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Lucifensin, a Novel Insect Defensin of Medicinal Maggots: Synthesis and Structural Study

Václav Čeřovský,* Jiřina Slaninová, Vladimír Fučík, Lenka Monincová, Lucie Bednárová, Petr Maloň, and Jitka Štokrová[a]

Recently, we identified a new insect defensin, named lucifensin that is secreted/excreted by the blowfly *Lucilia sericata* larvae into a wound as a disinfectant during the medicinal process known as maggot therapy. Here, we report the total chemical synthesis of this peptide of 40 amino acid residues and three intramolecular disulfide bridges by using three different protocols. Oxidative folding of linear peptide yielded a peptide with a pattern of disulfide bridges identical to that of native lucifensin. The synthetic lucifensin was active against Gram-positive bacteria and was not hemolytic. We synthesized three lucifensin analogues that are cyclized through one native disulfide bridge in different positions and having the remaining four cysteines substituted by alanine. Only the analogue cyclized through a Cys16–Cys36 disulfide bridge showed weak antimicrobial activity. Truncating lucifensin at the N-terminal by ten amino acid residues resulted in a drop in antimicrobial activity. Linear lucifensin having all six cysteine residues alkylated was inactive. Circular dichroism spectra measured in the presence of α-helix-promoting compounds showed different patterns for lucifensin and its analogues. Transmission electron microscopy revealed that *Bacillus subtilis* treatment with lucifensin induced significant changes in its envelope.

Introduction

The beneficial use of fly larvae in healing chronic, infected wounds has been known since ancient times,[1] but reintroduction of standard, routine maggot debridement therapy in clinical practice at hundreds of hospitals around the world dates back only to the 1990s and the systematic work of Sherman.[1,2] The action of wound-healing larvae results in removal of necrotic tissue (debridement), elimination of infecting microorganisms, disinfection of the wound, and stimulation of wound granulation and repair.[2,3] The application of green bottle fly larvae (*Lucilia sericata*) has become particularly important in the treatment of non-healing wounds infected with such multidrug-resistant pathogens as methicillin-resistant *Staphylococcus aureus* (MRSA).[4,5] Prompted by successful clinical experience, many researchers have focused on the fundamental healing principles of maggot therapy. A main focus of interest has been to investigate antimicrobial activity of the components of larval secretions and fecal waste products.[5–9] Early research has shown that larval excretions/secretions (ES) of *Lucilia sericata* contain a variety of alkaline components that inhibit bacterial growth and that the pH increase provides optimal conditions for the activity of larvae-secreted proteolytic enzymes that liquidize necrotic tissues.[2,3] It also has been proposed that larvae release antimicrobial substances into the wound as a response to infection. Some of these are bacteriostatic low-molecular-weight compounds that were already characterized some time ago.[2,3,10] In the past decade, several researchers have suggested that antimicrobial peptides originating from the larval immune system might be released into the wound and thus could contribute to wound healing.[5,7,11] These peptides belong to the large group of insect defensins.[12] Defensins of dipteran species are 4 kDa cationic peptides containing three disulfide bridges. They differ from one another by a few amino acid residues and are preferentially active against Gram-positive bacteria.[13–15] Surprisingly, the structural characterization of *Lucilia* defensin had not been reported until 2010, when we purified lucifensin from an extract of larval guts, determined its structure by mass spectrometry and Edman degradation (Figure 1), and then identified it in other larval tissues as well as in the larval ES.[16] Soon after, the primary sequence

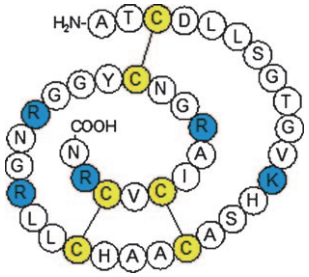

Figure 1. Primary structure of lucifensin. The basic amino acid residues (K, R) that play an important role in its antimicrobial activity are shown in blue; cysteines forming disulfide bridges are shown in yellow.

[a] *Dr. V. Čeřovský, Dr. J. Slaninová, Dr. V. Fučík, L. Monincová, Dr. L. Bednárová, Dr. P. Maloň, Dr. J. Štokrová*
Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic
Flemingovo 2, 166 10 Prague 6 (Czech Republic)
Fax: (+420) 2-2018-3578
E-mail: cerovsky@uochb.cas.cz

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of lucifensin was also published by Andersen and co-workers.[17] They had used molecular biology methods to confirm its sequence. We assume lucifensin to be the key antimicrobial component that protects the maggots when they are exposed to the highly infectious environment of a wound during maggot therapy, and that it contributes as a disinfectant and healing factor. To accelerate progress in lucifensin research, it will be necessary to produce sufficient quantities of lucifensin through chemical synthesis or recombinant methods. In this study, we report total chemical synthesis of lucifensin by using three different protocols employing 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS).

Several reports show that within the family of mammalian defensins, the order of disulfide connectivity or presence of three disulfide bridges is not necessary for the antimicrobial activity.[18,19] Mammalian defensins have been reported to retain antimicrobial activity even without disulfide bridges.[20–22] Inspired by those reports, we studied the importance of disulfide bridges and the importance of the N-terminal part of the lucifensin sequence for antimicrobial activity.

Results

Synthesis of linear lucifensin peptides

In the first approach, the linear precursor of lucifensin was prepared manually by stepwise Fmoc SPPS chemistry by using the *N*,*N*′-diisopropylcarbodiimide (DIPC)/1-hydroxybenzotriazole (HOBt) method in *N*,*N*′-dimethylformamide (DMF). The conversion of free amino groups during the coupling was monitored by bromphenol blue indicator. Because the coupling reaction slowed during growth of the peptide chain, the coupling times had to be extended from several minutes at the beginning of peptide assembly to several hours, thereby reaching completion of the 40-residue peptide within three weeks. This procedure resulted, after deprotection and cleavage from the resin, in a crude peptide showing an analytical HPLC profile dominated by a discrete peak of the required 40-residue peptide within a bunch of closely related impurities (Figure 2A). The determined monoisotopic molecular mass of the linear peptide, 4120.1 Da, was in good agreement with its calculated value of 4119.93 Da (Figure 3A). The N-terminally shortened linear precursor of Luc[des1–10, Ala30] analogue (Figure 4D) was prepared similarly (for HPLC and MS spectrum see Figures S7 and S8 in the Supporting Information). Secondly, to avoid the difficulties associated with sluggish couplings and the large amounts of impurities from Fmoc deprotections, we implemented fragment condensation of the protected N-terminal octapeptide (ATCDLLSG) fragment onto a 32-residue resin-bound peptide. The analytical HPLC profile of the deprotected condensation product still showed the presence of the unreacted 32-peptide (Figure 2B) in addition to the desired peptide. Moreover the presence of deletion peptides indicated their formation even before cycle 32 (Figure 2B). Thirdly, the linear lucifensin as well as the linear precursors of analogues with one disulfide bridge (Figure 4A–C) were prepared by using an automated peptide synthesizer and working with a standard protocol based on the 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorphosphate (HBTU)/HOBt/*N*,*N*-diisopropylethylamine (DIPEA) activation and *N*-methyl-2-pyrrolidone (NMP) as a solvent. Although, the assembly of the 40-residue lucifensin peptide was accomplished within a substantially shorter time (one week), the purity of the final crude peptide (Figure 2C) was not as good as that of the manually synthesized peptide. See also Figures S3—S6 for HPLC and MS spectra of analogues containing one disulfide bridge in Figure 4A–C.

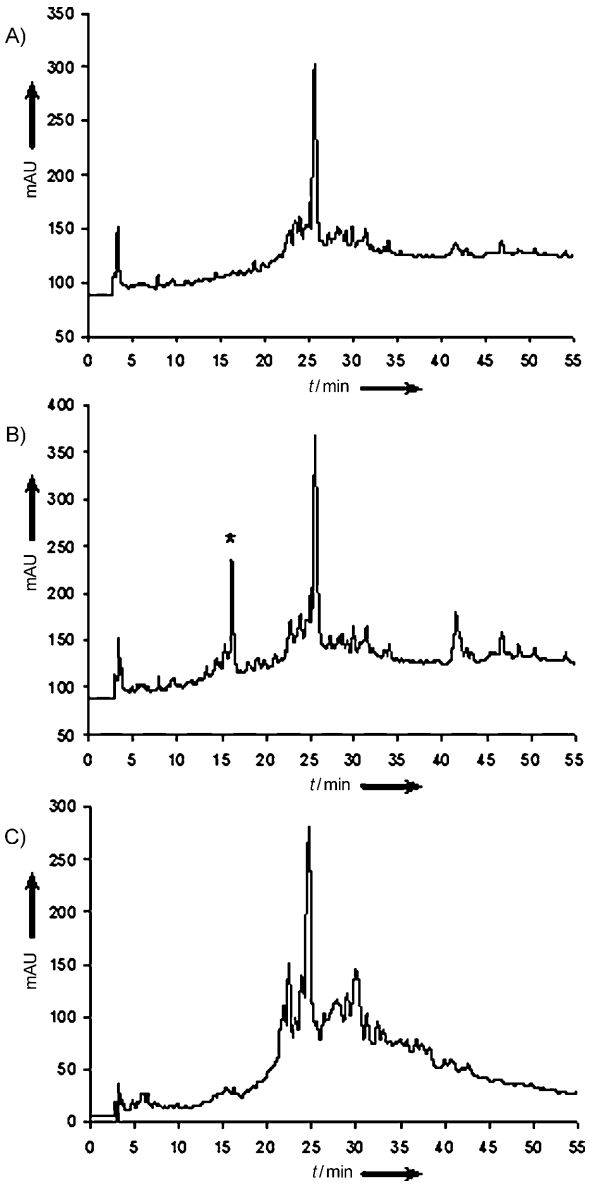

Figure 2. RP-HPLC profiles of the crude linear lucifensin peptide prepared by different SPPS synthetic strategies at 220 nm. A) Manual stepwise synthesis; B) 8 + 32 fragment condensation on the resin; C) Automated peptide synthesis by using Applied Biosystems peptide synthesizer. The dominant peak represents the required peptide of 40 amino acid residues. The peak labeled with an asterisk in panel B represents the unreacted 9–40 peptide fragment. An elution gradient ranging from 80% of solvent A to 50% of solvent B was applied for 60 min at a flow rate 1 mL min^{-1}. Solvent A: 5% MeCN/H_2O/0.1% TFA, solvent B: 70% MeCN/H_2O/0.1% TFA.

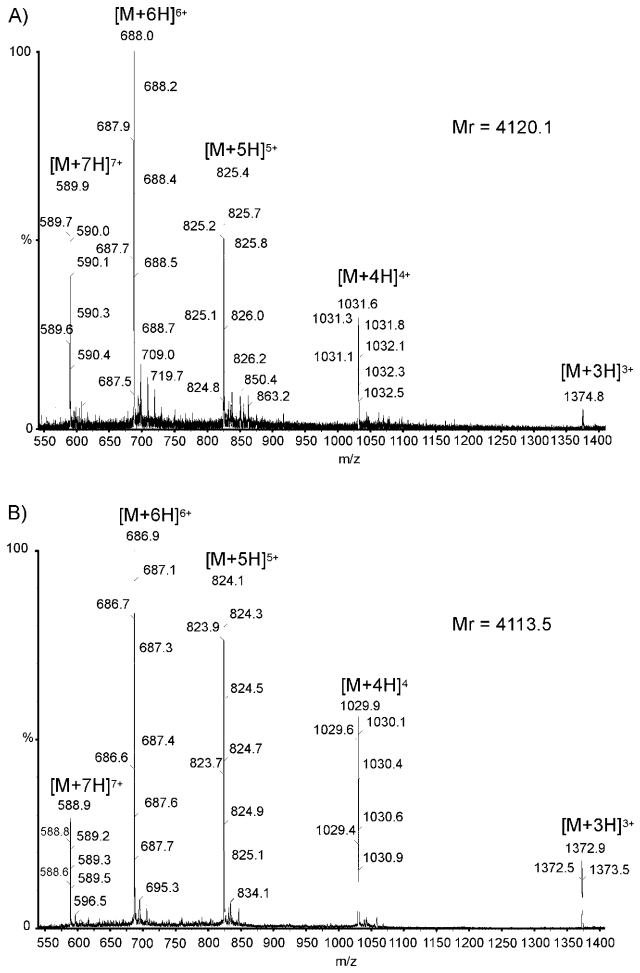

Figure 3. ESI-QTOF mass spectra of A) linear lucifensin and B) lucifensin.

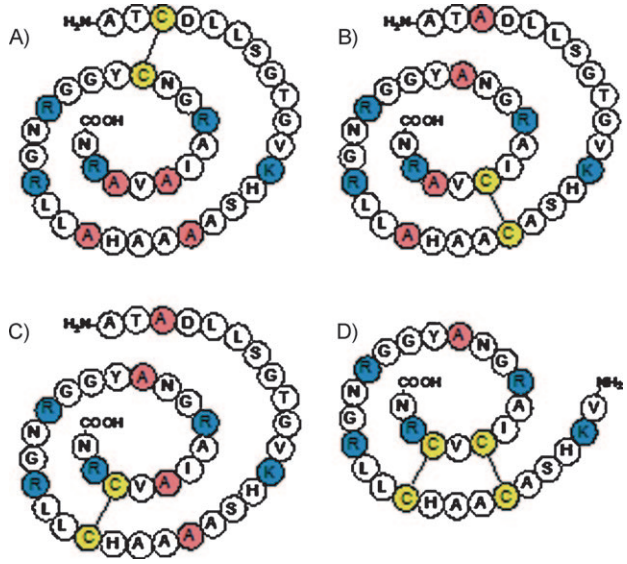

Figure 4. Primary structures of lucifensin analogues. A) Luc[C3–C30]; B) Luc[C16–C36]; C) Luc[C20–C38]; D) Luc[des1-10, Ala30]. Basic amino acid residues (K, R) playing an important role in peptides' antimicrobial activity are shown in blue, cysteines forming disulfide bridges are shown in yellow and their replacements by alanines are shown in red.

Oxidative folding

The time course of lucifensin folding as followed by HPLC is shown in Figure 5. As clearly demonstrated, the conversion of the linear peptide (peak 2) into lucifensin (peak 1) proceeded via an intermediate that eluted at a longer retention time (peak 3). ESI-MS analysis of lucifensin (Figure 3B) as well as the intermediate (not shown) revealed a loss of six mass units compared to the linear peptide (Figure 3A), thus suggesting the formation of three disulfide bridges; in the case of intermediate (peak 3) with incorrect pairing. This intermediate showed activity against *Micrococcus luteus* in a drop–diffusion test almost equal to that of lucifensin (not shown). After lyophilization of the mixture, the folded lucifensin was purified by preparative HPLC. The final product was analyzed by HPLC and ESI-MS (Table 1, and Figures S1 and S2). The determined molecular mass of 4113.5 Da was in good agreement with the calculated mass of 4113.89 Da (Figure 3B). As expected, in the case of the analogues with one disulfide bridge, (for their structures see Figure 4A–C) we did not observe formation of any intermediate during the oxidation of linear peptides into cyclic peptide (MS data of these analogues in Figures S4–S6). The folding of the Luc[des1–10, Ala30] analogue (structure Figure 4D) proceeded similarly to that of lucifensin; that is, via an incorrectly folded intermediate (for MS spectrum of final product see Figure S9).

Determination of disulfide bridges

To determine the connectivity of disulfide bridges, the lucifensin was digested with thermolysin, as described elsewhere.[23] Peptide fragments obtained by the digestion were separated by HPLC and then analyzed by ESI-MS. Among numerous fragments, we clearly identified three peptides containing cysteine (single disulfide bond; Figure 6). Their sequences as well as the sequences of other identified fragments clearly fit to the sequence of lucifensin, which has the expected pattern of disulfide bridges consistent with the conserved pattern of disulfide bridges found in other insect defensins (i.e., Cys1–Cys4, Cys2–Cys5, and Cys3–Cys6).

Alkylation of linear lucifensin peptide

The alkylation of linear lucifensin peptide was performed on a sub-milligram scale in order to obtain the material for the antimicrobial microassay illustrated in Figure 7. The reaction of linear peptide with iodoacetamide resulted in complete alkylation of all six SH groups over one hour (Figures S10 and S11).

Antibacterial activity

The antimicrobial activities of synthetic lucifensin and its analogues were tested preferentially against Gram-positive bacteria, *Micrococcus luteus (M.l.)*, *Bacillus subtilis (B.s.)*, and *Staphylococcus aureus (S.a.)* and one Gram-negative one *Escherichia coli (E.c.)*. Lucifensin was highly active against *M. luteus* and *B. subtilis*, whereas lower but significant activity was observed

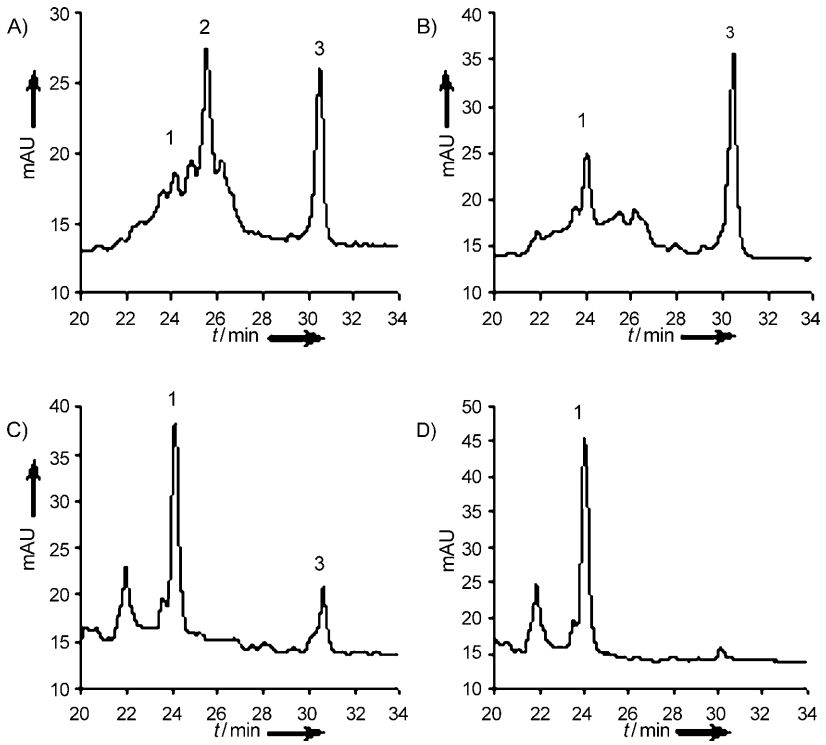

Figure 5. Time course for oxidative folding of lucifensin monitored by RP-HPLC at 220 nm. A) 5 min; B) 1 h; C) 2 h; D) 4 h. Peak 1, lucifensin; peak 2, linear peptide; peak 3, incorrectly folded lucifensin as an intermediate (active against *M. luteus*). An elution gradient ranging from 80% of solvent A to 50% of solvent B was applied for 60 min at flow rate 1 mL min^{-1}. Solvent A: 5% MeCN/H_2O/0.1% TFA, solvent B: 70% MeCN/H_2O/0.1% TFA.

Table 1. Antimicrobial activities of lucifensin and its analogues.

Peptide	Antimicrobial activity MIC [μM]			
	B. subtilis	*M. luteus*	*S. aureus*	*C. albicans*
Lucifensin	1.2	0.6	41	~100
Luc[C3–C30]	>100	>100	n.d.[a]	n.d.
Luc[C16–C36]	>100	23	n.d.	n.d.
Luc[C20–C38]	>100	>100	n.d.	n.d.
Luc[des1-10, Ala30]	>100	10	n.d.	n.d.

[a] Not determined.

against *S. aureus* (Table 1). No activity was detected against *E. coli*, thus confirming the generally recognized fact that insect defensins are more active against Gram-positive than Gram-negative bacteria.[13,15,24,25] The peptide showed slight antifungal activity against *Candida albicans (C.a)*. Lucifensin, having 87–90% sequence identity with other dipteran defensins, such as *Phormia terranovae* defensins A and B[14] and *Sarcophaga peregrina* sapecin,[13] shows a similar profile in antimicrobial activity (pathogenic *S. aureus* is not the most sensitive bacterium among those tested). This was also reported by Andersen et al. in his recent study on lucifensin.[17] As shown in Table 1, the lucifensin analogues that folded through one disulfide bridge were practically inactive even against the most sensitive bacteria, *M. luteus* and *B. subtilis*. The only exception is the Luc[C16–C36] analogue, which still shows noticeable activity as clearly demonstrated by the drop–diffusion assay on a Petri dish (Figure 7A). These results indicate that the presence of disulfide bridges in lucifensin is essential for its antimicrobial activity because it is necessary for preserving its three-dimensional structure. Similar requirements have been reported for other insect defensins.[23,24] This is not valid for the defensins belonging to the mammalian family, however, in which the presence of disulfide bonds is not a prerequisite for antimicrobial activity.[20,21,22] In this context, we tested the antimicrobial activity of linear nonfolded lucifensin and linear lucifensin having the SH group of all Cys residues blocked by alkylation. The results of this test (Figure 7B, C) clearly show that the antimicrobial activity of linear lucifensin (free –SH groups) was almost comparable to that of lucifensin, whereas the alkylated species was completely inactive when tested on two bacteria strains. These results indicate that linear lucifensin was oxidatively folded while carrying out the assay. HPLC analysis of linear lucifensin incubated in LB broth gave multiple peaks, one of which corresponded to lucifensin (not shown).

The Luc[des1–10, Ala30] analogue showed only negligible activity against *M. luteus* in the drop–diffusion assay but displayed noticeable activity when *M. luteus* growth was observed in a microdilution assay (Table 1).

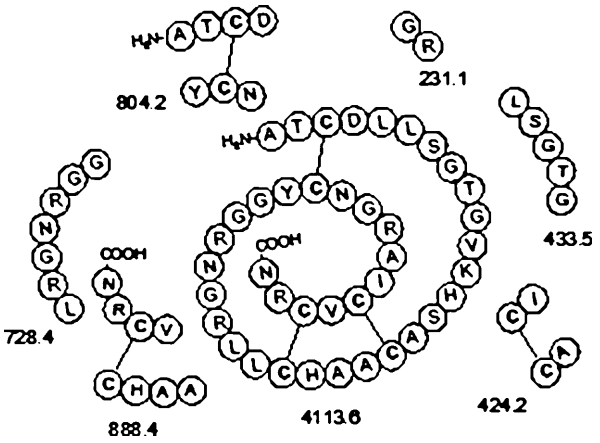

Figure 6. Lucifensin and its peptide fragments identified after thermolysin digestion by MS. The numbers denote calculated molecular masses of peptides that were identical to those obtained experimentally. The existence of three cystine-containing peptide fragments (bold letters) shown in the Figure confirmed the correctness of disulfide pairing.

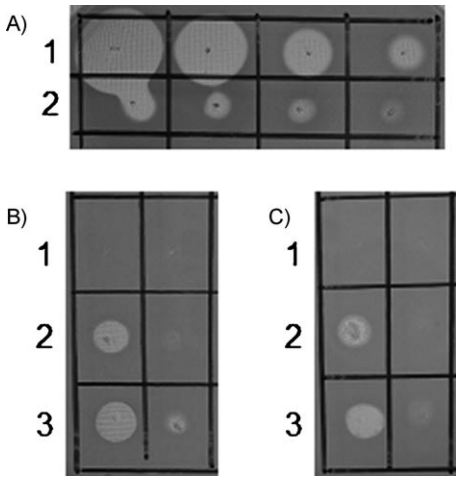

Figure 7. Antimicrobial activities of lucifensin, Luc[C16-C36], linear lucifensin and linear alkylated lucifensin in the drop–diffusion test. A) *Micrococcus luteus*, lane 1: lucifensin in twofold serial dilution starting at concentration 1 mg mL^{-1}; lane 2: Luc[C16-C36] at the same dilution but starting at the concentration 10 mg mL^{-1}. Luc[C3-C30] and Luc[C20-C38] were inactive in this test. B) *Micrococcus luteus*, lane 1: linear lucifensin with all six cysteines alkylated, lane 2: linear lucifensin, lane 3: lucifensin. The concentrations of the peptides were equal; in the right column ten times diluted. C) *Bacillus subtilis*, the same order as in (B).

CD analyses and structural features

UV CD spectra of lucifensin and of its analogues containing one disulfide bridge, Luc[C3–C30], Luc[C16–C36], and Luc[C20–C38], were acquired 1) in water, 2) in the presence of the helix-promoting additive 2,2,2-trifluoroethanol (TFE), and 3) in water containing increasing quantities of SDS as a very simple system intended to mimic a membrane environment. At first glance, the CD spectrum of lucifensin in water is somewhat different from those of its analogues containing only one disulfide bridge (Luc[C3–C30], Luc[C16–C36], and Luc[C20–C38]) (Figure 8A). A qualitative estimate taking into account just the general spectral shape would tempt one to interpret this difference as a slightly different content of random and α-helical conformations.

This approximate picture seems to be supported by the observation that upon addition of helix-supporting additives like TFE (Figure 8B) or SDS (Figure 8C–F) the conformation differences disappear, and all peptides adopt a conformation more resembling an α-helix. However, the absolute intensity of these spectra remains generally less than 15000 deg cm^2 mol^{-1} per unit of residue and thus contradicts such an interpretation. On the contrary, it gives evidence that even in these situations the α-helical content is not very high. We attempted to clarify this picture in at least a semiquantitative way by using a computational analysis of the spectra by following a numerical procedure (Dichroweb[26]). In accordance with our assumptions, the result for lucifensin showed α-helical content of about 15% and random conformation of about 30%, whereas the family of β-structures (parallel, antiparallel and turn; $a_\beta \approx 60\%$) was demonstrated to be the major structural component. Results of the numerical analysis were further supported by the IR spectra of lucifensin, in which the presence of β-structure (1688, 1678, 1641, and 1633 cm^{-1}) in addition to the α-helix and random structure (1658 cm^{-1}) was clearly identified in the amide I spectral region (not shown). The analogues of lucifensin containing one disulfide bridge showed a lower α-helical fraction ($a_H \approx 10\%$) under the same conditions and a higher β-structure fraction ($a_\beta \approx 65\%$) in their spectra. The differences seem to be a consequence of secondary structure stabilization caused by the presence of disulfide bridges in the primary structure of a natural peptide. Surprisingly, in the presence of TFE, lucifensin exhibited almost identical CD spectra as did the analogues containing one disulfide bridge (Figure 8B), with the helix fraction a_H at about 25%. This could mean that all these peptides possess very similar ability to form an α-helical conformation.

On the other hand, in a membrane-mimicking environment with different concentrations of SDS the studied peptides showed distinctly different behavior (Figure 8C–F). Although the peptides were partially α-helical in water, the addition of SDS at a concentration lower than the critical micelle concentration (cmc) changed the conformation of the peptides in favor of the β-structure. Further continuous addition of SDS resulted again in a partial formation of α-helical structure. For the lucifensin itself (Figure 8C), the α-helical fraction increases to its maximum ($a_H \approx 30\%$) already at around the cmc of 2–4 mM. With further increase in the SDS concentration above cmc (16 mM), the α-helical content decreases again, probably in favor of a structure combining polyproline II and β-sheet conformations. Knowing that by solely using CD spectra one cannot reliably demonstrate β-structure and distinguish between unordered and polyproline conformations, we obtained additional information by using infrared spectroscopy. IR spectra of lucifensin with different SDS concentrations below and above cmc were measured. When increasing the concentration of SDS, a similar trend was observed: an increase of α-helical structure was accompanied by a decrease of β-structure and β-turn conformations. The observed spectral changes confirm the formation of β-structure at concentrations of SDS below the cmc and the presence of polyproline structure for SDS concentrations above cmc (16 mM; not shown). The cause of polyproline structure formation was not investigated in detail. The analogues containing one disulfide bridge (Figure 8D–F) behaved similarly, displaying the increase of α-helical content around the cmc. In these cases, however, the relatively high α-helical content also remained above the cmc (16 mM). This indicates that in the absence of constraints imposed by three disulfide bridges, the analogues with one disulfide bridge are more flexible in adopting the α-helical conformation dictated by the environment.

Transmission electron microscopy (TEM)

To analyze the mechanism of lucifensin's action, we used TEM to study the structural changes on bacterial membrane after peptide treatment. *Bacillus subtilis* was used as a model bacterium and treated with lucifensin for 10 or 60 min. Bacterial cells were visualized by negative staining. Untreated bacteria revealed a normal shape with a smooth, electron-dense surface

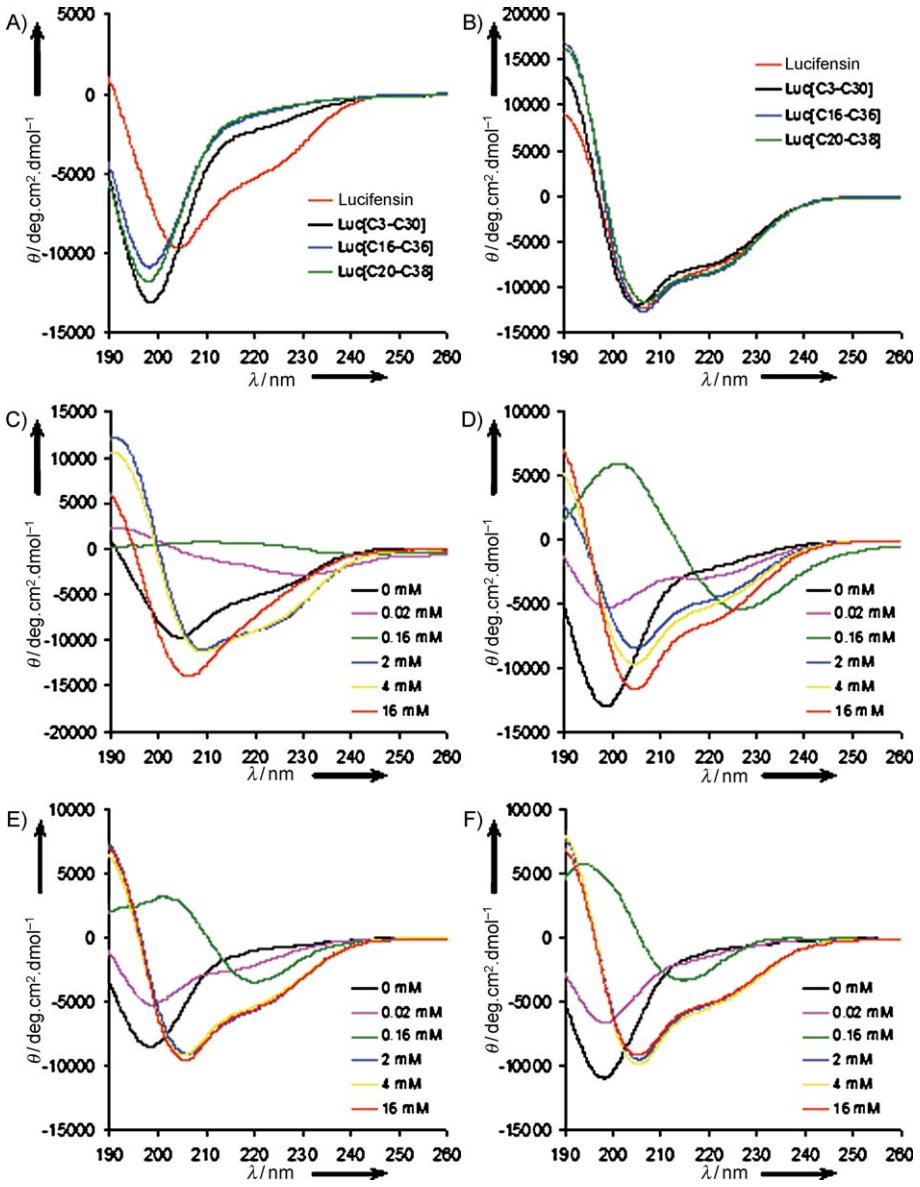

Figure 8. UV CD spectra of lucifensin and its one disulfide bridge containing analogues in A) H_2O, B) in 50% TFE, and in the presence of various concentrations of SDS (C–F). C) lucifensin; D) Luc[C3–C30]; E) Luc[C16–C36]; F) Luc[C20–C38].

and well-preserved membrane structure. The bacterial surface was well-connected with flagella (Figure 9A). After treatment with lucifensin, significant structural changes in the bacterial membrane were observed. After 10 min of treatment (Figure 9B), most of the bacterial cells lost their electron-dense character and the bacterial envelopes, the integrity of which was still preserved, revealed many small pores. The bacterial surface was still connected with numerous flagella. Also, slight leakage of cytoplasmic components was observed. Longer incubation with the peptide (60 min) led to further damage to the bacteria (Figure 9C, D). Even when some bacterial envelopes retained a certain degree of integrity, many more pores, but only rarely flagella, were found on the envelope surface. Extensive ruptures in the cell membrane of many bacteria led to their lysis and leakage of their cytoplasmic content. Distinctive complex circular structures (not shown), sometimes bounded by flagella, were often observed to be released from bacteria.

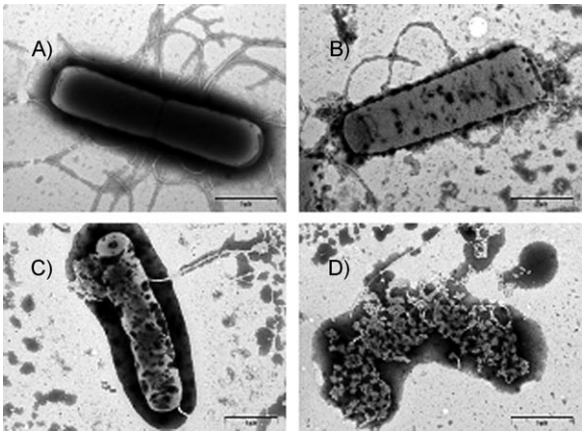

Figure 9. Electron micrographs of negatively stained *Bacillus subtilis* either A) untreated, or B) treated by lucifensin for 10 min or 60 min (C, D). Scale bars represent 1 μm.

Discussion

In 2010, we published the primary structure of lucifensin, the key antimicrobial factor of the *Lucilia sericata* immune system.[16] Later, the production of recombinant peptide resulting in a few milligrams of active peptide was reported by Andersen.[17] Although, numerous synthetic studies in the expanding field of defensins have been published during the last two decades, only a few of these papers report the synthesis of insect defensins.[24,27,28] Despite the generally accepted assumption that the synthesis and purification of a 40-residue peptide can be achieved routinely, the synthesis of a peptide with multiple disulfide bridges is not so straightforward. Challenged by our discovery of lucifensin (Figure 1) and seeing the need to fill a gap as to its structural study, we focused on the total chemical synthesis of lucifensin as well as on the synthesis of its three analogues containing only one native disulfide bond (Fig-

ure 4A–C). We also synthesized an N-terminally shortened analogue with two native disulfide bridges preserved (Figure 4D).

Generally, the presence of intramolecular disulfide bridges in most biologically active peptides is essential for their conformation and biological activity. However, the requirement of the presence of disulfide bridges in cationic antimicrobial peptides for their activity is not fully clarified, particularly within the family of defensins. Some linear analogues of defensins with cysteines substituted by other amino acids, omitted, or side-chain modified have exhibited biological activities similar to those of their native cyclic peptides. Some others, however, have not. For example, several linear analogues of human β-defensins 3 with cysteines substituted by various amino acid residues retained potent antimicrobial activity and exhibited decreased cytotoxicity to human epithelial cells compared to wild-type defensin.[21] Interestingly, reduced synthetic human β-defensin 1 (in the presence of dithiothreitol) was potent against some Gram-positive bacteria and *Candida albicans* whereas its native oxidized form was inactive.[22] Similarly, the synthetic linear human α-defensin HNP1 with six cysteine residues omitted from its sequence also displayed significant antimicrobial activity.[20] However, the linear analogue of HNP1 with all cysteines having side-chain residues protected by an acetamidomethyl group was inactive.[29] On the other hand, few reports show that, in the group of insect defensins which have different connectivity of disulfide bridges (Cys1–Cys4, Cys2–Cys5, Cys3–Cys6) than do mammalian defensins, the presence of disulfide bridges is essential for their antimicrobial activity. For example, the antimicrobial activity of sapecin was reduced by one order of magnitude when its disulfide bonds were cleaved.[23]

We have proven that linear lucifensin with cysteine residues blocked by alkylation was inactive in a drop–diffusion antimicrobial test. The main issue we wished to resolve by our study was to elucidate which of those three disulfide bridges in the lucifensin molecule plays the most important role in maintaining its conformation, reflected in its antimicrobial activity. We expected that the omission of the other two bridges would certainly negatively affect the antimicrobial activity of lucifensin. We supposed that the three-dimensional structure of lucifensin is similar to that of sapecin,[30] because the primary sequence of sapecin (40 residues) differs from the sequence of lucifensin by only four amino acid residues (Ile11, Asn12, Lys33, Val35). The conformation of sapecin was determined by ^{1}H NMR spectroscopy and simulated annealing calculations,[30] resulting in a structure that includes an N-terminal flexible loop (residues 4–12), a helical part (residues 15–23), and an antiparallel β-structure (residues 24–31 and 34–40). Significant antimicrobial activity of the Luc[C16–C36] analogue against the most sensitive bacterium *M. luteus* (Figure 7A) in comparison with the other two analogues containing one disulfide bridge clearly indicates that the disulfide bond between Cys16 and Cys36, which connects the 15–23 helical part of the molecule with the 34–40 strand is the most important one for maintaining the ordered structure of lucifensin. The α-helix and β-structure connected by two disulfide bridges form a common structural element typical for insect defensins known as the cysteine-stabilized αβ (CSαβ) motif and which is important for antimicrobial activity.[30,31]

The percentage of α-helical fraction in lucifensin and its analogues that was calculated from CD spectra (25%) measured in the presence of TFE is in good agreement with the secondary structure of sapecin that was determined from NMR spectroscopic data, just as the presence of β-sheet conformations assumed from lucifensin CD spectra when measured in water or a membrane-mimicking environment.

Slight differences are noticeable in the CD spectra between analogues in which the disulfide bond connects the α-helical part with the 34–40 strand (Luc[C16–C36] and Luc[C20–C38]; Figure 8E, F) versus the Luc[C3–C30] analogue (Figure 8D) in which the disulfide bond connects the terminal of the loop with the 24–31 strand. The CD spectra of the Luc[C3–C30] analogue underwent a slower change of helical formation upon increasing the SDS concentration around the cmc than did those of the other two analogues. This might indicate a supportive role of disulfide bonds 16–36 and 20–38 in maintaining the CSαβ motif, which also involve formation of the 15–23 helical segment.

Based on the three-dimensional model of sapecin, the N-terminal flexible loop (sequence 4–14) is supposed to adopt several orientations with respect to the αβ motif.[30] In lucifensin, the presence of Lys12 within the loop contributes to lucifensin's overall net cationic charge being +4 whereas the sapecin with Asn in position 12 has a net charge of +3. The importance for antimicrobial activity of the N-terminal loop in the molecule of insect defensins has been studied marginally. Chemically synthesized tenecin 1, for example, having a deleted N-terminal loop, did not exhibit antibacterial activity. It was shown that this loop plays an important role in increasing the molecule's α-helical content in a membrane-mimicking environment, which relates to the activity.[24] To follow the role of the lucifensin molecule's N-terminal loop in antibacterial activity, we synthesized an analogue of lucifensin (Luc[des1–10, Ala30]) that was truncated at its N terminus by ten amino acid residues. Because it is generally accepted that the presence of positively charged amino acid residues in antimicrobial peptides is a prerequisite for their activity, we retained the 11–12 part of the loop containing the cationic Lys12 residue in the truncated analogue to preserve its net cationic charge at +4. Briefly, the cationic antimicrobial peptides interact with the anionic phospholipids of bacterial cell membrane, then integrate into the lipid bilayer and disrupt the membrane structure by forming transmembrane pores or ion channels that lead to leakage of cytoplasmic components and ultimately cell death. Although deletion of the N-terminal part of the loop did not affect the net cationic charge of the analogue, its antimicrobial activity decreased. On the basis of NMR spectroscopy experiments, a putative mechanistic model for membrane permeabilization caused by sapecin has been proposed.[32] According to this model, sapecin oligomerizes in the bacterial membrane and thus forms the channels that result in membrane permeabilization. This putative model of sapecin oligomerization is based on electrostatic interaction between Asp4 of one sapecin molecule and Arg23 of another sapecin molecule because

these two residues are situated at opposite ends of the oligomerization site. We suppose that the absence of the Asp4 residue in truncated lucifensin analogue might be the key reason that its antimicrobial activity decreased. Because the putative model of sapecin oligomers provides innovative insights into membrane permeabilization caused by insect defensins, we also speculate that the rigidity of insect defensin molecules represented by the CS$\alpha\beta$ motif and supported by three disulfide bridges is the prerequisite for their oligomerization in microbial membrane. The softening of the lucifensin rigidity by omitting the disulfide bridges (the studied analogues) can make the oligomerization impossible in microbial membrane because the Asp4 and Arg23 residues cannot come into proper contact with one another to provide the necessary electrostatic interaction.

Conclusions

Lucifensin is a key antimicrobial peptide involved in the defense system of *Lucilia sericata* larvae against infection. Its discovery as a crucial disinfectant secreted/excreted by maggots to the wound broadened the understanding of the healing process of maggot therapy. Currently, in collaboration with clinicians, we continue in the detailed study of lucifensin with respect to wound healing. Nevertheless, the determination of lucifensin's three-dimensional structure by NMR spectroscopy, which we plan, is a prerequisite to correlate lucifensin structure and the antimicrobial activity and to understand its mode of action definitively. To continue the study and explore its pharmacological potential, the production of lucifensin in larger quantities either by total chemical synthesis or a recombinant method will be necessary. In this report, we have shown that its production by total chemical synthesis by using SPPS methodology is feasible in principle. The overall yield of the final product might be enhanced by optimizing the procedure or by using alternative synthetic approaches such as native chemical ligation.[33] As a homologue to such other dipteran defensins as sapecin and defensin A, lucifensin has a similar effect on Gram-positive bacteria but it is inactive against Gram-negative bacteria. The presence of three disulfide bridges in the lucifensin molecule is essential for maximal activity and points to the importance of the Cys16–Cys36 bridge as a main supporter of the CS$\alpha\beta$ motif stabilization. Our results also indicate that the order of the disulfide connectivity is not so important for its activity. The antimicrobial activity of lucifensin was dramatically affected by truncation of the N-terminal loop. Lucifensin might find application as a new agent in the treatment of such serious non-healing wounds as diabetic foot ulcers, pressure sores, and varicose ulcers.

Experimental Section

Materials: Fmoc-protected L-amino acids and the Fmoc-L-Asn(Trt)-Wang resin were purchased from IRIS Biotech GmbH (Marktredwitz, Germany). LB (Luria–Bertani) broth, LB agar and thermolysin were bought from Sigma–Aldrich. All other reagents, peptide synthesis solvents, and HPLC-grade MeCN were of the highest purity available from commercial sources.

Instruments: The HPLC 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, California) at a 1 mL min^{-1} flow rate using various solvent gradients ranging from solvent A (5% MeCN/H_2O/0.1% TFA) to solvent B (70% MeCN/H_2O/0.1% TFA) over 60 min. The elution was monitored by absorption at 220, 254, and 280 nm by using a diode-array detector. ChemStation software was used to control the instrument and evaluate UV spectra. Mass spectra (ESI-MS) of the peptides were acquired on a Micromass Q-Tof micro mass spectrometer (Waters) equipped with an electrospray ion source or on LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in the service facility of the institute. Bioscreen C instrument (Oy Growth Curves AB Ltd, Helsinki, Finland) was used for quantitative antimicrobial activity determination.

Peptide synthesis of linear precursor of lucifensin

Method A, Manual stepwise synthesis: The peptide was synthesized on the preloaded Fmoc-Asn(Trt)-Wang resin (100 mg, substitution on the resin: 0.47 mmol g^{-1}) in a 5 mL polypropylene syringe with a Teflon filter on the bottom by using the protocol of N^{α}-Fmoc chemistry. After swelling the resin in DMF and the Fmoc deprotection with 22% piperidine in DMF, the resin was acylated with a mixture of Fmoc-Arg(Pbf)-OH/HOBt/DIPC (4:5:7) in DMF. To provide a lower substitution on the resin, the reaction was quenched after 7 min by washing the resin (substitution on the resin 0.33 mmol g^{-1}), and the remaining amino groups were acetylated with HOAc/HBTU/*N*-methylmorpholine in DMF. Protected amino acids were coupled in fourfold excess in DMF as solvent and DIPC (7 equiv)/HOBt (5 equiv) as coupling reagents while utilizing nondestructive monitoring of the conversion of the free amino groups with bromphenol blue indicator. With the growing peptide chain, the reaction times of the coupling (2 h) as well as of the Fmoc deprotection (1×5 min + 1×20 min) were gradually prolonged. Val, Ile, and Thr(But) were double coupled. After the Fmoc deprotection of the 32-residue peptide, the peptide–resin was equally divided into two syringes. The resin in one syringe was left for completion of the synthesis via fragment condensation (Method B), and the peptide elongation by "stepwise" synthesis was completed in the other syringe (Method A). The linear lucifensin peptide was deprotected and cleaved from the resin by using a mixture of TFA/1,2-ethanedithiol/H_2O/thioanisol/triisopropylsilane (2 mL; 90:2.5:2.5:3:2) for 3.5 h and precipitated with *tert*-butyl methyl ether, yielding crude product (88 mg). The crude peptide was repeatedly purified by preparative HPLC on a Vydac C-18 column (250×10 mm) by using a gradient ranging from 80% of solvent A to 50% of solvent B over 60 min at a flow rate 3.0 mL min^{-1} resulting in 8.5 mg of material available for oxidative folding. The 30-residue linear peptide (Figure 4D), Luc[des1–10, Ala30], was also synthesized as described above resulting in 152 mg of crude peptide, which was purified by HPLC (19 mg) prior to oxidative folding. Measured molecular masses of the pure linear peptides were in good agreement with their calculated values as shown in Table S1.

Method B, Manual synthesis by 8+32 fragment condensation on the resin: The N-terminal 1–8 side-chain-protected peptide fragment was synthesized on 2-chlorotrityl chloride resin (200 mg) in the syringe by using the same protocol for the coupling steps as above. Boc-Ala-OH was used as the N-terminal amino acid residue. The protected peptide was cleaved from the resin by using a mixture

of CH_2Cl_2/TFE/HOAc (4 mL; 7:2:1) at RT for 60 and then again for 15 min. The combined solution was concentrated under vacuum, and the residue triturated with a mixture of *tert*-butyl methyl ether and *n*-hexane yielding the protected peptide (165 mg). The purity and identity of the peptide was checked by HPLC and MS after deprotection of the sample (about 1 mg). ESI-MS: *m/z* calcd for $C_{31}H_{54}N_8O_{13}S$: 778.35, found: 778.3. The mixture of Boc-1–8 side-chain-protected peptide fragment (82 mg, 0.064 mmol) and HOBt (11 mg, 0.08 mmol) in NMP (0.4 mL) was mixed with 32-residue peptide–resin in the syringe and then the resin was soaked with 2 M solution of DIPC in DMF (56 μL, 0.112 mmol) and agitated for 24 h. After washing the resin, the condensation reaction was repeated under the same conditions. The deprotection and cleavage of the 40-residue peptide from the resin was done as described in method A, yielding crude product (87 mg). This product was purified by HPLC prior to oxidative folding (13 mg).

Method C, Automated peptide synthesis: Crude linear 40-residue peptides of lucifensin (Figure 1), Luc[C3–C30], Luc[C16–C36] and Luc[C20–C38] (Figure 4 A, B, and C, respectively), were synthesized on 100 or 200 mg of preloaded Fmoc-Asn(Trt)-Wang Resin in an Applied Biosystems 433A peptide synthesizer by using the HBTU/HOBt/DIPEA activation protocol of Fmoc chemistry. The protected amino acids were coupled in tenfold excess in NMP as solvent. The resin substitution was reduced as described above prior to placing it into the instrument. The deprotection and cleavage as described in method A, yielded on average 160 mg of crude product per 100 mg of the resin. Measured molecular masses of the pure peptides were in a good agreement with their calculated values (Table S1).

Oxidative folding: The lyophilized linear peptides prepurified by HPLC were dissolved (at concentration 1 mg/4 mL) in 0.1 M NH_4OAc buffer, pH 7.8 (prepared by bubbling gaseous NH_3 into 0.1 M acetic acid) and stirred under open air at RT. The time course of the disulfide bond formation was monitored by HPLC. Typically, after 4–6 h of the folding reaction the solvent was removed by lyophilization and the desired folded peptides were further purified by preparative HPLC by using a gradient of solvents ranging from 80% of solvent A to 50% of solvent B at a flow rate 3 mL min^{-1} over 60 min. The final products were then lyophilized. Typically, we obtained 3–4 mg of final folded peptides starting from 10 mg of corresponding linear precursors.

Determination of disulfide bridges: Lucifensin and Luc[des1–10, Ala30] (about 0.05 mg) were dissolved in 0.1 M Mes buffer, pH 6.5 containing 2 mM $CaCl_2$ (50 μL). After addition of of thermolysin stock solution (1 μL; 1 mg mL^{-1}), the mixture was incubated at 35 °C for 16 h. The reaction was quenched by adding 10% TFA (2 μL) and then the entire mixture was subjected to fractionation by HPLC on a Vydac C-18 column (250×4.6 mm). The material was at first eluted with 2% MeCN/H_2O/0.1% TFA for 10 min and then with a linear gradient of solvents from 2% to 35% MeCN/H_2O/0.1% TFA over 60 min at a 1 mL min^{-1} flow rate. The elution was monitored by absorption at 220, 254, and 280 nm. The fractions were collected, the solvent evaporated in a Speed-Vac, and the peptide fragments were analyzed by MS to identify their sequences.

Alkylation of linear lucifensin peptide: HPLC-purified linear lucifensin peptide was dissolved in a solution of iodoacetamide (1 mg) in 0.05 M NH_4HCO_3 (50 μL). The mixture was shaken at RT in darkness for 1 h and then the reaction was quenched by 10% TFA (3 μL). The fully alkylated peptide (all 6 Cys residues) was isolated by HPLC as a single symmetrical peak. Its identity was confirmed by ESI-MS: *m/z* calcd for $C_{178}H_{296}N_{66}O_{57}S_6$: 4462.06, found: 4462.0.

Determination of antimicrobial activity: A simple qualitative estimate of antimicrobial properties was undertaken by using the drop–diffusion test on Petri dishes by the double-layer technique.[34] The Petri dishes (90 mm in diameter) contained 20 mL of LB agar (Sigma). Melted "soft" agar (2 mL), prepared from LB broth (Sigma) and 0.5% agar (Difco) containing about 10^7 colony-forming units of various bacteria (*M. luteus, B. subtilis, S. aureus*) was poured over the surface. Fresh bacterial cultures were always prepared in the LB broth and added when the melted soft agar cooled to about 45 °C. Antimicrobial-activity-containing materials (evaporated HPLC fractions) were diluted in H_2O (10 μL) and dropped (2 μL) onto the surface of the solidified upper layer. To compare the activity of lucifensin with its analogues (Figure 7) their amounts were determined from the HPLC peak areas. The plates were incubated at 37 °C. Clear zones of inhibition appeared within a few hours and remained clear for days. The potency was semiquantitatively estimated according to the diameter and clarity of the zones of inhibition. Quantitative minimum inhibitory concentrations (MICs) were established by observing bacterial growth in multiwell plates.[35,36] 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 (Helsinki, Finland). The absorbance was measured at 540 nm every 15 min and each peptide was tested at least three times in duplicate. Routinely, 5×10^3–10×10^3 CFU of bacteria were used per well. Tetracycline in a concentration range of 0.5–50 μM was tested as a standard.

CD spectra measurement: The circular dichroism (CD) experiments were carried out on a Jasco 815 spectropolarimeter (Tokyo, Japan). All peptide samples were measured in H_2O, in 50% (*v/v*) TFE, and in the presence of SDS at concentrations of 0.16 mM to 16 mM (below and above the critical micelle concentration) with the final peptide concentration 0.25 mg mL^{-1}. The spectra were collected from 190 nm to 300 nm as averages over four scans at RT by using a 0.1 cm path length. A 0.5 nm step resolution, 10 nm min^{-1} speed, 32 s response time, and 1 nm bandwidth were generally used. Following baseline correction, the final spectra were expressed as molar ellipticity θ [deg cm^2 $dmol^{-1}$] per residue. The secondary structure representation was calculated by using the circular dichroism analysis program Dichroweb.[26]

Transmission electron microscopy: *Bacillus subtilis* cells treated with lucifensin for either 10 or 60 min and untreated control cells were used for negative staining examination. Bacteria were adsorbed on parlodion-carbon-coated copper grids for 5 min. After short washing, the samples were negatively stained by floating on a drop of 0.25% phosphotungstic acid (PTA) with 0.01% BSA in dH_2O for 30 s. Excess stain was blotted off with a piece of filter paper and samples were air-dried. A JEOL JEM/1200EX transmission electron microscope operating at 60 kV was used for analysis.

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