This study presents purification, activity characterization, and \(^{1}\)H NMR study of the novel antifungal peptide EcAMP1 from kernels of barnyard grass \textit{Echinochloa crus-galli}. The peptide adopts a disulfide-stabilized \(\alpha\)-helical hairpin structure in aqueous solution and thus represents a novel fold among naturally occurring antimicrobial peptides. Micromolar concentrations of EcAMP1 were shown to inhibit growth of several fungal phytopathogens. Confocal microscopy revealed intensive EcAMP1 binding to the surface of fungal conidia followed by internalization and accumulation in the cytoplasm without disturbance of membrane integrity. Close spatial structure similarity between EcAMP1, the trypsin inhibitor VHTI from seeds of \textit{Veronica hederifolia}, and some scorpion and cone snail toxins suggests natural elaboration of different functions on a common fold.

Antimicrobial peptides (AMPs)\(^4\) are a structurally diverse group of generally small, positively charged peptides produced by various living organisms and demonstrating a wide spectrum of antimicrobial activity (1, 2). Natural sources of AMPs range from prokaryotes to higher animals, and their targets include bacteria, fungi, protozoa, and viruses. The mechanism of action of most known AMPs involves their direct or receptor-mediated interaction with microbial membranes (3–5). It has been generally accepted that membrane-disruptive AMPs kill microorganisms by provoking in different ways an increase in plasma membrane permeability. Non-membrane-disruptive peptides have been shown to target cell wall formation or traverse membranes and affect various internal cellular processes, for example, RNA, DNA, and/or protein biosynthesis. Some AMPs can combine disruptive and non-disruptive mechanisms of action (6). Moreover, mechanisms of action of the same peptide may differ depending on the target. Recent studies have also indicated that AMPs are multifunctional molecules; they can interact with host membrane receptors and influence diverse intracellular processes modulating the immune response of the host organism (7, 8).

Essential variety in detailed mechanisms of action and multifunctionality imply structural diversity among AMPs. The following structural groups are usually recognized: (i) linear peptides that form \(\alpha\)-helices in contact with membranes; (ii) disulfide-containing with predominance of \(\beta\)-structural elements; and (iii) linear non-\(\alpha\)-helix-forming, usually with a high content of certain amino acid residues (1, 2, 9). Most of the approximately 200 AMP spatial structures known at present (see the Antimicrobial Peptide Database v2.26 (10)) fall into one of the first two groups. Further classification is based on unique features in the sequences and/or structures of AMPs. For example, thionins, defensins, nonspecific lipid transfer proteins, and hevein- and knottin-like peptides have been identified in plants (11–13).

To characterize the array of AMPs produced by a plant under certain physiological conditions, we have carried out a systematic analysis of these peptides from different species (wheat AMPs are extensively reported in Ref. 14). Among dozens of AMPs produced by plants, we have identified peptides with unique features in their sequences, such as an unusual 4-cysteine motif. Further studies of one of these peptides, EcAMP1 isolated from barnyard grass and reported here, demonstrate that this peptide represents a novel disulfide-stabilized helical hairpin fold, unprecedented among naturally occurring AMPs. EcAMP1 exhibits high activity against fungi of the genus \textit{Fusarium}. This property makes it a new interesting candidate in protein engineering for pharmaceutical and agricultural applications.
**Plant Defense Peptide with Helical Hairpin Structure**

**EXPERIMENTAL PROCEDURES**

**Biological Material**—Seeds of *Echinochloa crus-galli* (L.) P. Beauv. (family Poaceae) were collected in the Krasnodarsky region (Russia) in 2005. The fungi *Colletotrichum graminicola*, *Dipodia maydis*, *Fusarium graminearum*, and *Fusarium verticilloides* were from the collection of Pioneer Hi-Bred International. *Alternaria alternata*, *Bipolaris sorokiniana*, *Fusarium oxysporum*, *Fusarium solani*, and the oomycete *Phytophthora infestans* were from the collection of the Timiryazev Agricultural Academy (Moscow, Russia). *Alternaria solani*, *Aspergillus niger*, *Phoma betae*, *Trichoderma album*, and the oomycetes *Pythium debaryanum* and *Pythium ultimum* were from the All-Russian Collection of Microorganisms at the G.K. Skryabin Russian Academy of Sciences (Pushchino, Moscow Region, Russia).

**Purification of EcAMP1**—Seeds of *E. crus-galli* (100 g) were ground into flour in a coffee mill and extracted with 10% acetic acid (~1 liter) in the presence of the proteinase inhibitor mixture for plant cell extracts (Sigma-Aldrich) at room temperature for 1.5 h under constant stirring. After centrifugation at 10,000 x g for 15 min, the supernatant was filtered through paper and evaporated (to the volume of 100 ml). Ice-cold acetone (700 ml) was added, and polypeptides were precipitated overnight at 4 °C, collected by centrifugation at 10,000 x g for 15 min, air-dried, and redissolved in 0.1% trifluoroacetic acid (TFA).

Desalting was performed by reversed-phase HPLC (RP-HPLC) on an Aquapore C8 column (10 x 10 mm; Applied Biosystems) using a quick step of acetonitrile concentration (0 – 60%). The retained fraction was dried, dissolved in 10 mM Tris-HCl, pH 7.2, and subjected to affinity chromatography on a Heparin-Sepharose 6 Fast Flow column (15 x 30 mm; GE Healthcare) equilibrated with the same buffer at a flow rate of 1 ml/min. After elution of the unadsorbed fraction, polypeptides were eluted with consecutive steps of salt concentration: 50, 100, and 500 mM NaCl; detection was performed at 280 nm. The obtained fractions were desalted as described above and dried. The fraction containing EcAMP1 eluted at 500 mM NaCl and was separated by size-exclusion chromatography on a Sephacryl S-100 HR column (20 x 495 mm; GE Healthcare). Elution was performed with 5% acetonitrile in 0.05% TFA at a flow rate of 60 ml/h, and effluent absorbance was monitored at 214 nm. The peptidic fraction was further separated by RP-HPLC on a Luna C8 column (10 x 250 mm; Phenomenex) using a linear gradient of acetonitrile concentration (8 – 40% in 60 min) at a flow rate of 1.5 ml/min, and detection was performed at 214 nm.

**Mass Spectrometry**—Peptides were analyzed by matrix-assisted laser desorption/ionization (MALDI) MS. Mass spectra were acquired on an Ultraflex TOF-TOF mass spectrometer (Bruker Daltonics, Germany) as described (15).

**Determination of EcAMP1 Amino Acid Sequence**—Purified peptide was reduced (by dithiothreitol) and alkylated (by 4-vinylpyridine) using a common procedure described earlier (16). The reduced-alkylated EcAMP1 was subjected to automated Edman sequencing on a Procise model 492 protein sequencer (Applied Biosystems) according to the manufacturer’s protocol.

**Peptide Concentration Measurements**—Absorption spectra were recorded on a U-3210 spectrophotometer (Hitachi, Japan). The GPMAW program (Lighthouse data, Odense, Denmark) was used to calculate the molar extinction coefficient of EcAMP1 at 280 nm (ε280 = 5930 M⁻¹cm⁻¹). Alternatively, peptide concentration was determined by RP-HPLC on a Luna C18 column (4.6 x 150 mm; Phenomenex) calibrated with bovine insulin (Sigma-Aldrich).

**Enzyme Assay**—Trypsin (Hoffmann-La Roche, Basel, Switzerland) activity with N-α-benzoyl-DL-arginine-p-nitroanilide as substrate was measured spectrophotometrically at 410 nm by monitoring p-nitroaniline release. 15 μl of aqueous enzyme solution (5 x 10⁻⁶ M) was mixed with 1.5 ml of solution containing the substrate (3.3 x 10⁻⁴ M), 50 mM CaCl₂, 0.1 M Tris-HCl (pH 8) and the tested compound at different concentrations and further incubated for 1 h at 37 °C. The reaction was stopped by the addition of 30% cold acetic acid (0.5 ml). Bovine pancreatic trypsin inhibitor (Sigma-Aldrich) was used as the positive control. No tested compound was added in the negative control.

**Peptide Cleavage by Trypsin**—EcAMP1 (~2 nmol) was dried and redissolved in 20 μl of 50 mM NH₄HCO₃, pH 8.0. Trypsin solution (0.5 μl) in the same buffer (0.5 mg/ml) was added to the sample followed by incubation at 37 °C for 4 h. Reaction products were separated by RP-HPLC on a Luna C18 column (4.6 x 150 mm) in a linear gradient of acetonitrile concentration. Detection was performed at 214 nm.

**Antifungal Activity Measurements**—Antifungal activity of EcAMP1 was tested against several fungi using microtiter plate assays essentially as described (17). Wells were filled with 10 μl of 2-fold serial dilutions of the peptide and mixed with 90 μl of half-strength potato dextrose broth containing conidia or zoosporangia (~10⁴/ml). The inhibition of spore germination was evaluated by measuring the absorbance at 620 nm. Morphological changes were recorded using a light microscope. Antifungal activity was expressed by the peptide concentration causing a 50% inhibition of fungal growth (EC₅₀). The presented values are means ± S.E. of three independent measurements.

**EcAMP1 Fluorescent Tagging**—Dried peptide (10 nmol) was dissolved in 200 μl of 50 mM Na₂HPO₄, pH 7.2, 5 μl of 5.7 mM 5(6)-carboxytetramethylrhodamine (TMR) N-succinimidyl ester (Pierce) in dimethyl sulfoxide was added, and the mixture was incubated at 37 °C for 45 min. Reaction products were separated by RP-HPLC on a Luna C₁₈ column (4.6 x 150 mm) in a linear gradient of acetonitrile concentration. Effluent absorbance was monitored at 254 nm. Concentration of the TMR-labeled peptide was determined by measuring its solution absorbance at 555 nm (ε₅₅₅ = 65,000 M⁻¹cm⁻¹).

**Confocal Laser Scanning Microscopy**—*F. solani* conidia suspension (~10⁶ spores/ml, 385 μl/well) was placed into an 8-well Lab-Tek chambered cover glass (Nalge Nunc International), and the spores became attached to the bottom of the chamber. Spores were incubated with 4 μM equimolar mixture of EcAMP1 and EcAMP1-TMR or 2 μM EcAMP1-TMR. The peptide concentrations used corresponded to 50% (EC₅₀) and 30% inhibitory effect, respectively. In some experiments, 5(6)-
Plant Defense Peptide with Helical Hairpin Structure

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Chemical modification followed by MS analysis demonstrated that EcAMP1 contained 4 cysteine residues, all of which were involved in disulfide bonding, because the measured monoisotopic molecular mass of the peptide (4271.7 Da) increased by 424 Da after reduction and alkylation (mass of the pyridylethyl group is 105.1 Da) and did not change if alkylation increased by 424 Da after reduction and alkylation (mass of the pyridylethyl group is 105.1 Da) and did not change if alkylation of the unreduced peptide was performed. The complete amino acid sequence of EcAMP1 consisting of 37 amino acid residues was determined by Edman degradation as follows: GSGRG-

Cys-25. Other types of cysteine pairing contradict the pattern of NOE contacts obtained for EcAMP1. No restraints for hydrogen bonds were added.

NMR Spectroscopy—1.4 mg of EcAMP1 was dissolved in 0.5 ml of H2O containing 5% D2O or D2O (99.6% deuterium, Cambridge Isotope Laboratories). 1H NMR spectra were obtained at 20 °C and pH 3.1, 3.4, 4.1, 4.6, 5.3, 5.7, and 6.6. All NMR experiments were performed on Bruker Avance-800, Avance-700, or Avance-600 spectrometers equipped with pulsed field gradients. Double-quantum filtered COSY (18), total correlation spectroscopy (19) with a mixing time of 70 ms, and NOESY (20) with mixing times of 40, 50, 100, and 200 ms were recorded (see supplemental Figs. 1 and 2 for fingerprints of TOCSY and NOESY spectra). Water suppression was achieved using the Watergate technique (21). Unless otherwise stated, a relaxation delay of 4.0 s was used (26) in COSY spectra of EcAMP1.

Spatial Assignment and Spatial Structure Calculation—Complete proton resonance assignment was performed by a standard procedure (22) using the CARA (23) and XEASY (24) programs (see supplemental Table 1 for proton chemical shifts). Volumes of cross-peaks in NOESY spectra were measured using an algorithm of non-linear least squares approximation for line shapes of cross-peak sections in both directions of two-dimensional spectra implemented in the XEASY program. The 3JH,NH coupling constants were determined from line shape analysis of NOESY cross-peaks with the INFIT procedure (25). The 3JHH coupling constants were measured using the ACME program (26) in COSY spectra of EcAMP1 solution in D2O (relaxation delay of 4.0 s).

Spatial structure calculations were performed for EcAMP1 at pH 6.6 using the simulated annealing in torsion angle space protocol as implemented in the CYANA program version 2.0 (27). Upper interproton distance restraints were derived from NOESY (with a mixing time of 40 ms) cross-peaks via a 1/r6 calibration. Methyl and methylene protons were stereo-specifically assigned using the GLOMSA procedure implemented in CYANA. Torsion angle φ and χ critical restraints were derived based on qualitative analysis of spin-spin coupling constants and NOE intensities. Additional restraints were introduced for two disulfide bonds between residues Cys-7–Cys-29 and Cys-11–Cys-25. Other types of cysteine pairing contradict the pattern of NOE contacts obtained for EcAMP1. No restraints for hydrogen bonds were added.

carboxyfluorescein (CF) as a soluble fluorescent probe for cell wall and membrane integrity was added to a concentration of 50 μM. Interactions of EcAMP1-TMR with spores were studied with the Fluoview FV1000 confocal laser scanning microscope (Olympus, Germany). An UPLSAPO (60×O/1.35 NA) objective (Olympus) was used for the measurements. CF and EcAMP1-TMR fluorescence was excited at 488 and 559 nm, respectively. Fluorescence emission was collected in the range of 497–558 nm (CF) or 579–679 nm (EcAMP1-TMR). Lateral and axial resolutions were ~0.2 and ~1 μm, respectively.

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Plant Defense Peptide with Helical Hairpin Structure

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Echinocloa crus-galli, Zea mays, Macadamia integrifolia, Curcubita maxima, Luffa aegyptiaca, Veronica hederifolia, and Fagopyrum esculentum.

The EC50 values were in the range of 1–10 μM. The peptide induced morphological changes in some of the affected fungi only at higher concentrations (>20 μM). A. niger, C. graminicola, D. maydis, and T. album were all insensitive to the peptide at the concentrations used in our assays (Table 1). EcAMP1 activity is in qualitative agreement with results reported for the related antifungal peptide MBP-1 from maize (Zea mays) (31). MBP-1 was active against Alternaria longipes and several Fusarium and Sclerotinia species, but practically ineffective against Aspergillus flavus. For example, MBP-1 inhibited F. graminearum spores germination with similar activity to EcAMP1 (EC50 of ~4 μM).

Our results are indicative of a certain specificity of EcAMP1 action. Close inspection with a light microscope revealed that the peptide had a deleterious effect on the rate of hyphae elongation from the germinated conidia (length of F. solani hyphae reached 0.8–1.3 mm after incubation for 48 h in control and 0.3–0.6 mm if 4 μM EcAMP1 was added), whereas the germination itself was not affected in all Fusarium species. We believe that the newly discovered peptide may serve as a lead for development of antifungal substances to combat fusariosis.

EcAMP1 did not exhibit trypsin inhibitory activity. Instead, it was readily cleaved by trypsin at arginine residues to yield a number of fragments that were separated by RP-HPLC and identified by MALDI MS and Edman sequencing.

EcAMP1 Mode of Action—To visualize interactions of EcAMP1 with fungal spores, a fluorescently labeled peptide was used in confocal laser scanning microscopy experiments. Selective labeling of the peptide was easily achieved because the N-terminus contains the only amino group of the peptide. Biological activities of EcAMP1 and EcAMP1-TMR against F. solani were found exactly equivalent, thus justifying the application of EcAMP1-TMR in the intended studies.

At concentration equal to EC50 (4 μM), the interaction of EcAMP1 with F. solani spores featured protracted kinetics and was characterized by time-dependent changes in the peptide cellular distribution (Fig. 3). In 1 h after the addition of EcAMP1 to the spores, it was found to be intensively and uniformly bound to the spore envelope including septa. During the next several hours, penetration and accumulation of EcAMP1 in the cytoplasm occurred. In 6 h, EcAMP1 stained cytoplasmic cellular structures much brighter than the spore envelope. As analyzed with optical microscopy, this intracellular accumulation of EcAMP1 was not accompanied by any changes in the spore morphology. Essentially the same results were obtained with F. solani hyphae (not shown). Moreover, as probed with CF, having a hydrodynamic diameter of ~1 nm, the cytoplasmic membrane preserved integrity at all stages of the peptide-fungus interaction; CF was not able to penetrate into the spores and hyphae treated with EcAMP1 (data not shown).

Interestingly, at lower concentration (2 μM) producing ~30% spore germination inhibition, EcAMP1 localized predominantly to the cell wall region, and only a minor fraction of the peptide was internalized in 6 h after addition (Fig. 3). This might indicate intracellular localization of the specific target of EcAMP1 action.

The data obtained indicate that the mode of EcAMP1 action is inhibition of hyphae elongation without cytoplasmic mem-

TABLE 1

| Fungus | EC50 (μM) |
|--------|----------|
| A. alternata | 16.0 ± 2.3 |
| A. solani | 14.0 ± 2.1 |
| A. niger | >32 |
| B. sorokiniana | 18.2 ± 2.7 |
| C. graminicola | >10 |
| D. maydis | >10 |
| F. graminearum | 4.5 ± 1.4 |
| F. oxysporum | 8.5 ± 1.6 |
| F. solani | 4.0 ± 1.2 |
| F. verticillioides | 8.1 ± 2.3 |
| P. betae | 6.0 ± 1.5 |
| P. infestans | 16.3 ± 2.5 |
| P. debaryanum | 12.0 ± 1.7 |
| P. ultimum | 14.4 ± 2.1 |
| T. album | >32 |

FIGURE 2. EcAMP1 amino acid sequence alignment with similar peptides. Sequences of the following peptides are shown: EcAMP1 from E. crus-galli (P86698; this work); antimicrobial peptides MBP-1 from Z. mays (P28794 (31)) and 2d from M. integrifolia (Q95IPS (32)); C2 peptide processed from the PV100 protein in Cucurbita maxima (Q9ZW13 (54)); ribosome-inactivating peptide luffin P1 from Luffa aegyptiaca (P56568 (55)); trypsin inhibitors VhTI from V. hederfolia (P85981 (34)) and BWI-2b from Fagopyrum esculentum (no UniProt entry (33)). Each peptide length is indicated in the right column. Cysteine residues are in bold and shaded. Disulfide pairing has been determined for EcAMP1, VhTI, and BWI-2b.
brane lysis. The interaction of EcAMP1 with fungal cells may have a multitarget character. At the first stage, the peptide binds to one or several abundant components of *F. solani* cell surface, producing a uniform intense staining if labeled. At this point, we cannot infer the molecular identity of these components, which can reside in both the plasma membrane (for example, lipids) and the cell wall (for instance, chitin or glucan) (35). One cannot exclude that the target of EcAMP1 action is located in the plasma membrane or in the cell wall. Alternatively, intensive binding at the cell surface is just a prerequisite for subsequent peptide internalization. At the second stage, EcAMP1 is taken up by the fungus and accumulates in vesicular structures inside the conidium (probably vacuoles, or lipid bodies) (36). This may either represent an attempt of the fungus to circumvent the peptide action or testify in favor of an intracellular target of EcAMP1.

To our knowledge, such a peculiar behavior of plant AMP is reported for the first time. Most AMPs follow a membrane-disruptive mechanism (5), whereas EcAMP1 leaves fungal cells intact. Plant defensins DmAMP1 from dahlia (*Dahlia merckii*), RsAFP2 from radish (*Raphanus sativus*), and HsAFP1 from coral bells (*Heuchera sanguinea*) target specific binding sites on fungal cells (mannosyldiinositolphosphorylceramide from *Saccharomyces cerevisiae* for DmAMP1 (37) and glucosylceramide from *Pichia pastoris* for RsAFP2 (38)), leading to membrane permeabilization and probably triggering other processes and eventually decreasing viability (39). Among plant defense peptides exhibiting a non-disruptive mechanism of action, MsDef1, a defensin from alfalfa (*Medicago sativa*), was found to block L-type Ca\(^{2+}\) channels in mammalian cells. It was suggested that MsDef1 also targets Ca\(^{2+}\) channels in susceptible fungi and in this way interferes with hyphal elongation (40). Psd1 defensin from pea (*Pisum sativum*) was found to enter *Neurospora crassa* cells, localize to the nuclei, and interfere with the cell cycle, probably interacting with cyclin F (41). To the contrary, EcAMP1 localized to some vesicular structures inside *F. solani* cells. The principal targets (both extracellular and intracellular) of EcAMP1 are yet to be identified.

**EcAMP1 Spatial Structure**—The unique pattern of NOE contacts obtained for EcAMP1 at pH 6.6 (Fig. 4) demonstrates that the peptide forms a helical hairpin. The location of residues in a helical conformation is confirmed by the hydrogen-deute-
The spatial structure of EcAMP1 at pH 6.6 consists of two antiparallel \( \alpha \)-helices formed by the residues 7–14 and 22–30 that are stabilized by two disulfide bonds 7–29 and 11–25 and linked together by a type I \( \alpha \)-turn (residues 15–18) and a 310-helix turn (residues 19–21) (Figs. 4, bottom, and 5). N- and C-terminal tails (residues 1–6 and 31–37) are unstructured.

Identification of residues in 310- and \( \alpha \)-helical conformations was based on typical patterns of NOE contacts and backbone hydrogen bonds in the NMR ensemble of EcAMP1 identified by MOLMOL (hydrogen bonds were not restrained in structure calculation). The obtained pattern of hydrogen bonds correlates adequately with data on hydrogen-deuterium exchange measured at pH 3.4 (Fig. 4).

The 310-helix turn (residues 19–21) is stabilized by a salt bridge between Glu-18 and Arg-21 (Fig. 5B). The salt bridge formation is confirmed by three lines of argumentation. 1) Unambiguously assigned NOE contacts between \( \beta \)-protons of Glu-18 and \( \gamma \) - and \( \delta \)-protons of Arg-21 were detected. 2) The \( \epsilon \)-proton of Arg-21 exchanges slower with the solvent in contrast to \( \epsilon \)-protons of other arginine residues in EcAMP1; all cross-peaks from \( \epsilon \)-protons of other arginines in EcAMP1 undergo severe line broadening at pH 4.6–6.6, except those from the \( \epsilon \)-proton of Arg-21. 3) The minimum distance between atoms O1 or O2 of Glu-18 and atoms N of N\( \alpha \),N\( \beta \), or N\( \gamma \) of Arg-21 is less than 4 Å in 8 of 20 structures from the NMR ensemble (ensemble structures were not energy-minimized).

Despite the lack of secondary structure, some conformational preferences were found for the C-terminal tail of EcAMP1. In particular, the strong down-field shift of 9.9 ppm at pH 6.6 for the HN atom of Cys-7 along with its pH dependence (0.5 ppm in the pH range of 3.1–6.6) suggest the formation of a transient hydrogen bond with a carboxylate group. Hydrogen bond between backbone amide of Cys-7 and C-terminal or side chain carboxylate group of Asp-37 agrees well with the NOE contacts obtained for EcAMP1; the addition of restraints for this hydrogen bond in structure calculation does not change the target function significantly (0.37 ± 0.07 and 0.30 ± 0.07 Å² for structures with and without the hydrogen bond, respectively).

A small hydrophobic core between the helices of EcAMP1 is formed by the side chains of residues Met-12, Pro-19, Val-22,
Val-26, cysteines, and the aliphatic part of the Arg-8 side chain (Fig. 5A). The side chain of His-15 is buried into the hydrophobic core; it has a total of 6% accessible surface area as calculated with the MOLMOL program. The side chain of Trp-20 has NOE contacts that cannot be fulfilled by a single conformation. It is mobile and exposed to aqueous solution.

Analysis of pH dependences of chemical shifts in pH range of 3.1–6.6 (data not shown) gave a pK_a value of 5.5 for His-15 and pK_a values in the range of 3.4–4.0 for carboxylate groups. In EcAMP1, carboxylates are located nearly each other, which precludes the determination of their precise pK_a values.

**Comparison of EcAMP1 with Three-dimensional Structures of Other Peptides**—As outlined in the Introduction, the most widely accepted classification, although not irrefutable, envisages three major groups among AMPs: (i) linear peptides that form α-helices in contact with membranes; (ii) disulfide-containing with predominance of β-structural elements; and (iii) linear non-α-helix-forming, usually with a high content of certain amino acid residues (1, 2, 9). EcAMP1 clearly does not fit into any of these categories; it is a disulfide-containing α-helical peptide. To top that off, it represents a novel fold among AMPs, namely a disulfide-stabilized antiparallel α-helical hairpin. This type of fold, although unprecedented in AMPs, has been noted for several peptides from different sources (see below). The first example of an isolated α-helical hairpin stapled by two disulfide bridges, in particular, was noted for an excised fragment of p8_MTCP1, a protein coded by the human oncogene MTCP1 (42).

A similar fold occurs in some toxins including κ-hefutoxin-1 and OmTx1–3 from the scorpions *Heterometrus fulvipes* and *Opisthacanthus madagascariensis* (43, 44), flf14a-c and vil14a from cone snails *Conus floridanus floridensis* and *Conus villicepini* (45), and neurotoxin B-IV from the marine worm *Cerbratulus lacteus* (46). The mentioned scorpion toxins and conopeptides are very similar to EcAMP1; they are short molecules (22–27 residues) characterized by the same peculiar cysteine spacing C1–Xn–X–C3–C4. B-IV is longer (55 residues), and its two helices are cross-linked by four rather than two disulfide bridges. Interestingly, a similar disulfide-stabilized antiparallel α-helical hairpin fold has been assigned to the phytotoxic protein PcF (52 residues) from *Phytophthora cactorum* (47), but the disulfide connectivities differ significantly from those in EcAMP1.

**Plant Defense Peptide with Helical Hairpin Structure**

**TABLE 2**

| Parameter [Unit] | Value |
|------------------|-------|
| **NMR distance and dihedral restraints** | |
| No. of restraints | 100/20 |
| Target function [Å] | 0.30 ± 0.07 |
| Restraint violations (sum/max) | 1.7 ± 0.3/0.22 |
| Upper [Å] | 0.01/0.01 |
| Lower [Å] | 0.8 ± 0.2/0.15 |
| Angle [degree] | 0/0 |
| Mean r.m.s.d.* | 0.17 ± 0.06 |
| Backbone atoms of residues 7–30 | |
| Heavy atoms of residues 7–30 | 1.27 ± 0.18 |

**Ramachandran analysis**

- Residues in most favored regions [%] | 80.0 |
- Residues in additionally allowed regions [%] | 19.2 |
- Residues in generously allowed regions [%] | 0.8 |
- Residues in disallowed regions [%] | 0.0 |

* r.m.s.d., root mean square deviation.
* All glycine residues are located in N- and C-terminal tails and excluded from the analysis.
* Residues from N- and C-terminal tails.

**FIGURE 5. Spatial structure of EcAMP1.** A, ribbon diagram of the 20 best CYANA structures superimposed on the backbone atoms of residues 7–30. Buried side chains are displayed and labeled; those forming the hydrophobic core (Cys-7, aliphatic part of Arg-8, Cys-11, Met-12, Pro-19, Val-22, Cys-25, Val-26, and Cys-29) are colored green, and the side chain of His-15 is colored magenta. The side chain of Trp-20 is labeled and colored blue. Disulfide bridges are depicted as yellow sticks. The backbone H4 atom of Cys-7 involved in a transient hydrogen bond with a C-terminal carboxylate is depicted by a red sphere. The N and C termini are labeled. B, as compared with A, the structure is rotated as depicted in between the panels, and the side chains of Glu-18 and Arg-21 forming a salt bridge are shown, labeled, and colored red.
Plant Defense Peptide with Helical Hairpin Structure

The influenza hemagglutinin fusion domain has recently been shown to adopt an α-helical hairpin structure in a membrane-mimicking environment, but the observed structure is due to tight packing of glycine residues lying at the interface between helices, as in many membrane proteins (48). Among AMPs with a determined three-dimensional structure, only those of pardaxin (from the Red Sea Moses sole Parachaerus marmoratus) and MSI-594 (a synthetic variant of magainin, AMP from the frog Xenopus laevis) resemble EcAMP1. Both molecules are disulfide-free. Pardaxin forms a conventional helix-hinge-helix motif (49) and MSI-594 assumes an extended helix (50) in dodecylphosphocholine micelles, but they both fold into an antiparallel α-helical hairpin in lipopolysaccharide micelles (51, 52) with a peculiar hydrophobic core between the helices reminiscent of EcAMP1 (see above).

Most intriguingly, an α-helical hairpin with the same cysteine motif as in EcAMP1 is part of the fold characteristic of plant AMPs called thionins (53). Moreover, if excised from the parent AMP, the α-helical hairpin fragment retains much of the biological activity. One may therefore speculate on possible parallelism or convergence between thionins and 4-cysteine motif-containing AMPs.

Comparative analysis of three-dimensional structures deposited in the Protein Data Bank (PDB) revealed considerable similarity of EcAMP1 with the trypsin inhibitor VhTI from seeds of V. hederifolia (34). VhTI and EcAMP1 share the same cysteine motif in their sequences (Fig. 2) and are highly similar in the helical region. Moreover, both peptides feature exactly the same location of proline in their sequences (at position 19). The disulfide bridge 11–25 has similar conformations classified as g^nnnt (see “Experimental Procedures”) in both structures. The disulfide bridge 7–29 is in g^nnnt and tnnng conformations in EcAMP1 and VhTI, respectively. However, the crucial basic residue mediating recognition of VhTI by trypsin (Arg-15) is missing in EcAMP1. The latter peptide consequently shows no inhibitory activity against trypsin and is cleaved as a normal proteinaseic substrate.

Conclusion—In this study, we described EcAMP1, an AMP from E. crus-galli seeds with an unprecedented three-dimensional structure. This peptide is the first example of AMP with a disulfide-stabilized α-helical hairpin fold. In different plants, a number of EcAMP1 homologues featuring the same peculiar cysteine motif in the sequence were found. These peptides exhibit different functions (antimicrobial, enzyme-inhibitory, ribosome-inactivating) and group to a novel family of plant defense peptides; further structure-function investigations of the α-helical hairpin fold are anticipated. Moreover, EcAMP1 showed strong antifungal action toward species of the Fusarium genus; the mode of action of this peptide differs from other known AMPs. Therefore, EcAMP1 presents an attractive molecular scaffold for both fundamental studies and design of novel antimicrobials.

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