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Structure-activity relationships of the intramolecular disulfide bonds in coprisin, a defensin from the dung beetle

Jaeho Lee[1], Daeun Lee[1], Hyemin Choi[2], Ha Hyung Kim[3], Ho Kim[4], Jae Sam Hwang[5], Dong Gun Lee[2] & Jae Il Kim[1,]*

[1]School of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, [2]School of Life Sciences, KNU Creative BioResearch Group (BK21 plus program), College of Natural Sciences, Kyungpook National University, Daegu 702-701, [3]College of Pharmacy, Chung-Ang University, Seoul 156-756, [4]School of Life Sciences and Biotechnology, College of Natural Science, Daejin University, Pocheon 487-711, [5]Department of Agricultural Biology, Natural Academy of Agricultural Science, RDA, Suwon 441-707, Korea

Defensins, which are small cationic molecules produced by organisms as part of their innate immune response, share a common structural scaffold that is stabilized by three disulfide bridges. Coprisin is a 43-amino acid defensin-like peptide from *Copris tripartitus*. Here, we report the intramolecular disulfide connectivity of cysteine-rich coprisin, and show that it is the same as in other insect defensins. The disulfide bond pairings of coprisin were determined by combining the enzymatic cleavage and mass analysis. We found that the loss of any single disulfide bond in coprisin eliminated all antibacterial, but not antifungal, activity. Circular dichroism (CD) analysis showed that two disulfide bonds, Cys20-Cys39 and Cys24-Cys41, stabilize coprisin's α-helical region. Moreover, a BLAST search against UniProtKB database revealed that coprisin's α-helical region is highly homologous to those of other insect defensins. [BMB Reports 2014; 47(11): 625-630]

INTRODUCTION

The innate immune system is the first line of defense against bacterial, fungal, and viral pathogens (1). This defense system is essential for host survival, in a world full of potentially dangerous microbes. As invertebrates do not have an adaptive immune system (2), the innate immune system is particularly important in these organisms., For example, insects protect themselves from pathogens through the production of antimicrobial peptides, the socalled defensins, in response to bacterial infection, or bodily injury (3, 4). The primary structures of insect defensins show a high degree of sequence conservation, with homologies ranging from 58% to 95% (5).

Most insect defensins share a common structural feature that is composed of a short amphipathic α-helix, followed by a C-terminal antiparallel β-sheet structure that is stabilized by two disulfide bridges (*i.e.* the cysteine-stabilized α/β motif) (6). The disulfide bridge pattern of insect defensins is also conserved. When their six cysteine residues are numbered 1 to 6 from the N-terminus, the bonds are 1-4, 2-5, and 3-6. These structural characteristics of insect defensins affect both their activity and stability. For example, a structure-activity study of tenecin 1 from *Tenebrio molitor* revealed the importance of the α-helical structure for the activity of this defensin (7). Moreover, the cysteine-stabilized α/β motif in drosomycin from *Drosophila melanogaster* accounts for its remarkable resistance to proteases, and its heat tolerance (8). On the other hand, little is known about the functional role of disulfide bridges in insect defensins.

Recently, a novel insect defensin, coprisin, was isolated from the dung beetle *Copris tripartitus* (9). Coprisin shows a high degree of sequence homology with sapecin (75%) and tenecin 1 (80%), and potent bactericidal activity against Gram-negative and Gram-positive bacteria and fungi. The solution structure of coprisin, which was recently solved using NMR (10), contains a cysteine-stabilized α/β motif, like other defensins. However, the disulfide bond pattern of coprisin could not be determined, because the bond pattern of other insect defensins was used as a constraint to resolve coprisin's solution structure. In addition, earlier studies on coprisin did not focus on the function of its three disulfide bonds (9-12).

The present study therefore investigated the disulfide bond pattern, and the functions of the three disulfide bonds of coprisin. In addition, the active site of insect defensins was investigated, by making comparisons among a set of coprisin-like antimicrobial peptides.

*Corresponding author. Tel: +82-62-715-2494; Fax: +82-62-715-2484; E-mail: jikim@gist.ac.kr

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RESULTS

Synthesis of coprisin and its Cys-to-Ala analogs

Coprisin contains six cysteines that form three disulfide bonds. We employed the standard Fmoc solid-phase peptide method

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to synthesize wild-type coprisin and its three Cys-to-Ala analogs, Cop [Ala^{3,34}], Cop [Ala^{20,39}], and Cop [Ala^{24,41}] (Table S1). Three Cys-to-Ala analogs were designed, on the basis of an enzymatic analysis for disulfide connectivity of wild-type coprisin. To form the disulfide bonds, synthesized linear peptides (0.02 mM) were stirred slowly in 0.1 M ammonium acetate (pH 7.8), at room temperature for 1 day (13). The purity of the synthesized linear peptides was >90%, as measured by analytical HPLC. The theoretical molecular weight (4471.2 Da) of the oxidized coprisin was in good agreement with the molecular weight of 4471.2 Da, measured using MALDI-TOF MS. The theoretical masses (4409.1 Da) of Cop [Ala^{3,34}], Cop [Ala^{20,39}], and Cop [Ala^{24,41}] were also consistent with the measured molecular weights (4409.2 Da).

Disulfide bond parings of coprisin

To assign the connectivity of disulfide bonds for wild-type coprisin, the oxidized peptide was enzymatically digested, after which the resultant peptide fragments were separated by HPLC, and characterized by MALDI-TOF MS. In this assay, we chose to digest coprisin using thermolysin. Given coprisin's sequence, thermolysin would be expected to generate peptide fragments that are linked by a single disulfide bond. Thermolysin digestion of coprisin yielded four fragments, F1, F2, F3, and F4 (Fig. 1A, Fig. S1). The F1 fragment eluted early (17% acetonitrile) on RP-HPLC, and had a mass of 413.3 Da, indicating the presence of a disulfide bond between C2 and C5. The F2 fragment had a mass of 863.3 Da, and was composed of LHC linked to VCRN. The mass of the F3 fragment was 912.2 Da, and was composed of VNHSACA linked to VC. The F4 fragment had a mass of 1680.4 Da, and was composed of fragments connected by the first disulfide bonds. Thus, the disulfide bond bridges of coprisin are C1-C4, C2-C5, and C3-C6 (*i.e.* Cys3-Cys34, Cys20-Cys39, and Cys24-Cys41).

Conformational studies

The secondary structures of coprisin and the three Cys-to-Ala analogs were analyzed using circular dichroism (CD) spectroscopy, with a membrane-mimicking environment that was achieved through the addition of 50 mM SDS to sodium phosphate buffer (Fig. 1C). Consistent with previously reported CD spectra (10), coprisin's spectra showed two minima around 208 and 222 nm, which indicate the presence of α-helix. In addition, the 208 nm band was of larger magnitude, and more prominent than the 222 nm band, which is characteristic of the α + β class of proteins, and indicates that coprisin contains α-helix and β-strands (14).

To investigate the structure-function relations of the disulfide bridges, we used three coprisin analogs, in which a single disulfide bond was deleted, by substituting two cysteine residues with alanine (Cop [Ala^{3,34}], Cop [Ala^{20,39}], and Cop [Ala^{24,41}]). When measured under the same conditions, the CD spectrum of Cop [Ala^{3,34}] was similar to that of coprisin. By contrast, the CD spectra of Cop [Ala^{20,39}] and Cop [Ala^{24,41}] revealed more flexible conformations than the wild-type peptide (Fig. 1C). These data suggest that two disulfide bonds (Cys20-Cys39 and

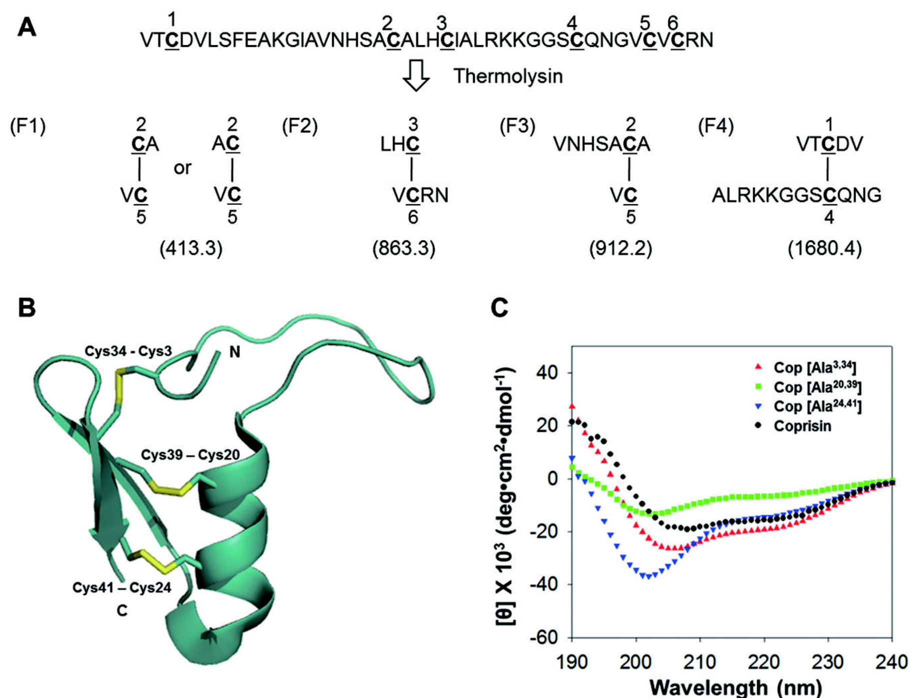

Fig. 1. Scheme for identifying the intramolecular disulfide bonds within coprisin. (A) Coprisin was digested with thermolysin, after which the peptide fragments (F1-F4) were purified, using RP-HPLC. The molecular weights of the fragments were measured using MALDI-TOF mass spectroscopy, and are given in parentheses. (B) Solution structure of coprisin (PDB ID: 2LN4) in 10% D_2O (pH 4.3). The three disulfide bridges are shown in yellow. (C) CD spectra for 50 μm coprisin, Cop [Ala^{3,34}], Cop [Ala^{20,39}], and Cop [Ala^{24,41}] were collected in the presence of 50 mM SDS in 50 mM sodium phosphate buffer (pH 7.4).

Cys24-Cys41) play major roles in stabilizing the α-helical structure within coprisin, while the first disulfide bond (Cys3-Cys34) appears to be involved in forming the N-terminal loop. These findings are consistent with the three-dimensional structure of coprisin, which has two disulfide bridges (Cys20-Cys39 and Cys24-Cys41) located in the αhelical region, and one (Cys3-Cys34) located between the N-terminal loop and the β-strand (Fig. 1B).

Functional characterization of coprisin and its Cys-to-Ala analogs

To assess the importance of the disulfide bonds to coprisin function, we examined the antimicrobial and antifungal activities of the wild-type coprisin, and the aforementioned analogs, against a representative set of Gram-negative (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonus aeruginosa*) and Gram-positive (*Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*) bacterial strains, as well as four fungal strains (*Candida albicans*, *C. parapsilosis*, *Malassezia furfur*, *Trichosporon beigelii*). In the liquid growth inhibition assays, the ability of serial dilutions of coprisin, Cop [$Ala^{3,34}$], Cop [$Ala^{20,39}$], and Cop [$Ala^{24,41}$] to inhibit cell growth *in vitro* was evaluated. The positive control was melittin, a well-known antimicrobial peptide. As summarized in Table 1, coprisin inhibited growth of bacterial strains in the range 0.8 to 3.1 μM, and fungal strains in the range of 5 to 10 μM. These values are similar to previously reported MICs (11, 15). By contrast, Cop [$Ala^{3,34}$], Cop [$Ala^{20,39}$], and Cop [$Ala^{24,41}$] did not inhibit bacterial growth at any concentration up to 100 μM, although antifungal activity was slightly reduced. Thus, the three disulfide bonds appear to be essential for coprisin's antibacterial activity, but not its antifungal activity.

Table 1. Antimicrobial activities of coprisin and its three Cys-to-Ala analogs

Microorganism	Minimal inhibitory concentration (μM)				
	Coprisin	Cop [$Ala^{3,34}$]	Cop [$Ala^{20,39}$]	Cop [$Ala^{24,41}$]	Melittin
Gram-negative bacteria					
E. coli	3.1	>100	>100	>100	1.6
S. typhimurium	3.1	>100	>100	>100	1.6
P. aeruginosa	3.1	>100	>100	>100	1.6
Gram-positive bacteria					
S. aureus	0.8	>100	>100	>100	0.8
S. epidermidis	1.6	>100	>100	>100	1.6
B. subtilis	1.6	>100	>100	>100	0.8
Fungal strains					
C. albicans	10	12.5	12.5	12.5	3.1
C. parapsilosis	10	12.5	12.5-25.0	12.5	6.3
M. furfur	5	25.0	25.0	25.0	6.3
T. beigelii	10	25.0	25.0	50.0	6.3

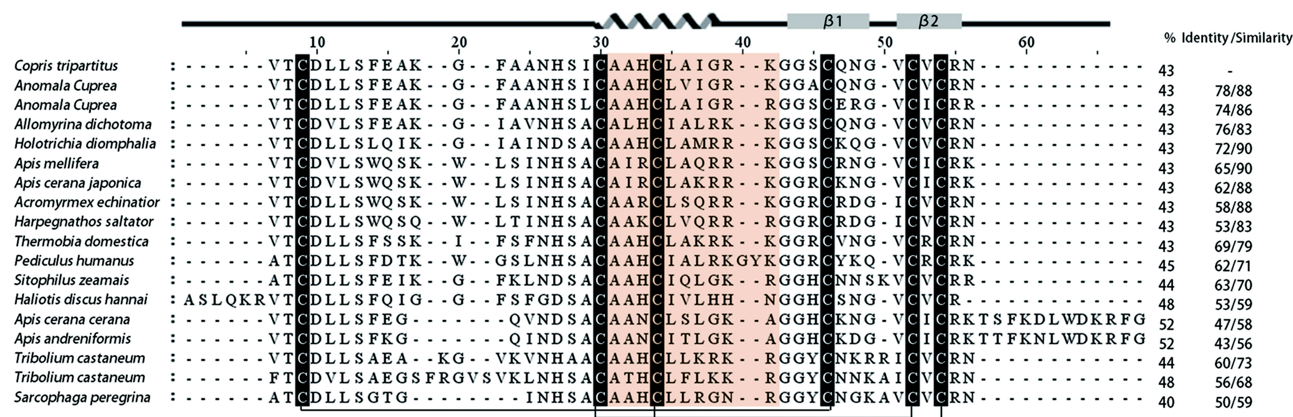

Fig. 2. Multiple sequence alignment of insect defensins. These sequences were aligned, using the ClustalW multiple alignment tool. The ranges of sequence identity and similarity of aligned sequences are 50%-78% and 59%-90%, respectively. The α-helical regions of the defensins are highlighted by a pink box.

Location of the active site related to antimicrobial activity

Interestingly, Cop [Ala^{3,34}] had no antibacterial activity, despite having a secondary structure similar to that of coprisin. We therefore sought to identify the active site of coprisin. In an earlier report, it was suggested that the active site is the amphipathic α-helical region of insect defensins (16). To test whether the antimicrobial activity of coprisin originates from the amphipathic α-helical region, we synthesized 18 short peptides that were derived from the α-helical regions of a set of insect defensins, including coprisin (Fig. 2, Table S2). The primary sequence identity and similarity between coprisin and other insect defensins lie in the range from 50% to 78%, and 59% to 90%, respectively. As expected, N1, which was derived from coprisin, showed antimicrobial activity against selected Gram-negative bacteria (MIC = 15 μm), Gram-positive bacteria (MIC = 7.5-15 μm), and drug-resistant bacteria (MIC = 3.8-15 μM). Although the MICs for N1 were about 10-fold higher than those for coprisin, N1 has the potential to serve as an antimicrobial peptide. Most of the other peptides that were tested also showed broad-spectrum antimicrobial activity against the bacterial strains, in the range of 3.8 to 30 μM. Three exceptions were N12, N14, and N15, which at concentrations up to 30 μM, did not show antibacterial activity. Thus 15 of the 18 tested short peptides corresponding to the α-helical region apparently exhibited a meaningful antibacterial activity. This strongly suggests the active site for its antibacterial activity may be the α-helical region of insect defensin.

DISCUSSION

In this study, we investigated the structure-activity relationships of coprisin. To obtain information about its structure, we determined the disulfide bond structure, and analyzed its CD spectrum. Enzymatic digestion and mass measurements revealed that coprisin has the same disulfide bond pairings as other insect defensins (17). Analysis of its CD spectrum showed that the structure of coprisin contains α-helix and β-sheet. The CD spectrum further showed that two disulfide bonds (Cys20-Cys39 and Cys24-Cys41) are important for stabilizing the α-helix, which was lost in Cop [Ala^{20,39}] and Cop [Ala^{24,41}], along with the peptides' antibacterial activity. On the other hand, Cop [Ala^{3,34}] also did not show antibacterial activity, despite retaining its α-helical region. In Cop [Ala^{3,34}], it was the N-terminal loop that was lost. This raises the question, why does Cop [Ala^{3,34}] not inhibit bacteria growth? It may be that the loss of either the flexible N-terminal loop, or the α-helical region of coprisin, affects oligomerization of the peptide. Coprisin shows a high degree of similarity with sapecin, which is the only insect defensin whose oligomerization mechanism is known. In sapecin, two residues (Asp^{4} and Arg^{28}) mediate oligomer formation through electrostatic interaction (5). These residues are conserved in coprisin, which prompts us to speculate that coprisin also forms oligomers. If so, loss of the N-terminal loop or the α-helix could reduce the likelihood of electrostatic interaction between Asp^{4} and Arg^{28} of coprisin. Alternatively, the N-terminal loop may be important for the activity itself. It was previously reported that even though the α-helical region and all three disulfide bonds were retained, deletion of the N-terminal loop of tenecin 1, an insect defensin from *T. molitor*, eliminated the peptide's antimicrobial activity, (7). Tenecin 1 and coprisin have the same number of residues in their N-terminal loops, and show >80% sequence homology. It therefore seems likely that the N-terminal loop of coprisin also contributes to its antimicrobial activity.

Interestingly, we found that irrespective of the α-helical content, the antifungal activities of the three Cys-to-Ala analogs were sustained. One reported observation that provides a possible explanation was made in *C. albicans*, where coprisin induces apoptosis without membrane disruption, after being taken up into the cells via active transport (11). By contrast, when

Table 2. Antimicrobial activities of 9-mer peptides derived from insect defensins

Microorganism	Minimal inhibitory concentrations (μM)																		
	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	CopA3
Gram-negative bacteria																			
E. coli	15	7.5	15	15	30	15	30	>30	>30	>30	>30	>30	>30	>30	>30	7.5	15	15	7.5
S. typhimurium	15	7.5	15	15	30	7.5	7.5	15	7.5	30	30	>30	>30	>30	>30	15	15	30	15
P. aeruginosa	15	7.5	15	15	30	7.5	7.5	30	7.5	30	30	>30	15	>30	>30	15	15	30	15
Gram-positive bacteria																			
S. aureus	7.5	7.5	15	7.5	30	7.5	7.5	30	15	30	30	>30	>30	>30	>30	15	15	30	15
S. epidermidis	15	7.5	7.5	15	30	7.5	7.5	15	7.5	30	30	>30	15	>30	>30	7.5	15	15	7.5
B. subtilis	7.5	7.5	7.5	7.5	15	7.5	7.5	30	15	30	30	>30	15	>30	>30	15	7.5	>30	15
E. faecium	15	7.5	30	15	>30	15	3.8	>30	>30	30	7.5	>30	>30	>30	>30	15	>30	>30	7.5
E. faecalis	15	15	>30	15	>30	>30	30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	>30	3.8
Drug resistant bacteria																			
VRE (*E. faecium*)	3.8	3.8	7.5	15	7.5	3.8	15	>30	30	>30	>30	>30	>30	>30	>30	>30	>30	>30	7.5
VRE (*E. faecalis*)	15	30	>30	>30	>30	>30	30	>30	30	>30	15	>30	>30	>30	>30	>30	>30	>30	7.5
MRSA	7.5	7.5	15	30	30	7.5	7.5	30	15	30	>30	>30	>30	>30	>30	15	15	>30	15

applied to bacteria, coprisin induces membrane disruption (10). These findings suggest the mode of action of coprisin differs between bacteria and fungi.

Generally, the primary structures of insect defensins are highly homologous. In addition, structural studies have also demonstrated that these peptides share a common structural feature, the cysteine-stabilized α/β motif. This is composed of a short amphipathic α-helix, followed by Cterminal antiparallel β sheets that are stabilized by three disulfide bridges (19). In their analyses of the structure-function relationships of these peptides, several groups have shown that short linear fragments corresponding to the α-helical region possess antimicrobial activity (16, 19, 20). Our present findings that were obtained using 18 short peptides derived from the α-helical regions of a set of insect defensins (Table 2) are consistent with those earlier reports. However, three of the short peptides, N12, N14, and N15, did not show antimicrobial activity. Given what we know about the parameters important for activity, and the primary structures of these three peptides, as compared to the active peptides, we suggest that the net positive charge and the number of the hydrophobic residues could be critical parameters that affect antimicrobial activity.

In sum, we determined a disulfide bond pattern of coprisin, and showed that it is the same as in other insect defensins. In addition, mutation studies showed that all three disulfide bonds are essential for antibacterial activity, and that coprisin's modes of action against bacteria and fungi likely differ. Finally, we determined that the α-helical region is the active site in insect defensins.

MATERIALS AND METHODS

Peptide synthesis and disulfide formation

All peptides were purchased from Anygen (Korean, Gwangju). These peptides were manually synthesized, using the solid-phase peptide synthesis method with Fmoc chemistry. The peptides were cleaved from the resin using trifluoroacetic acid containing various scavengers, and purified using preparative RP-HPLC (Shimadzu, Tokyo, Japan). The purity of the peptides was verified by analytical RP-HPLC, and correct peptide masses were confirmed using MALDITOF MS (Shimadzu). For disulfide bond formation, linear coprisin and the Cys-to-Ala mutants, Cop [$Ala^{3,34}$], Cop [$Ala^{20,39}$], and Cop [$Ala^{24,41}$], were dissolved to a concentration of 25 μM in 0.1 μM ammonium acetate solution (pH 7.0), and allowed to react at room temperature for 24 h, with gentle stirring. The course of the reaction was monitored using HPLC. The yields of coprisin and the three cysteine mutants were $>$90%.

Antimicrobial activity

Using broth microdilution assays, we assessed the antimicrobial activities of each peptide, against six selected organisms obtained from the Korean Collection for Type Cultures (KCTC), at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). These included three Gram-negative (*E. coli* KCTC 1682, *S. typhimurium* KCTC 1926, and *P. aeruginosa* KCTC 1637) and three Gram-positive (*S. aureus* KCTC 1621, *S. epidermidis* KCTC 1917, and *B. subtilis* KCTC 3068) strains. Briefly, single colonies of bacteria were inoculated into medium (LB broth), and cultured overnight at 37°C. An aliquot of the culture was then transferred to 10 ml of fresh medium, and incubated for an additional 3-5 h at 37°C, until the mid-logarithmic phase. Two-fold dilution series of the peptides in 1% peptone was then prepared, after which the serial dilutions (100 μl) were added to 100 μl of cells [5×10^5 colony-forming units (CFU)/ml)], in 96-well microtiter plates (F96 microtiter plates; Nunc, Odense, Denmark), and incubated for 16 h at 37°C. The lowest concentration of peptide that completely inhibited growth was defined as the MIC. MICs were determined as the average of triplicate measurements, in three independent assays.

Fungal strains and antifungal susceptibility test

Candida albicans (ATCC 90028) and *C. parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Malassezia furfur* (KCTC 7744) and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC), at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). With the exception of *M. furfur*, the fungal strains were cultured in YPD broth (Difco), with aeration at 28°C. *Malassezia furfur* was cultured at 32°C in a modified YM broth (Difco), containing 1% olive oil. The cell suspensions were adjusted to obtain standardized populations, by measuring the turbidity with a spectrophotometer (DU530; Beckman, Fullerton, CA, USA). Fungal cells at log phase (2×10^6/ml) were inoculated into 100 μl of YPD or YM broth per well, in microtiter plates. The MIC values were then determined in three independent assays.

Determination of disulfide bond pairings

Intramolecular disulfide bond pairings within coprisin were identified through enzymatic cleavage, using thermolysin and MALDI-TOF MS measurements. Coprisin was digested with thermolysin (Promega, USA) in 100 mM ammonium acetate (pH 6.2) with 2 mM $CaCl_2$, for 1 h at 60°C. The ratio of coprisin to thermoysin was 10 : 1 (w/w). The resultant mixture was subjected to RP-HPLC separation, after which the collected fragments were analyzed, using MALDITOF MS.

CD analysis

The CD spectra of the peptides were recorded, using a Jasco J-710 CD spectrophotometer (Jasco, Tokyo, Japan), with a 1 mm path-length cell. Wavelengths were measured from 190 nm to 240 nm (bandwidth, 1 nm; step resolution, 0.1 nm; speed, 50 nm/min; response time, 0.5 s). The CD spectra were collected for the peptides in the presence of 50 mM SDS micelles (pH 7.4) at 25°C. The spectra were averaged over 4 scans, and were

expressed as molar ellipticity [h] versus wavelength.

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