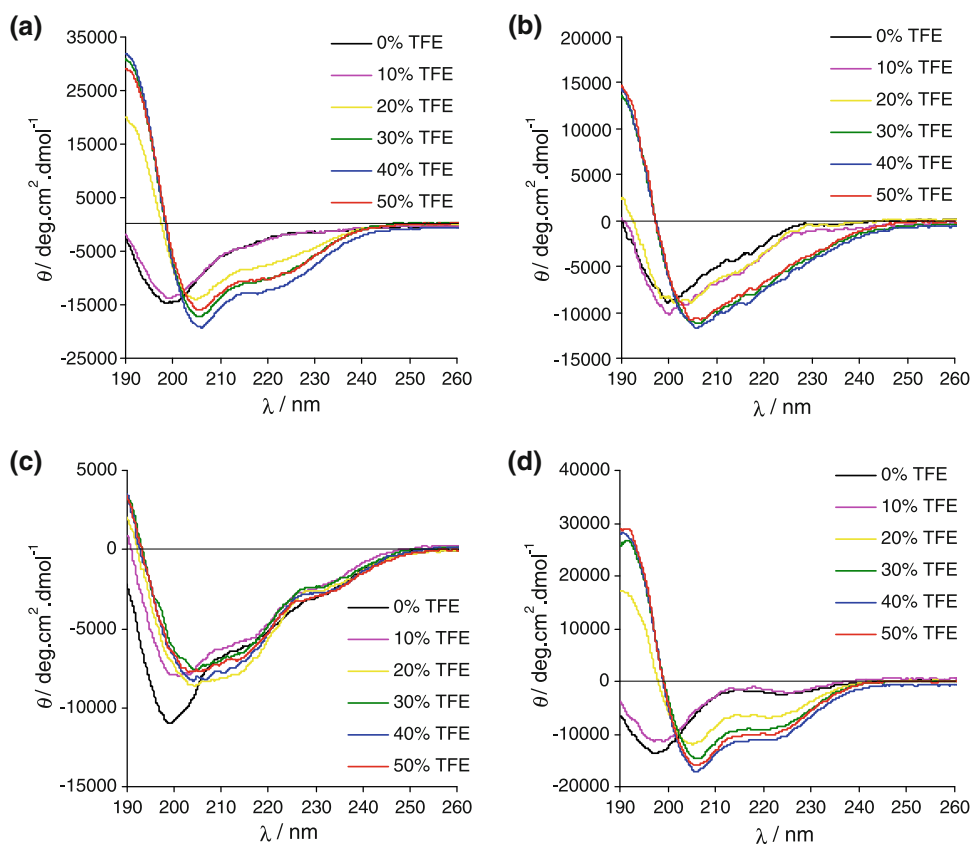
The circular dichroism (CD) experiments were carried out on a Jasco 815 spectropolarimeter (Tokyo, Japan). All peptide samples were measured in water, in a TFE/water mixture (10, 20, 30, 40 and 50% v/v of TFE), and in the presence of SDS at concentrations of 0.16–16 mM (below

and above the critical micelle concentration) with the final peptide concentration 0.25 mg/mL. The spectra were collected from 190 to 300 nm as averages over four scans at room temperature using a 0.1 cm path length. A 0.5 nm step resolution, 50 nm/min speed, 2 s response time and 1 nm bandwidth were generally used. Following baseline correction, the final spectra (Fig. 2) were expressed as molar ellipticity θ ($\text{deg}/\text{cm}^2/\text{dmol}$) per residue. The peptide helical content was calculated assuming a two-state model according to a method proposed by Backlund et al. (1994) and Rohl and Baldwin (1998). This approach is limited to a situation where random or α -helical conformation is present.

Nuclear magnetic resonance (NMR) spectroscopy

The NMR spectra of halictines HAL-1 and HAL-2 were measured using the Bruker AVANCE 600 MHz spectrometer with a cryoprobe. Each peptide (about 5 mg) was dissolved in an $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixture (9:1). A trace amount of dioxane was added as an internal reference and the pH was adjusted to 3.84 (HAL-1) and 2.80 (HAL-2) using HCl. The series of proton 1D, 2D-COSY, 2D-TOCSY, and 2D-NOESY was measured at temperatures 5, 27, and 40°C . After a complete set of NMR experiments, $\text{CF}_3\text{CD}_2\text{OH}$ (TFE) was added to produce a 30% TFE solution and the

Fig. 2 UV-CD spectra of HAL-1 (a) and selected analogs of halictines in the presence of various concentrations of TFE. **b** HAL-1/7, **c** HAL-1/28, **d** HAL-2/8



same series of NMR spectra was taken again at 5, 27, and 40°C. A spin-lock time of 90 ms was used for 2D-TOCSY spectra and a mixing time of 300 ms for 2D-NOESY experiments. A typical 2D NMR data set consisted of 2 K data points in the t_2 dimension with 512 increments in the t_1 and a spectral width of 6,600 Hz in both dimensions.

Results

Purification and sequence determination

RP-HPLC purification of the venom extract obtained from six venom reservoirs gave a profile with relatively few intense peaks. Antimicrobial activity was detected only in two dominant peaks at t_R 32.2 and 32.7 min (Fig. 1) and labeled as HAL-1 and HAL-2, respectively. The UV spectrum corresponding to these peaks confirmed that these peptides contain Trp within their sequences. The internally calibrated ESI-Q-TOF mass spectrum of the component eluted in the HAL-1 peak showed doubly and singly charged peaks $[M + 2H]^{2+}$ at m/z 705.4 and $[M + H]^+$ at m/z 1,409.9. The mass spectrum of the HAL-2 compound showed the $[M + 4H]^{4+}$ ion at m/z 363.9, the $[M + 3H]^{3+}$ ion at m/z 484.9, and the $[M + 2H]^{2+}$ at m/z 726.9 (see Supplementary Material). The mass spectra were deconvoluted using in-house software, resulting in the monoisotopic masses of $1,408.8 \pm 0.1$ and 1451.7 ± 0.1 for HAL-1, and HAL-2, respectively. The Edman degradation (see Supplementary Material) gave the entire sequence in 14 cycles as follows: Gly-Met-Trp-Ser-Lys-Ile-Leu-Gly-His-Leu-Ile-Arg (HAL-1) and Gly-Lys-Trp-Met-Ser-Leu-Leu-Lys-His-Ile-Leu-Lys (HAL-2). The deconvoluted masses signify that these two peptides are C-terminally amidated. The peptide composition was further confirmed from accurate masses obtained on the LTQ Orbitrap XL. The electrospray spectra were deconvoluted using the Xtract utility giving masses of singly charged peptides (HAL-1: 1,409.8129, -4.9 ppm for $C_{65}H_{109}O_{13}N_{20}S$; HAL-2: 1,452.8890, 1.3 ppm for $C_{69}H_{118}O_{13}N_{19}S$). In addition, the peptide fragments obtained by α -chymotrypsin digestion were consistent with determined sequence.

Peptide synthesis

Halictines and their analogs were prepared by standard peptide coupling chemistry using bromphenol blue indicator for monitoring the conversion of free amino groups during coupling. Final products were obtained by preparative RP-HPLC purification to provide analytical HPLC purity ranging from 96 to 99%. The retention times for the purified peptides and the results of MS analyses confirming their identities are provided in Tables 1 and 2.

CD analyses and structural features

In water, halictines exhibit CD spectra characteristic of an unstructured peptide with a broad minimum at 200 nm containing approximately 14% (HAL-1) and 10% (HAL-2) of α -helix (Table 3). The CD spectra undergo a considerable change upon increasing the concentration of TFE, demonstrating a predominantly α -helical structure as indicated by the appearance of typical bands at 207 and 221 nm. The spectra (Fig. 2a, HAL-1) at 30, 40, and 50% TFE show a maximum α -helical content of 38% for HAL-1 and 40% for HAL-2, at 40% TFE (Table 3). A similar formation of α -helical structure was observed in the anisotropic environment of SDS micelles (spectra not shown), reaching maximal α -helical fractions of 51% (HAL-1) and 48% (HAL-2) at 4 and 2 mM SDS concentrations, respectively (Table 3). CD spectra of the structurally related HAL-1/10 display practically the same profile as those of HAL-1, indicating more pronounced propensity to form an α -helix (Table 3, spectra not shown). This is due to the exchange of Ser4 by the α -helix promoting Lys residue. As expected, the substitution of D-Leu for Leu7 restrained the α -helix formation of the HAL-1/7 analog in the presence of TFE (Fig. 2b) but not in the presence of SDS (spectra not shown). However, the substitution of *N*-methyleucine (MeL) for Leu7 totally prevented HAL-1/28 from forming an α -helix either in the presence of TFE (Fig. 2c) or of SDS. Surprisingly, the replacement of Trp3 in HAL-2 by D-Trp resulted in noticeably good propensity for the HAL-2/8 analog to form an α -helix in the anisotropic environment of the SDS micelles as well as in the presence of TFE (Fig. 2d). On the other hand, the spectra (not shown) of HAL-2/7 clearly reflect the presence of the strong helix breaker β -alanine (β A) in the position 6.

NMR spectroscopy

The resonance assignment of proton signals in peptides HAL-1 and HAL-2 was achieved by the standard sequential resonance assignment strategy (Billeter et al. 1982) using 2D-COSY, 2D-TOCSY, and 2D-NOESY spectra. The spectra collected under different temperatures allowed unambiguous resonance assignment in case of an overlap of amide proton signals and determination of temperature coefficients of amide NH protons. The NMR spectra confirmed the structure and purity of peptides.

Secondary structure

The observed inter-residual NOEs, $^3J(NH, \alpha H)$ coupling constants, chemical shift index values (CSI), and temperature

Table 1 Amino acid sequences, MS data, physical properties, and biological activities of HAL-1 and its analogs

Acronym	Sequence	Monoisotopic molecular mass (Da)		Charge	t_R (min)	μ_H	H	Antimicrobial activity MIC (μM)				Hemolytic activity LC ₅₀ (μM)
		Calculated	Found					<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	
HAL-1	GMWSKILGHLIR-NH ₂	1,408.8	1,408.8	+3	32.24	0.380	−0.004	0.8	7.7	3.8	45.0	82
HAL-1/22	GMWSKILGHLIR-NH ₂	1,408.8	1,408.9	+3	32.14	0.380	−0.004	0.8	9.1	3.3	36.7	52
HAL-1/24	MWSKILGHLIR-NH ₂	1,351.8	1,351.6	+3	27.84	0.425	−0.019	5.0	45.0	50.0	>100	>200
HAL-1/25	WSKILGHLIR-NH ₂	1,220.8	1,220.6	+3	25.03	0.475	−0.047	18.3	>100	>100	>100	>200
HAL-1/26	GMWSKILGHLI-NH ₂	1,252.7	1,252.6	+2	33.46	0.254	0.155	3.5	15.5	7.0	100	122
HAL-1/27	GMWSKILGHL-NH ₂	1,139.6	1,139.7	+2	29.37	0.260	0.098	22.5	>100	>100	>100	>200
HAL-1/6	GMWSKILGHLIK-NH ₂	1,380.8	1,380.6	+3	31.71	0.323	0.051	1.3	15.8	7.2	65.0	132
HAL-1/4	GMWSKILGHLKR-NH ₂	1,423.8	1,423.6	+4	27.41	0.361	−0.157	2.2	100	28.3	93.0	>200
HAL-1/9	GMWSKILGKLIR-NH ₂	1,399.9	1,400.0	+4	32.70	0.416	−0.063	0.9	9.2	3.7	26.3	143
HAL-1/18	GMWSKILKHLIR-NH ₂	1,479.9	1,480.0	+4	31.37	0.455	−0.109	0.9	3.7	2.3	13.5	45
HAL-1/5	GMWKKILGHLIR-NH ₂	1,449.9	1,449.8	+4	29.44	0.386	−0.074	0.9	8.4	1.8	16.3	93
HAL-1/12	GKWSKILGHLIR-NH ₂	1,405.9	1,406.0	+4	26.81	0.426	−0.118	2.0	23.3	3.7	71.7	>200
HAL-1/17	KMWSKILGHLIR-NH ₂	1,479.9	1,480.1	+4	27.81	0.473	−0.109	2.1	41.7	6.3	73.3	>200
HAL-1/10	GMWKKILGKLIR-NH ₂	1,440.9	1,440.1	+5	29.91	0.416	−0.133	0.8	15.0	2.3	13.1	>200
HAL-1/20	GKWSKILGKLIR-NH ₂	1,396.9	1,396.8	+5	27.84	0.473	−0.176	1.7	21.7	2.3	28.3	>200
HAL-1/19	GKWKKILGHLIR-NH ₂	1,446.9	1,446.9	+5	25.10	0.416	−0.188	1.2	28.3	2.7	30.0	>200
HAL-1/21	GKWKKILGKLIR-NH ₂	1,438.0	1,438.0	+6	25.60	0.458	−0.246	1.3	60.0	1.8	14.2	>200
HAL-1/2	GMWSKILGPLIR-NH ₂	1,368.8	1,368.9	+3	31.37	0.361	0.023	3.6	>100	30.0	>100	>200
HAL-1/1	GMWSKILPHLIR-NH ₂	1,448.8	1,448.6	+3	28.97	0.391	−0.023	3.3	>100	20.0	>100	>200
HAL-1/3	GMWSKIPGHLIR-NH ₂	1,392.8	1,392.6	+3	23.43	0.336	−0.054	>100	>100	25.0	>100	>200
HAL-1/11	GMWPKILGHLIR-NH ₂	1,418.8	1,418.8	+3	30.17	0.377	0.012	3.0	75.0	33.3	>100	>200
HAL-1/28	GMWSKIMeLGHLIR-NH ₂	1,422.8	1,422.6	+3	26.89	–	–	70.0	>100	>100	>100	>200
HAL-1/7	GMWSKILGHLIR-NH ₂	1,408.8	1,408.7	+3	29.23	0.380	−0.004	2.4	36.2	20.3	>100	>200
HAL-1/8	GMWSKILGHLIR-NH ₂	1,408.8	1,409.0	+3	28.96	0.380	−0.004	11.9	>100	>100	>100	>200
HAL-1/29	GMWSKILGHLIR-NH ₂	1,408.8	1,408.6	+3	28.98	0.380	−0.004	5.3	>100	46.7	>100	>200
HAL-1/15	GMWSKLLGHLIR-NH ₂	1,408.8	1,408.8	+3	33.70	0.366	−0.038	1.3	7.7	3.0	20.2	62
Anoplin	GLLKRIKTLL-NH ₂	1,152.8	1,152.9	+4	29.78	0.336	−0.113	5.0	>100	20	40	>200
Indolicidin	ILPWKWPWPWRR-NH ₂	1,905.1	1,905.2	+4	34.16		−0.132	1.0	70	13.3	>100	>200
Tetracycline								7.3	0.4	1.1	55	>200

Bold letters show replaced amino acid residues

coefficients of NH proton chemical shifts ($\Delta\delta_{NH}/\Delta T$) were used as the indicators of the secondary structure.

NMR spectra of HAL-1 and HAL-2 in water

The selected proton NMR data in water solution at 27°C are summarized in Tables 1 and 2 in the Supplementary Material. Their NOESY spectra contain only intra-residual and sequential NOEs between neighboring residues. The $^3J(NH,\alpha H)$ coupling constants appear in the range of 5.9–8.1 Hz, and chemical shifts of H α protons are very close to the random coil values (Wishart and Nip 1998) ($\Delta\delta_{\alpha H}$ is either slightly positive or slightly negative but in general lower than 0.10 ppm except for being −0.18 ppm for Gly-1 and −0.12 ppm for residue 4 in both peptides and −0.23 ppm for Ser-5 in HAL-2). The observed large $\Delta\delta_{NH}/$

ΔT values (in the range of −6 to −11 ppb, with the exception of His-9 in HAL-1 and residue 4 in both peptides which have values of −1 to −2.2 ppb) are typical for random coil peptides (Merutka et al. 1995). All these observation are characteristic of flexible peptides adopting multiple conformations in water solution. The spectra obtained at lower temperature (5°C) show a small decrease of $^3J(NH,\alpha H)$ (typically by 0.2–0.5 Hz) that can indicate some propensity to form a helical conformation.

NMR spectra of HAL-1 and HAL-2 in 30% TFE solution

The proton NMR data in 30% TFE solution at 27°C are presented in Tables 3 and 4 in the Supplementary Material. The NOESY spectra show—in addition to the intra-residual

Table 2 Amino acid sequences, MS data, physical properties, and biological activities of HAL-2 and its analogs

Acronym	Sequence	Monoisotopic molecular mass (Da)		Charge	<i>t_R</i> (min)	μ _H	<i>H</i>	Antimicrobial activity MIC (μM)				Hemolytic activity LC ₅₀ (μM)
		Calculated	Found					<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	
HAL-2	GKWMSLLKHILK-NH ₂	1,451.9	1,451.7	+4	32.68	0.366	−0.071	1.0	8.1	2.5	42.1	78.1
HAL-2/22	GKWMSLLKHILK-NH ₂	1,451.9	1,451.9	+4	32.72	0.366	−0.071	1.3	11.1	2.6	15.8	54
HAL-2/4	GKWMSLLK K ILK-NH ₂	1,442.9	1,442.8	+5	33.22	0.410	−0.129	1.2	6.3	2.7	13.8	126
HAL-2/3	GKWMS K LKHILK-NH ₂	1,466.9	1,466.8	+5	25.19	0.360	−0.207	3.7	>100	45.0	>100	>>200
HAL-2/2	GKW M LLKHILK-NH ₂	1,492.9	1,492.9	+5	30.61	0.436	−0.141	0.8	4.5	2.5	10.7	87.4
HAL-2/1	GKW K SLLKHILK-NH ₂	1,448.9	1,449.0	+5	27.51	0.370	−0.184	1.2	20.0	3.7	34.2	>200
HAL-2/5	K KWMSLLKHILK-NH ₂	1,523.0	1,523.0	+5	28.57	0.454	−0.176	1.1	28.3	3.0	25.0	133
HAL-2/26	GKWMSLLK H ILK-NH ₂	1,451.9	1,452.1	+4	31.31	0.366	−0.071	1.5	22.5	9.0	62.5	>200
HAL-2/7	GKWMS β ALKHILK-NH ₂	1,409.8	1,409.8	+4	25.47	—	—	11.7	>100	>100	>100	>200
HAL-2/8	GKWMSLLKHILK-NH ₂	1,451.9	1,451.9	+4	32.04	0.366	−0.071	1.7	10.8	3.3	31.7	99
HAL-2/18	GKWMSLLKH I WK-NH ₂	1,524.9	1,525.0	+4	30.39	0.360	−0.084	1.3	12.2	5.0	25.0	87
HAL-2/19	GKWMSLLK H WLK-NH ₂	1,524.9	1,525.1	+4	32.06	0.342	−0.101	1.3	13.8	3.5	25.0	106
HAL-2/20	GKWMSL W KHILK-NH ₂	1,524.9	1,525.1	+4	30.25	0.354	−0.084	1.4	27.0	8.0	32.5	>100
HAL-2/21	GKWMS W LKHILK-NH ₂	1,524.9	1,525.1	+4	32.82	0.363	−0.084	1.5	10.1	3.9	13.5	87
HAL-2/10	GKW W SLLKHILK-NH ₂	1,506.9	1,507.0	+4	33.60	0.367	−0.062	0.6	4.7	1.6	50.0	33
HAL-2/23	GKAMSLLKHILK-NH ₂	1,336.8	1,336.7	+4	28.15	0.356	−0.081	9.3	>100	40.0	>100	>200
HAL-2/24	GKFMSLLKHILK-NH ₂	1,412.9	1,412.7	+4	32.79	0.385	−0.051	1.5	43.3	5.8	30.0	>200
HAL-2/25	GK N aMSLLKHILK-NH ₂	1,463.0	1,463.1	+4	35.31	—	—	1.2	5.5	1.5	12.0	50
HAL-2/14	GKWMSLLK Q ILK-NH ₂	1,442.9	1,442.7	+4	34.53	0.384	−0.095	1.8	10.8	2.5	50.0	110
HAL-2/6	GKWMS F LKHILK-NH ₂	1,485.9	1,485.8	+4	32.54	0.367	−0.064	1.1	6.4	4.0	21.8	69
HAL-2/15	GKWMSAL K HILK-NH ₂	1,409.8	1,409.6	+4	28.46	0.361	−0.094	2.7	130	20.0	100	>200
HAL-2/12	GKW M HLKHILK-NH ₂	1,501.9	1,501.7	+4	28.94	0.378	−0.083	1.1	12.5	2.2	46.7	109
HAL-2/13	GKWMTLLKHILK-NH ₂	1,465.9	1,465.7	+4	32.95	0.359	−0.064	1.3	5.3	2.7	38.3	86
HAL-2/9	GKW Q SLLKHILK-NH ₂	1,448.9	1,448.8	+4	29.97	0.365	−0.150	2.2	25	8.0	56.7	>200
HAL-2/11	GKWLSLLKHILK-NH ₂	1,433.9	1,433.9	+4	33.30	0.369	−0.048	0.9	5.5	1.8	31.7	65
HAL-2/16	S KWMSLLKHILK-NH ₂	1,481.9	1,481.7	+4	32.52	0.394	−0.106	1.1	9.2	2.3	50.0	87
HAL-2/17	H KWMSLLKHILK-NH ₂	1,531.9	1,531.7	+4	29.57	0.404	−0.118	1.5	38.3	3.5	100	>200

Bold letters show replaced amino acid residues

and neighboring sequential NOEs—those NOEs typical for helical conformation (see Fig. 3). Most of the observed $^3J(\text{NH}, \alpha\text{H})$ couplings are lower than 6 Hz and most of the signals of H α protons are significantly shifted upfield, thus indicating the presence of helical conformation. The $\Delta\delta_{\text{NH}}/\Delta T$ values are in general lower than in water solution, but they vary in a rather broad range (+0.8 to −9.4 ppb). Slightly positive or negative values of the $\Delta\delta_{\text{NH}}/\Delta T$, indicating H-bonded amide protons, were observed only for residues 5, 8, 9, and 12 in HAL-1 and residues 2, 9, and 12 in HAL-2.

Viewing the NMR data in detail reveals certain periodical changes of some NMR parameters—chemical shifts of NH protons (δ_{NH}), difference between chemical shifts of NH protons in 30% TFE and in water ($\Delta\delta_{\text{NH}}$), and temperature coefficients of NH protons chemical shifts ($\Delta\delta_{\text{NH}}/\Delta T$)—along the peptide chain (see Figs. 1 and 2 in the

Supplementary Material). Such periodical changes are described in the literature (Zhou et al. 1992; Kuntz et al. 1991; Wishart and Sykes 1994) for amphipathic helical peptides with curvature of helix due to a shortening of hydrogen bonds on the hydrophobic side and a lengthening of hydrogen bonds on the hydrophilic side (Suh et al. 1996; Zhou et al. 1992). Such type of curved helical conformation extending from residue 4 to residue 11 is obviously present also in 30% TFE solution of our peptides HAL-1 and HAL-2.

Biological activity

We have determined MIC values for synthetic peptides against four bacteria: *B. subtilis*, *E. coli*, *S. aureus*, and *P. aeruginosa*. As standards, we used the antibiotic tetracycline and two peptides described in the literature: anoplin (Konno et al. 2001) and indolicidin (Selsted et al. 1992),

Table 3 Calculated α -helical fractions (f_h) of halictines and selected analogs determined by CD spectroscopy in the presence of varying concentrations of SDS and TFE (Backlund et al. 1994; Rohl and Baldwin 1998)

Peptide	α -Helical fraction (f_h)								
	TFE (%)						SDS (mM)		
	0	10	20	30	40	50	2	4	8
HAL-1	0.14	0.15	0.26	0.32	0.38	0.32	0.46	0.51	0.37
HAL-1/22	0.14	0.13	0.25	0.35	0.31	0.31	0.52	0.37	0.41
HAL-1/10	0.15	0.17	0.27	0.36	0.35	0.41	0.63	0.63	0.50
HAL-1/28	0.09	0.19	0.21	0.19	0.20	0.20	0.24	0.25	0.21
HAL-1/7	0.13	0.16	0.16	0.25	0.26	0.24	0.41	0.46	0.39
HAL-1/8	0.11	0.12	0.13	0.16	0.17	0.17	0.21	0.21	0.21
HAL-1/29	0.12	0.12	0.17	0.18	0.21	0.21	0.39	0.39	0.31
HAL-1/2	0.14	0.16	0.21	0.26	0.30	0.28	0.44	0.37	0.37
HAL-1/1	0.11	0.12	0.16	0.21	0.24	0.22	0.27	0.27	0.31
HAL-1/3	0.13	0.15	0.17	0.16	0.18	0.19	0.21	0.18	0.17
HAL-1/11	0.12	0.14	0.17	0.21	0.24	0.24	0.43	0.18	0.18
HAL-2	0.10	0.13	0.27	0.37	0.40	0.36	0.48	0.47	0.43
HAL-2/22	0.11	0.14	0.31	0.39	0.39	0.41	0.57	0.48	0.52
HAL-2/7	0.10	0.11	0.12	0.20	0.22	0.22	0.45	0.38	0.37
HAL-2/8	0.14	0.14	0.26	0.31	0.36	0.33	0.56	0.57	0.55

synthesized in our laboratory. All the values are given in Tables 1 and 2.

Biological activities of halictines, their all D-isomers and truncated analogs

Both HAL-1 and HAL-2 show very comparably high antimicrobial potency against the two Gram-positive and two Gram-negative bacteria (Tables 1 and 2), with the Gram-negative bacterium *P. aeruginosa* being the most resistant. They also display noticeable hemolytic activity against rat red blood cells (Tables 1 and 2) and human red blood cells (102 μ M, HAL-1; 91 μ M, HAL-2) that would not be acceptable for their consideration in therapeutic applications. CD spectra indicate (Fig. 2) that both peptides have a similar tendency to adopt an amphipathic α -helical secondary structure in the anisotropic environment, even though the NMR study indicates that HAL-1 shows slightly better propensity to form α -helix than HAL-2. Both peptides show very similar values of hydrophobicity and hydrophobic moment (Tables 1 and 2), and they can adopt an “ideal” amphipathic helix that has six hydrophilic residues on one side and six hydrophobic residues on the opposite side of the helix (Fig. 4). HAL-2, however, has one more net positive charge than does HAL-1, and the angle ($\Phi = 20^\circ$) subtended by the polar Lys5 and Arg12 residues in HAL-1 is markedly lower than the angle ($\Phi = 120^\circ$) subtended by Lys2 and Lys8 of HAL-2.

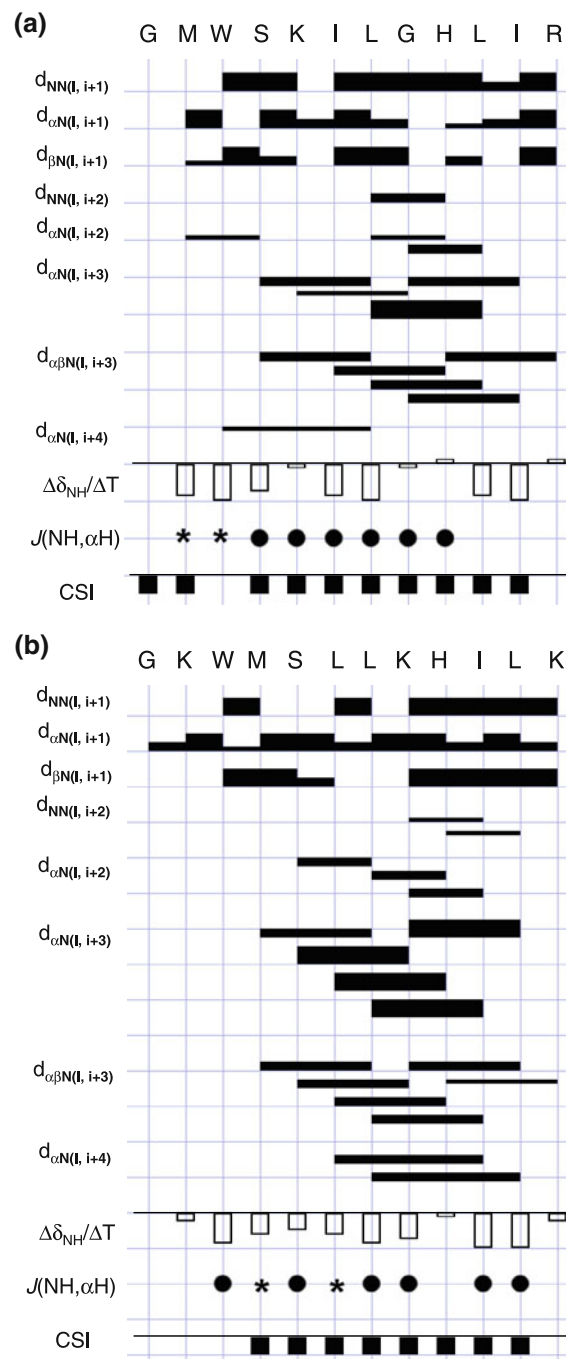


Fig. 3 Secondary structure determination of HAL-1 (a) and HAL-2 (b) in 30% TFE. The intensities of NOE cross-peaks are indicated by the thickness of the lines. The open boxes indicate relative sizes of temperature coefficients $\Delta\delta_{NH}/\Delta T$. The filled circles are drawn when $J(NH, \alpha H) < 6$ Hz; undetermined $J(NH, \alpha H)$ are shown with an asterisk. The filled squares above and below the horizontal line represent CSI values of +1 and -1, respectively

Nevertheless, these two peptides with different physico-chemical parameters exhibit basically the same biological activities. Amphipathic properties and the entire hydrophobicity of these molecules probably play decisive role in

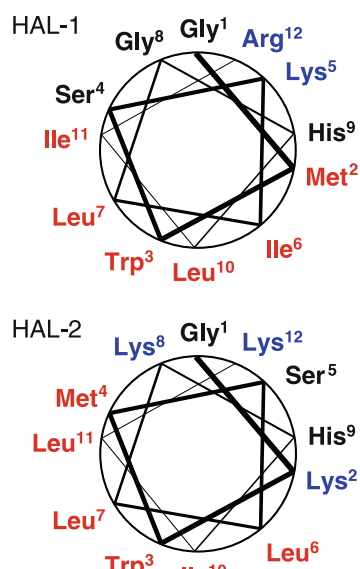


Fig. 4 Wheel diagram of halictines. Sector of hydrophobic amino acids is shown in red. The hydrophilic amino acid sector containing basic amino acid residues Lys and Arg (in blue) is shown in black and blue

the interactions with cell membrane. A slight increase in antimicrobial potency of all D-analogs (HAL-1/22, HAL-2/22) against *P. aeruginosa* may reflect their stability against proteases produced by this bacterium. Truncation of halictines from both termini by one residue resulted in sharp decrease of biological activities.

Biological activities of the analogs

Effect of the net positive charge

To evaluate the effect of increasing cationicity on biological activity while maintaining amphipathic character, we prepared a series of HAL-1 analogs (Table 1) in which one, two or three amino acid residues in the hydrophilic segment of the α -helix, including Met2 and Ile11 situated on the perimeter of the hydrophobic segment, were substituted by Lys (Fig. 4). Generally, the single replacement of the amino acid residues by Lys in the hydrophilic segment of α -helix slightly decreased hemolytic activity but produced no increase of antimicrobial potency with exceptions of three analogs (HAL-1/5, HAL-1/18, and HAL-1/9), which showed enhanced potency against Gram-negative bacteria, especially *P. aeruginosa*. Other substitutions by single Lys made in hydrophilic segment mostly rendered in parallel decrease of both activities. The double replacement of amino acid residues by Lys, combining the improved properties of HAL-1/5 and HAL-1/9 analogs, produced the HAL-1/10 analog with enhanced activity against

P. aeruginosa and substantial reduction of hemolytic activity. A triple substitution of amino acid residues (Met2, Ser4 and His9) by Lys produced a highly cationic (net charge +6) analog (HAL-1/21) with high potency against *P. aeruginosa* and negligible hemolysis.

The replacement of amino acid residues in the hydrophilic segment of the α -helix of HAL-2 (Gly1, Ser5, His9 including Met4 situated on one of the perimeters of the hydrophobic segment) by Lys produced analogs (HAL-2/5, HAL-2/2, HAL-2/4 and HAL-2/1, respectively) with slightly increased potency against *P. aeruginosa* and improved hemolytic properties. The two most potent analogs were those obtained by substitution of Ser5 and His9. On the other hand, the replacement of Leu6 situated on the opposite perimeter of the hydrophobic segment by Lys (HAL-2/3) substantially decreased the antimicrobial as well as hemolytic activity.

Effect of α -helical structure disruption

It has been described in the literature that increasing the helicity of α -helical AMPs results in an increase of hemolytic activity (Cornut et al. 1994; Conlon et al. 2007). Since halictines are noticeably hemolytic against rat blood cells, we synthesized several analogs in an effort to eliminate the hemolytic activity by disrupting the helical structure. This was achieved by substitution of different residues in the sequence of HAL-1 by the helix-breakers proline and *N*-methylleucine (MeL) or by replacing the centrally positioned hydrophobic residues in the sequence with their D-enantiomers. All these changes resulted in significant decrease of hemolytic activity, however, accompanied by undesirable decrease of antimicrobial potency.

In HAL-2, the substitution of Trp3 by enantiomeric D-Trp (HAL-2/8) caused practically no changes in antimicrobial as well as hemolytic properties. This correlates well with the results of the CD study, which surprisingly showed that this change in position 3 did not affect the propensity of the peptide to form α -helical structure in an anisotropic environment (Fig. 2d). Replacing His9 by D-His (HAL-2/26) already showed a slight decrease of biological activities. The incorporation of β -alanine into the central part of the molecule (position 6) generated practically inactive HAL-2/7 analog.

Effect of hydrophobicity

To evaluate the effect of hydrophobicity on biological activity, we designed several analogs of HAL-2 series with enhanced or decreased hydrophobicity while maintaining net positive charge and amphipathic character of the peptide. Replacing each residue of the hydrophobic segment of

the α -helix by Trp produced five analogs (HAL-2/10, HAL-2/21, HAL-2/20, HAL-2/19, HAL-2/18) with decreased hydrophobicity compared with HAL-2 (Table 2) which retained antimicrobial as well as hemolytic activity roughly comparable with those of the parent peptide. Only the analog containing the Trp modification in position 6 (HAL-2/21) showed noticeably increased potency against *P. aeruginosa* and unchanged hemolytic activity. Replacing the original Trp at position 3 by more hydrophobic and bulky L-3-(1-naphthyl)alanine (1Nal) resulted in the HAL-2/25 analog having increased (3.5-fold) potency against *P. aeruginosa* while other activities were unchanged. The replacement of Trp3 by the less bulky but hydrophobic Phe residue (HAL-2/24) improved the hemolytic activity but deteriorated the antimicrobial activity against *S. aureus*. Decreasing peptide hydrophobicity by replacing Trp with Ala (HAL-2/23) resulted in a significant decrease of potency against all bacteria tested.

As shown in Table 2, modest increase of the hydrophobicity of HAL-2 did not significantly affect the biological activity of the analogs. However, modest reduction of the hydrophobicity decreased hemolytic potency of HAL-2 analogs while the diminution of antimicrobial activity was not so profound. Reducing the hydrophobicity in position 6 (the hydrophobic segment) resulted in large decrease in hemolytic activity, but with a concurrent decrease of antimicrobial activity against all bacteria tested—and especially against *S. aureus* (HAL-2/15). On the other hand, enhancing the hydrophobicity at this position (HAL-2/6) caused a twofold increase in the potency against *P. aeruginosa* with no change of other activities.

Discussion and conclusions

The two newly isolated peptides from the venom of the wild eusocial bee *H. sexcinctus*, which we have named halictines (HAL-1 and HAL-2), show no significant homology to other known antimicrobial peptides categorized in the antimicrobial peptide database <http://aps.unmc.edu/AP/main.php> (Wang et al. 2009) and in Swiss-Prot and GenBank™/EMBL Data Banks (Blast program in Swiss-Prot). Only the N-terminal hexapeptide of HAL-1 possesses an identical sequence with the N-terminal part of amphibian dermaseptin (Wechselberger 1998). Both halictines display comparable antimicrobial activity against each of four bacteria (*B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*) in the μ M range. In addition, they show potent activity against the opportunistic yeast pathogen *C. albicans*, with MIC values of 6–7 μ M and potency to kill several cancer cells (author's unpublished results). Thus, the halictines (HAL-1 and HAL-2) may be considered as new antimicrobial peptides.

When the sequences of halictines are compared with the sequences of other α -helical AMPs obtained from natural sources, some obvious positional conservation in terms of residue types can be observed. In particular, features common to these types of AMPs can be seen in the Gly at position 1, Ser at position 4 (HAL-1), Lys at position 5 (HAL-1), Lys at position 8 (HAL-2), large aliphatic residues at positions 6 and 10, the presence of aromatic residue near the N-terminus, and C-terminal amidation. Generally, the distribution of amino acid residues in the halictine sequences fits very well to the statistical analysis diagram of the residue distribution of α -helical AMPs from natural sources as proposed by Tossi et al. (2000) in his review. Plotting the sequences of halictines in an α -helical wheel projection (Schiffer and Edmundson 1967) reveals two ideal amphipathic α -helices in which all the hydrophilic amino acid residues are situated on one side of the α -helix, whereas the hydrophobic amino acid residues are on the opposite side (Fig. 4). CD spectroscopic measurements of the halictines and some analogs in the presence of TFE or SDS confirmed the presence of a significant amount of α -helical structure.

Comparing antimicrobial and hemolytic activities of halictines and some of their analogs with other AMPs of similar size (the amphipathic α -helical peptide anoplin and the Trp-rich peptide indolicidin) shows that under our testing conditions, halictines exhibit better antimicrobial properties than those of the aforementioned peptides. Those properties are comparable to those of tetracycline, with the exception of how they act against *S. aureus* (Table 1). The therapeutic potential of halictines would, however, be limited due to their noticeable hemolytic activity. Systematic structural modification of these newly discovered naturally occurring AMPs is a further logical step in designing new and therapeutically valuable analogs.

Modification of peptide structure by a selected amino acid residue substitution generally results in the change of several structural parameters affecting biological activity. An increase of peptide cationicity, which is essential for the electrostatic attraction of AMPs to the negatively charged phospholipids of bacterial cell membrane, should in general promote an increase of antimicrobial potency without increasing toxicity against eukaryotic cells that contain in their membranes higher proportions of zwitterionic phospholipids together with uncharged cholesterol (Jiang et al. 2008). The net positive charges of HAL-1 and HAL-2, respectively, are +3 and +4. We anticipated that increasing cationicity by targeted substitution of amino acids in HAL-1 and HAL-2 sequences by Lys might be accompanied by an enhanced propensity of the analogs to form α -helix because Lys is an α -helix inducer. But, as already described in some cases, this combination could cause an undesirable increase of hemolytic activity of the

analogs (Jiang et al. 2008). That, however, was only the case of the HAL-1/18 analog and reflects the considerable difference in the helix-forming propensity between Gly and Lys. The gradual increase in the net positive charge through the incorporation of cationic Lys residues into various positions resulted in reduced antimicrobial activity against *S. aureus* (Table 1), similar to what we previously observed in the cases of other AMPs (Čeřovský et al. 2009). This may reflect a potential defense of resistant *S. aureus* against AMPs induced by a reduced net negative charge of its cell envelope causing the repulsion of strongly cationic HAL analogs (Peschel and Collins 2001) and by its secreted proteolytic activity specific for basic residues of HAL analogs and other AMPs (Sieprawska-Lupa et al. 2004).

It has been postulated that strong hemolytic activity of AMPs correlates with high hydrophobicity, high amphipathicity, and high helicity (Wieprecht et al. 1997; Chen et al. 2005). An attempt to diminish hemolytic activity while preserving antimicrobial activity by incorporating such helix-disturbing components as proline, β -alanine, *N*-methylleucine or *D*-amino acids into the peptide sequence indeed caused a sharp drop of hemolytic activity. This was accompanied, however, by a decrease in antimicrobial activity. Contrary to our results, some examples in the literature indicate that insertion of the Pro residue into the central part of α -helical amphipathic AMPs, which causes a slight bending of the helix, might lead to analogs with improved properties (Suh et al. 1999; Shin et al. 2001). It seems, however, that this design may be valid for longer peptides only.

As several examples in the literature show, the interactions of α -helical peptides with non-polar C-18 stationary phase groups during RP-HPLC are similar to those between AMPs and biological membranes (Bütner et al. 1992; Chen et al. 2007). Although RP-HPLC separates peptides mainly by hydrophobic interactions, the induced peptide secondary structures also influence their retention times. The ability of linear cationic AMPs to structure into a well-defined amphipathic α -helix is a prerequisite for their antimicrobial activity. The hydrophilic face of a peptide interacts with negatively charged membrane, induces the α -helix formation, and then the hydrophobic face of the amphipathic α -helical peptide must be capable of partitioning in the hydrophobic environment of the membrane lipid. It is generally accepted that increasing the hydrophobicity of the nonpolar face of the amphipathic α -helical peptides would increase not only their antimicrobial activity but also their hemolytic activity (Chen et al. 2007). The quantitative measure of peptide amphipathicity is the hydrophobic moment μ_H , which is the vector sum of individual amino acid hydrophobicities (H) normalized to an ideal helix (Pathak et al. 1995). In

this work, the mean hydrophobicity (H) of peptides was calculated by averaging hydrophobicities of each amino acid in the peptide chain on the basis of the Eisenberg consensus scale of hydrophobicity (Eisenberg et al. 1984). The hydrophobic moment (μ_H) was calculated according to the formula given in Pathak et al. (1995). As described for model α -helical peptides, their RP-HPLC retention times can be correlated to hydrophobicity and amphipathicity, and then to antimicrobial activity or hemolytic activity.

In this study, the hemolytic activities of peptides correlate quite well with their calculated hydrophobicities (H) and with their retention times measured by RP-HPLC. Practically all peptides showing weak hemolytic activity ($LC_{50} > 200 \mu M$) eluted with retention time < 30 min while all peptides with retention times > 30 min were more or less hemolytic. The lower retention times of some peptides reflect in addition the influence of α -helical structure distortion. On the other hand, this does not mean that all peptides with retention times < 30 min should exhibit low antimicrobial activity. Several analogs which eluted due to their increased cationicity (note the high hydrophilicity value of Lys in the Eisenberg consensus scale) at lower retention times exhibited appreciable antimicrobial activities. No other clear and significant correlation between retention times, hydrophobic moments and antimicrobial activities was observed within the series of studied peptides. We can see that the problem originates from the discrepancies between the Eisenberg consensus scale—which is an average of several other scales estimated by nonchromatographic methods (partitioning between two immiscible liquid phases)—and the ranking of hydrophobicity values of individual amino acids in peptides based on retention times measured on an RP-HPLC column (Biswas et al. 2003). In this work, for example, the replacement of Ile by Leu, Leu by Phe, Leu by Trp, and Ile by Trp produced six analogs (HAL-1/15, HAL-2/6, HAL-2/18, HAL-2/19, HAL-2/20, HAL-2/21) with retention times indicating the order of hydrophobicity being Leu $>$ Ile $>$ Trp and Leu $>$ Phe, whereas the Eisenberg scale shows the order of hydrophobicity to be Ile $>$ Phe $>$ Leu $>$ Trp. In addition, the determination of antimicrobial activity was carried out at a different pH value (neutral) than was the chromatography, which is conducted at a low pH value where the hydrophilicity of basic amino acid residues is substantially higher.

To conclude, equal changes in the structure of HAL-1 and HAL-2 lead to similar activity changes in both series. Among the potentially most promising analogs in both series are those with increased net positive charge (HAL-1/5, HAL-1/10, HAL-2/2, HAL-2/4), in which the suitable amino acid residues were replaced by Lys. This improvement essentially related to the antimicrobial activity

against *P. aeruginosa* and the hemolytic activity. These analogs might be promising leads for further research.

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