Cupiennin 1d*: the cytolytic activity depends on the hydrophobic N-terminus and is modulated by the polar C-terminus

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Abstract To investigate structural features modulating the biological activity of cupiennin 1 peptides from the spider *Cupiennius salei*, three truncated cupiennin 1d analogs were synthesized. The fact that their growth inhibiting effect on Gram-negative and Gram-positive bacteria, their lytic activity with human red blood cells and their insecticidal effect on *Drosophila melanogaster* correlates with structural properties shows that the hydrophobic N-terminal chain segment includes the major determinants of structure and activity. The polar C-terminus seems to modulate peptide accumulation at negatively charged cell surfaces via electrostatic interactions and has no important effect on the peptides' amphipathic secondary structure.

Key words: Antimicrobial peptide; Bactericidal activity; Cupiennin 1; Hemolysis; Insecticidal activity; *Cupiennius salei*

1. Introduction

Cytolytic peptides [1] are an important component of the innate immune system of invertebrates [2] and vertebrates [3] and play a defensive and/or offensive role against external aggressors. In the hemolymph of arthropods various antimicrobial peptides have been identified as responses to microbial invasion [2,4–6]. In the venom glands of some insects [7–13], scorpions [14–19] and spiders [20–22] structurally different cytolytic peptides are exclusively and constitutively available. These polycationic α-helical peptides differ considerably in their amino acids sequences and molecular masses. They all contain no disulfide bridges and are linear peptides composed of 10 to 48 amino acid residues and exhibit different cytolytic activities to bacteria, protozoa, fungi and erythrocytes. Some peptides cause mast cell [9,10,13,18] and granulocytes degranulation [16]. An additional insecticidal activity has been reported for ponericins from the ant *Pachycondyla goeldii* [12], oxyopinins and cupiennins from spider venom [21,22].

The only structural exception concerns scorpine from the venom of *Pandinus imperator* which is a defensin-like antimicrobial peptide composed of 75 amino acid residues and three disulfide bridges [15].

Recently, a new family of highly cationic, antimicrobial peptides has been purified from the venom of the subtropical wandering spider *Cupiennius salei* (Ctenidae) [22]. The cupiennin 1 family is constituted by four peptides. The C-terminally amidated peptides with minor differences in the amino acid composition consist of 35 residues. In addition to high antibacterial activity the peptides are insecticidal and hemolytic at micromolar concentrations. The rather unique amphipathic motif of the potentially helical cupiennin 1 peptides is generated by a ribbon of charged residues which forms a right handed spiral on the helix surface. This remarkable charge distribution starting with Lys7 and ending with Lys32 raises the question of the functional role of the N- and C-terminal chain regions of the cupiennins.

Sequence modification of antimicrobial peptides have been shown to result in distinct structural modifications and pronounced changes in activity and selectivity [23,24]. Studies of truncated endogenous peptides have identified structural determinants and regions with antimicrobial or hemolytic activity that are much shorter than the parent peptide [25].

In this study we analyzed the role of N- and C-terminal segments of cupiennin 1d* (cupiennin*: synthesized cupiennin) for their antimicrobial, hemolytic and insecticidal activity as well as for their peptide structure. Our working hypothesis was that the terminal regions might influence helix formation and modify peptide interaction with different membrane types and thus be the crucial factor in the activity spectrum of the cupiennins.

2. Materials and methods

2.1. Materials

Trifluoroethanol (TFE) and acetonitrile (LiChrosolv®, for chromatography) were purchased from Merck. 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl phosphatidyl-DL-glycer-

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Abbreviations: CD, circular dichroism; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethoxy-carbonyl; *H*, hydrophobicity; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl phosphatidyl-DL-glycerol; SUVs, small unilamellar vesicles; TFE, trifluoroethanol; cupiennin*, synthesized cupiennin

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ol (POPG) were purchased from Avanti Polar Lipids, Inc., Alabaster, AL, USA. 9-Fluorenylmethoxy-carbonyl (Fmoc) amino acids were obtained from Novabiochem (Bad Soden, Germany). All other chemicals were of analytical or reagent grade.

2.2. Isolation of cupiennin 1a and peptide synthesis

Venom of *C. salei* was obtained by means of electrical stimulation from a permanent breeding line established in our laboratory [26]. Cupiennin 1a was purified using gel filtration, ion-exchange chromatography and reverse-phase high-performance liquid chromatography as described [22]. Solid-phase peptide synthesis was performed using Fmoc chemistry on a Millipore 9050 continuous flow peptide synthesizer (Millipore Corp., Milford, MA, USA). Peak fractions were purified on a semipreparative C_{18} column (10×250 mm, 7 μm, 300Å, Vydac, Holland, MI, USA). Purity of the cupiennins was assessed by quantitative amino acid analysis, N-terminal sequence analysis and electrospray ionization mass spectrometry (ESI-MS; single-stage quadrupole instrument: VG Platform, Micromass, Manchester, UK).

2.3. Biological activity

Escherichia coli ATCC 25 922, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27 853 were cultured in Mueller Hinton broth. The antibacterial activity of the peptides was determined using a microtiter broth dilution assay [27]. The bacterial inoculum prepared from mid log phase cultures was diluted to a final concentration of $1.7–3.8\times10^5$ CFU/ml. Peptides (0.04–100 μM), non-treated growth control and a sterility control were tested in triplicate as described [22].

Peptide (0.08–100 μM) induced hemolysis was determined using fresh human red blood cells (hRBC). Release of hemoglobin was monitored by changes in the absorbance at 541 nm (Jasco,V-550, Japan). The positive control (100% hemolysis) was human erythrocytes in water and the negative control (0% hemolysis) was human erythocytes in phosphate buffered saline as described in [22]. The peptide concentration inducing 50% hemolysis (EC_{50}) was derived from dose response curves using sigmoidal curve fitting software (Graph Pad Prism, 3.0; Graph Pad Software, Inc. USA).

Drosophila melanogaster were used to determine the insecticidal activity of the peptides (1–400 pmol/mg fly). Each of three peptide concentrations was tested on 20 flies and the same number of flies was used as control (injecting 0.05 μl of insect ringer). Calculations of the lethal doses LD_{50} (50% of the test flies died of intoxication 24 h post injection) were performed as mentioned elsewhere [28].

2.4. Circular dichroism (CD) measurements

For CD measurements aliquots of the peptide solutions (prepared in 5 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 7.2 containing 150 mM NaF) were diluted with buffer or TFE or mixed with aliquots of a suspension of small unilamellar vesicles (SUVs) to give a final peptide concentration of 50 μM and the desired solvent composition or lipid to peptide molar ratio (C_l/C_p). SUVs were prepared as described [29]. Measurements were performed in 0.1 cm cells between 195 and 260 nm at room temperature using a J 720 spectrometer (Jasco, Japan). The helicity (error 5%) was calculated from the band intensity at 222 nm according to Chen et al. [30].

3. Results

3.1. Peptide design and structural features

Three cupiennin 1d peptides were synthesized, structurally characterized and compared with cupiennin 1a, the most abundant cupiennin 1 in the venom of *C. salei* [22] (Fig. 1A). Cupiennin 1d* (ESI-MS 3794.65 ± 0.44, theoretically 3795.55 Da) is an analog of the natural cupiennin 1d with Gln at the non-amidated C-terminus instead of the amidated C-terminal Glu. Cupiennin $1d^{*C}$ (ESI-MS 2694.74 ± 0.20, theoretically 2695.25 Da) was generated by deletion of nine amino acid residues at the cupiennin 1d* C-terminus. Cupiennin $1d^{*NC}$ (ESI-MS 2233.11 ± 0.06, theoretically 2233.73 Da) represents the 6–26 central region of cupiennin 1d* lacking the N-terminal penta- and C-terminal nonapeptide.

Elimination of the C-terminal nonapeptide reduced the cationic peptide charge by +2. As a consequence, the angle (Φ) of 220° subtended by the cationic residues in cupiennin 1d* decreases to 160° for the truncated derivatives (Fig. 1B). Simultaneously, the peptide hydrophobicity (H) calculated on the basis of the Eisenberg consensus scale of hydrophobicity for

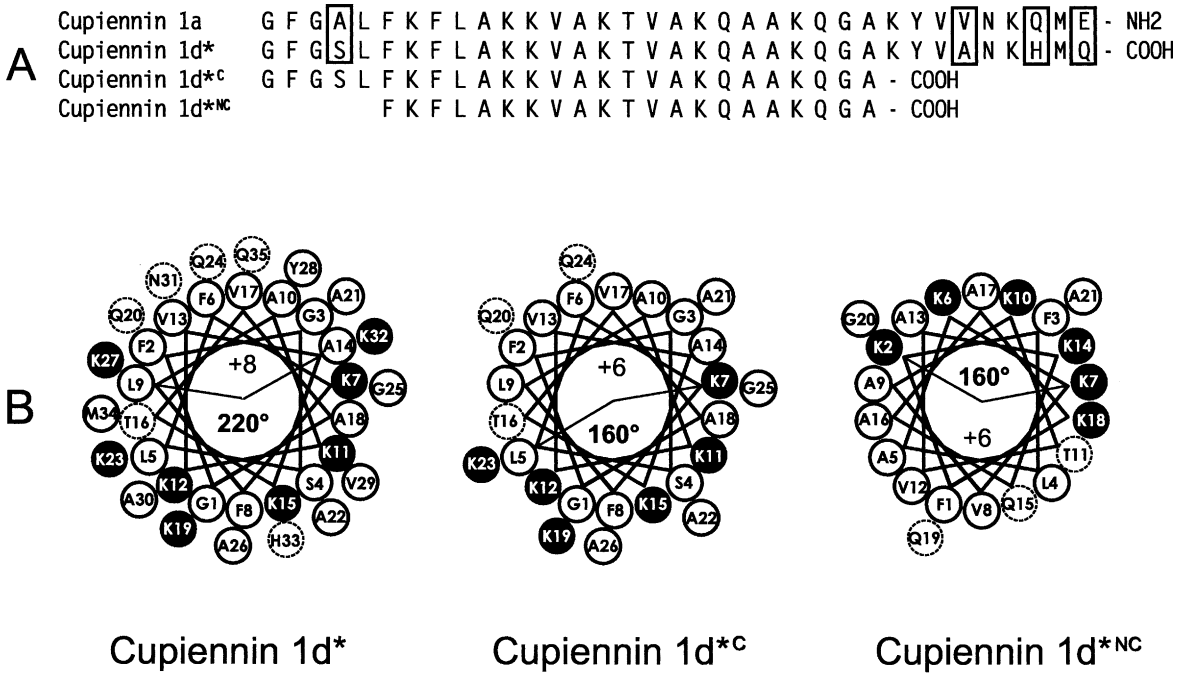

Fig. 1. Amino acid sequence and α-helical wheel projection of cupiennins. A: Comparison of the amino acid sequences of cupiennin 1a, and the synthetic cupiennins 1d*, $1d^{*C}$ and $1d^{*NC}$. Amino acid exchanges between cupiennin 1a and 1d are boxed. B: Helical wheel projection of cupiennin 1d*, $1d^{*C}$ and $1d^{*NC}$. Polar amino acids are marked with dashed circles and lysine residues with black circles. Φ is the denoted angle subtended by the cationic residues and given together with the net charge of the peptides in the center of the wheel.

Table 1
Structural properties of cupiennin 1a, 1d*, 1d*^{C} and 1d*^{NC}

Peptide	Number of residues	Molecular mass (Da)	Net charge[a]	Φ (deg)[b]	H[c]	μ[c]	α (%)[d] (TFE 30%)	α (%)[d] (TFE 50%)	α (%) POPG		α (%) POPC	
									$C_l/C_p = 52$	$C_l/C_p = 260$	$C_l/C_p = 52$	$C_l/C_p = 260$
Cupiennin 1a	35	3798.59	+8	220	−0.138	0.0226	94	100	99	100	5	21
Cupiennin 1d*	35	3795.55	+8	220	−0.152	0.0282	100	100	100	96	5	23
Cupiennin 1d*^{C}	26	2695.25	+6	160	−0.095	0.0738	98	100	87	83	3	26
Cupiennin 1d*^{NC}	21	2233.73	+6	160	−0.175	0.1160	77	84	40	41	0	1

[a]The net charge of the cupiennins was calculated assuming that His is not charged under physiological conditions.
[b]Φ is the angle subtended by the cationic residues on the helix surface of the cupiennins.
[c]The mean hydrophobicity, H, and the hydrophobic moment, μ, were calculated using the Eisenberg consensus scale [31].
[d]The helicity, α, of the cupiennins (50 μM; in phosphate buffer 30% and 50% TFE) was calculated according to [30].

Table 2
Bactericidal, hemolytic and insecticidal activity of cupiennins 1a, 1d*, 1d*^{C} and 1d*^{NC}

Peptide	Bactericidal activity MIC[a] (μM)				Hemolytic activity	Insecticidal activity
	E. coli (ATCC 25922) 3.1×10^5 CFU[b]/ml	*P. aeruginosa* (ATCC 27853) 3.8×10^5 CFU/ml	*S. aureus* (ATCC 29213) 2.6×10^5 CFU/ml	*E. faecalis* (ATCC 29212) 1.7×10^5 CFU/ml	EC_{50}[c] (μM)	LD_{50}[d] (pmol/mg fly)
Cupiennin 1a[e]	0.31–0.63	0.63–1.25	0.63–1.25	2.50–5.00	24.4 (22.9–26.0)	5.9 (4.2–8.3)
Cupiennin 1d*[e]	0.31–0.63	0.31–0.63	0.31–0.63	1.25–2.50	14.5 (14.4–14.7)	7.9 (6.4–9.9)
Cupiennin 1d*^{C}	1.25–2.50	0.63–1.25	10–20	>20	50.4 (49.7–51.1)	30.7 (28.7–33.0)
Cupiennin 1d*^{NC}	>160	>160	>160	>160	>100	>400

[a]The minimal inhibitory concentration (MIC) of cupiennins was determined by a liquid growth assay after 24 h. MIC's are expressed as final concentration intervals [*a*]−[*b*], where [*a*] is the highest concentration at which bacteria are growing and [*b*] is the lowest concentration causing 100% growth inhibition.
[b]CFU, colony forming units.
[c]Hemolytic activity (EC_{50}) was determined as the percentage of released hemoglobin spectroscopically measured at 541 nm after 1 h incubation at 37°C.
[d]Insecticidal activity (LD_{50}) was checked in a bioassay on *D. melanogaster* where peptides were dissolved in insect ringer and 0.05 μl injected into the flies.
[e]Data from [22].

the individual amino acid residues [31] increased from −0.152 for cupiennin 1d* to −0.095 for cupiennin 1d*^{C}. Conversely, elimination of the more hydrophobic N-terminal part reduced H of cupiennin 1d*^{NC} to −0.175. The mean hydrophobic moment, μ, follows the order: 1d*^{NC} (0.116) > 1d*^{C} (0.0738) > 1d* (0.0282) (Table 1).

3.2. Biological activity

The activities of cupiennin 1a and 1d* were similar. Compared to cupiennin 1d*, the antimicrobial activity of cupiennin 1d*^{C} decreased by one dilution step for *P. aeruginosa* (0.63–1.25 μM) and by two dilution steps for *E. coli* (1.25–2.50 μM). The susceptibility of the Gram-positive bacteria *S. aureus* (10–20 μM) and *E. faecalis* (> 20 μM) to cupiennin 1d*^{C} differed by three to five dilution steps compared with cupiennin 1d*. Cupiennin 1d*^{NC} was inactive up to the tested concentration of 160 μM (Table 2). The hemolytic activity followed the order: cupiennin 1d* (14.5 μM) > 1d*^{C} (50.4 μM) > 1d*^{NC} (> 100 μM). The insecticidal effects on *D. melanogaster* showed the same pattern. Compared to cupiennin 1d*, the activity of cupiennin 1d*^{C} was reduced by a factor of about four and no activity was found for cupiennin 1d*^{NC} administration below 400 pmol/mg fly (Table 2).

3.3. Conformational studies

CD spectra of the peptides in TFE and in the presence of lipid vesicles were recorded to determine the ability of the peptides to assume a helical conformation and to obtain information about their affinity to lipid bilayer. All sequences are highly flexible in buffer (Fig. 2). Addition of TFE resulted in helix formation. The spectra of cupiennin 1a, 1d* and 1d*^{C} reflect complete helicity while α of 1d*^{NC} was found to reach only 84% in buffer/TFE (1/1, v/v) (Table 1). The helical order: cupiennin 1d* ≥ 1d*^{C} ≥ 1a > 1d*^{NC} correlated with the order derived from a secondary structure prediction method yielding helicities of cupiennin 1d* (71%) > 1d*^{C} (69%) > 1a (54%) > 1d*^{NC} (52%) [32].

All peptides strongly interact with negatively charged POPG vesicles. The lipid bound cupiennin 1a, 1d* and 1d*^{C} revealed very high helicity while cupiennin 1d*^{NC} was at 40% much less helical (Table 1, Fig. 2). The data correlated with the helical propensity. Additionally, the low α of cupiennin 1d*^{NC} has to be associated with the reduced hydrophobicity of the fragment and strong electrostatic interactions with the charged lipid head groups which inhibit peptide interaction with the hydrophobic region of the lipid bilayer [29]. Hydrophobic interactions between peptide side chains and lipid acyl

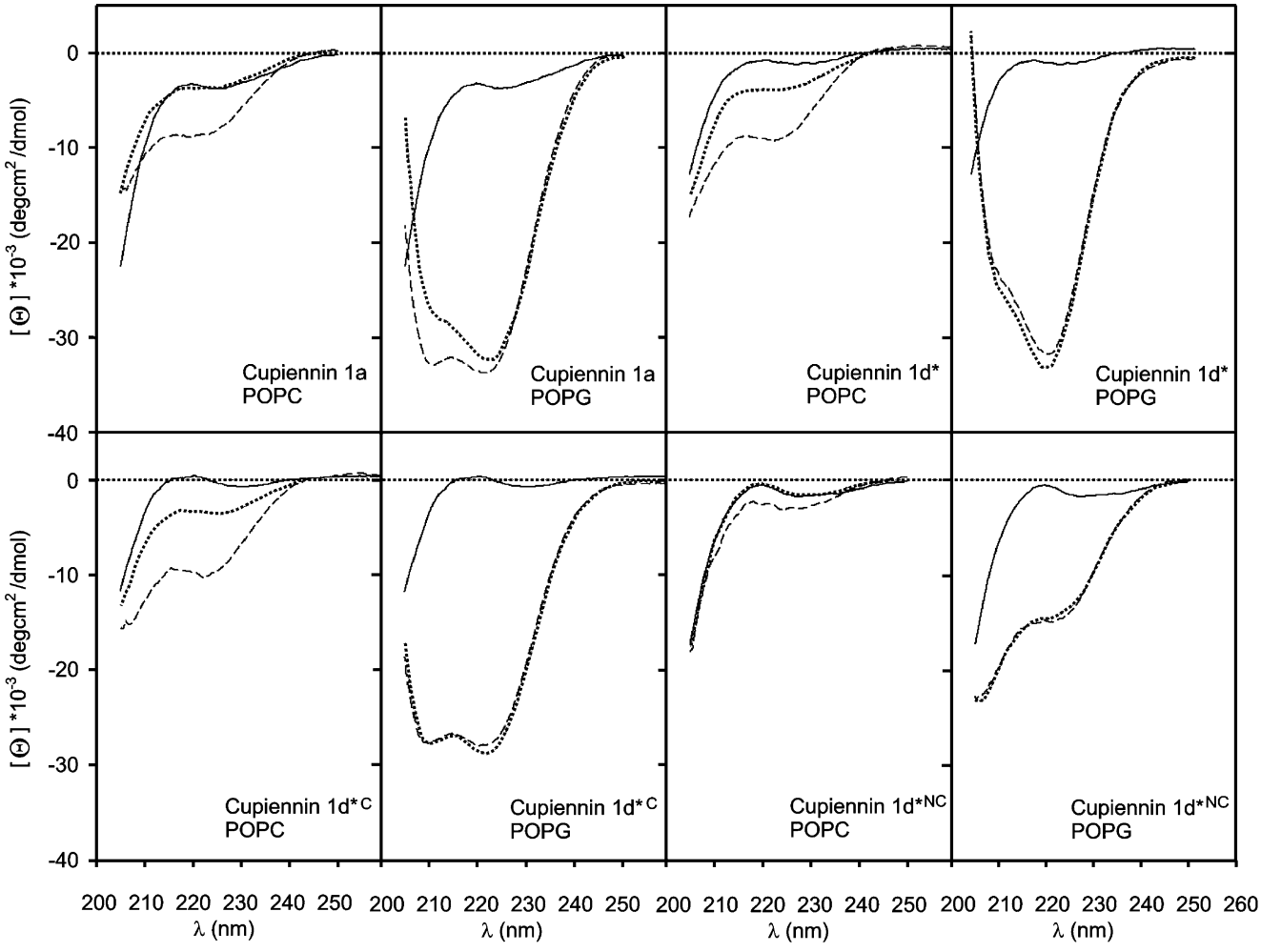

Fig. 2. CD spectra of cupiennin 1a, 1d*, 1d*^{C} and 1d*^{NC} in buffer (solid line) (5 mM sodium phosphate, 150 mM sodium fluoride, pH 7.2) and in the presence of POPC and POPG–SUVs. The lipid concentration (C_l) is 50 μM. The lipid to peptide molar ratio is 52 (dotted line) and 260 (dashed line). Θ is the mean ellipticity per residue.

chains appear to be the primary forces driving structure formation on model membranes [33]. The affinity of cupiennin 1a, 1d* and 1d*^{C} to POPC vesicles is low, therefore electrostatic interactions do not play a role. The slight increase of α with increasing lipid to peptide ratio led to the assumption that even at $C_l/C_p = 260$ only a small amount of peptide was in the vesicle bound state. The least hydrophobic cupiennin 1d*^{NC} did not bind at all under the conditions used (Table 1, Fig. 2).

4. Discussion

Studies of a variety of natural peptides and chemically modified analogs suggest that the two step process of membrane permeation, accumulation of the peptides at the membrane surface and insertion into the lipid layer, depends on the lipid composition of the target cells and is distinctly modulated by the structural properties of the peptides [34–36]. The importance of cationic peptide charge consists in the recognition of negatively charged bacterial membranes and high peptide accumulation on the membrane surface leading to membrane disruption. The effective permeabilization of neutral membranes depends more on a deep penetration of peptides into the hydrophobic membrane interior. Thus, the effect is closely related to peptide hydrophobicity, amphipathicity, quantified by the hydrophobic moment and the size of hydrophobic domains [37,38].

Many antimicrobial peptides occur in the C-terminus amidated form and their lytic activity decreases when the C-terminal amide is removed [39,40]. Our studies show that the exchange of the C-terminal, amidated Glu of cupiennin 1a against Gln in the 1d* analog has minor consequences for this activity. The activities are clearly related to nearly identical structural features: the peptides have the same size, their global charge is identical, H and μ are comparable and peptide helicities are indistinguishable.

All investigated peptides possess high affinity to negatively charged lipid matrices as revealed by CD spectroscopy. Thus, high peptide accumulation on the highly cationic outer membrane of Gram-negative bacteria resulting in an effective self promoted uptake [1] may provide the basis for the pronounced activity of cupiennin 1a, 1d* and 1d*^{C}. Moderate intrinsic hydrophobicity and a non-charged N-terminal segment seem to be responsible for the permeabilization of the inner target membrane after binding via electrostatic interactions. Electrostatic interactions also drive peptide binding to the negatively charged lipid matrix of Gram-positive bacteria. In contrast to Gram-negative bacteria, the effect is more strongly modulated by the global peptide charge. Impaired binding with reduction of charge from +8 to +6 might be one explanation for the more than 10-fold reduced activity of cupiennin 1d*^{C} towards *S. aureus* and *E. faecalis* compared with cupiennin 1d*. Disturbed amphipathicity due to a slightly reduced helicity of the lipid bound peptide (Table 1) may additionally decrease its insertion into the membrane. The low activity of cupiennin 1d*^{NC} towards bacterial strains underlines the important role of the hydrophobic N-terminal pentapeptide for the biological effect. This activity pattern correlates with structural characteristics. N-terminal truncation conferred pronounced hydrophilicity on the remaining fragment, thus reducing the contribution of hydrophobic interactions to activity. Furthermore, helix formation was hampered and the related reduction of the theoretically high hydrophobic moment had an additional activity impairing effect.

The highly cationic peptides also displayed substantial activity towards the neutral lipid matrix of eukaryotic cells, where the effect is determined by hydrophobic peptide membrane interactions. The activity pattern, which was comparable to that towards prokaryotic cells, points to a dominating role of the hydrophobic N-terminal pentapeptide. Its insertion into the lipid matrix generates membrane lysis; its elimination results in drastically reduced activity. Structure–activity studies on cecropin A showed a loss in antibacterial activity by deletion of the first two N-terminal amino acids [41]. Deletion of the three N-terminal amino acids of magainin-2 amide caused a slight decrease of antibacterial activity but removal of the four N-terminal amino acids resulted in a dramatic decrease in activity [42]. Melittin reveals an activity pattern comparable to cupiennins. After reduction of the first seven hydrophobic N-terminal amino acid residues the remaining 8–26 melittin peptide failed to lyse membranes [43,44]. Actually, the nature of the peptides is similar: the N-terminal part of the chain is highly hydrophobic. Surprisingly, despite enhanced H and μ, the hemolytic and insecticidal activities of cupiennin 1d*^{C} were reduced by a factor of about three to four compared to the parent analog. One reason might by a slightly reduced helicity of the lipid bound peptide (Table 1) which results in a disturbed amphipathicity and reduced hydrophobic interactions.

In conclusion, cupiennin 1d* and its truncated analogs were ideal objects for assessing the structural implications of the peptide characteristics and the effects of these on the various peptide activities. These studies show that the major determinant of structure and activity of the cupiennin 1 peptides is located in the hydrophobic N-terminal chain segment. The role of the charged C-terminus consists in modulating peptide accumulation at negatively charged cell surfaces via electrostatic interactions rather than modifying the amphipathic secondary structure of the peptides.

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