Trypsin inhibitory loop is an excellent lead structure to design serine protease inhibitors and antimicrobial peptides

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ABSTRACT The disulfide-bridged hendecapeptide (CWTKSIPPKPC) loop, derived from an amphibian skin peptide, is found to have strong trypsin inhibitory capability. This loop, called the trypsin inhibitory loop (TIL), appears to be the smallest serine protease inhibitor known. A series of synthetic peptides derived from this loop also exhibits trypsin inhibitory activity; some peptides even exhibit both antimicrobial and trypsin inhibitory activities. Antimicrobial peptides are attractive candidates for producing novel antibiotics, but their sensitivity to trypsin-like proteases appreciably limits their application. Bifunctional peptides with both antimicrobial and trypsin inhibitory activities could be ideal candidates for clinical antibiotics, since these reported synthetic peptides have shown resistance against trypsin. The crystal structure of a complex of trypsin with one TIL derivative is solved. The concept of TIL is introduced in this paper. Novel trypsin inhibitors or antimicrobial peptides can be designed readily on the basis of the TIL. Furthermore, functional analysis and a precursor comparison suggest that serine protease inhibitors may have a common ancestor with antimicrobial peptides.—Li, J., Zhang, C., Xu, X., Wang, J., Yu, H., Lai, R., Gong, W. Trypsin inhibitory loop is an excellent lead structure to design serine protease inhibitors and antimicrobial peptides. FASEB J. 21, 2466–2473 (2007)

Key Words: trypsin resistance

The growing problem of resistance to conventional antibiotics has stimulated the need to develop new human therapeutics. The gene-encoded antimicrobial peptides (AMPs) play an important role in innate immunity against noxious microorganisms (1–3). Since they cause much less drug resistance of microbes than conventional antibiotics, AMPs now attract considerable attention for the development of new antibiotics (1). However, the main obstacle impeding the systematic application of AMPs are the relatively large doses required, which are nearly toxic (1, 4). In addition, the hydrolysis of AMPs by trypsin-like proteases results in low absorbance efficiency. Therefore, a bifunctional peptide with both antimicrobial and trypsin inhibitory activities would be an ideal candidate as future anti-infective agents for clinical use.

Serine proteinase inhibitors are widely found in animals, plants, and microorganisms (5). Many pathogens are known to produce extracellular proteinases, which are reported to serve an active role in the development of various diseases. Several lines of evidence suggest that a major function of proteinase inhibitors is to combat the proteinases of pests and pathogens (5, 6). A few small proteinase inhibitors have been found in plants, including SFTI-1 from sunflower seeds, which is composed of 14 amino acids (7), and three macrocyclic trypsin inhibitors from squash seeds, composed of 34 amino acids (8). No similar small proteinase inhibitor has been found in animals. These proteinase inhibitors and antimicrobial peptides both serve defensive roles and exert a direct effect on pests. It is still not known whether there is an evolutionary connection between them.

In this paper we report the occurrence of a small serine protease inhibitor with antimicrobial capability in the frog, Odorrana grahami. Based on the disulfide-bridged hendecapeptide (CWTKSIPPKPC) loop of this
serine protease inhibitor, a series of excellent lead compounds of small bifunctional peptides that have both trypsin inhibitory and antimicrobial activities has been designed that could be applied to developing novel oral or other anti-infective agents. The crystal structure of the complex of trypsin-ORB2 is also solved. The possible evolutionary relationship between serine proteinase inhibitors and antimicrobial peptides is considered.

MATERIALS AND METHODS

Collection of frog skin secretions and peptide purification

Adult specimens of *O. grahami* of both sexes (*n*=30; weight range 30–40 g) were collected in the Yunnan Province of China. Skin secretions were collected and a lyophilized skin secretion sample of *O. grahami* (1.5 g, total OD280 nm of 400) was dissolved in 10 ml 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, Arlington Heights, IL, USA; 2.6×100 cm) gel filtration column equilibrated with 0.1 M phosphate buffer, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml. The absorbance of the eluate was monitored at 280 nm and the antimicrobial activities of fractions were determined as indicated below. The protein peaks containing antimicrobial activity were pooled (30 ml), lyophilized, and resuspended in 2 ml 0.1 M phosphate buffer, pH 6.0. The product was further purified on a C18 reverse-phase, high-performance liquid chromatography (RP-HPLC, Hypersil BDS C18, 30×0.46 cm) column (*Fig. 1B*).

Structural analysis

Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491 (Foster City, CA, USA). The actual molecular weight of the purified peptides was determined by fast atom bombardment mass spectrometry according to a previous report (9).

SMART cDNA synthesis and screening of cDNA encoding ORB

cDNA was synthesized using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA). The cDNA synthesized was used as a template for PCR to screen for the cDNAs encoding antimicrobial peptides. Two oligonucleotide primers were used in PCR reactions: S1 (5′-CCTAA (G/C) ATGTGACGACG-3′) in the sense direction, a specific primer designed according to the signal peptide sequences of antimicrobial peptides from ranid frogs; and primer II A, as mentioned in the “SMART cDNA Synthesis” manual, in the antisense direction. The DNA polymerase used was Advantage polymerase from Clontech. The PCR conditions were 2 min at 94°C, followed by 30 cycles of 10 s at 92°C, 30 s at 50°C, and 40 s at 72°C. Finally, the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA). DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Bioassays

Antimicrobial assays were performed according to our previous methods (9). Trypsin inhibitory activity was tested with the method described by Zhang et al. (10).

Cocrystallization with trypsin

Bovine β-trypsin was bought from Sigma (Catalog No. T1426; St. Louis, MO, USA). It was dissolved to ~60 mg/ml together with 10 mg/ml inhibitor ORB2K in 3 mM CaCl2, 10 mg/ml benzamidine, 0.02% sodium azide, 15% ethylene glycol, and 4% PEG4K. Crystals of ORB2K-trypsin complex were obtained at 289K using 0.5M CaCl2, 0.5M (NH4)2SO4, 0.1M HEPES-Na pH7.5, and 25% PEG4K employing the hanging drop vapor diffusion method. Diffraction data were collected at a mar345db detector system with a Rigaku MicroMax007 microfocus X-ray generation system in The Institute of High Energy Physics, Chinese Academy of Sciences. The data were processed with DENZO and SCALEPACK (11). Molecular replacement was done with Molrep (12) using the model of SFTI-1-trypsin complex (PDB code 1SFI). The final model was refined to 1.7 Å resolution by Refmac5 (13). The peptide ORB2K was fitted into the map manually. All manual adjustments were done in Coot (14). The stereochemical qualities of the final model were checked with PROCHECK (15).

Transmission electron microscopy

Transmission electron microscopy was utilized to study the possible mechanisms of ORB1 acting on Gram-positive bac-
teria, using the methods described by Friedrich et al. (16) with minor modifications. Exponential-phase bacteria were treated with the peptide of 10 × MIC for 30 min at 37°C, then centrifuged at 300 g for 10 min. The bacterial pellets were fixed with 2.5% buffered glutaraldehyde for 1 h. The cells were then postfixed with 1% buffered osmium tetroxide for 1 h, stained en bloc with 1% uranyl acetate, dehydrated in a graded series of ethanol, and finally embedded in white resin. Thin sections (LKB-V, Sweden) on copper grids were stained with 1% uranyl acetate and lead citrate. The buffer used was 0.1 M sodium cacodylate, pH 7.4. The resin and grids were purchased from Marivac (Halifax, Nova Scotia, Canada). Microscopy was performed with a JEM1011 microscope under standard operating conditions.

Synthetic peptides

All peptides used in this work were synthesized by AC Scientific Inc. (Xi An, China), with confirmed purity higher than 95% by HPLC and MALDI-TOF mass spectrometry. All peptides were dissolved in water.

RESULTS AND DISCUSSION

Identification of the smallest animal serine protease inhibitor

A short peptide ORB (AALKGCWTKSIPPKPCFGKR) was purified from skin secretions of the diskless-fingered odorous frog, O. grahami (Fig. 1A, B). ORB exerts strong antimicrobial activity but weak inhibitory activity against trypsin. The minimal inhibitory concentrations (MIC) of ORB for Staphylococcus aureus (ATCC2592), Escherichia coli (ATCC25922), Bacillus dysenteria, and Candida albicans (ATCC2002) are 3.20, 5.83, 1.84, and 2.40 μg/ml, respectively. The inhibitory constant for ORB against trypsin is 3.06 × 10⁻⁴. It is the smallest serine protease inhibitor ever found in animals. Based on the structure of ORB, the disulfide-bridged hendecapeptide (CWTKSIPPKPC) loop of ORB was synthesized and named ORB-C (Table 1). ORB-C was found to contain strong trypsin inhibitory capability but had lost its antimicrobial capability. ORB-C is the smallest serine protease inhibitor. The disulfide-bridged hendecapeptide loop CWTKSIPPKPC was named trypsin inhibitory loop (TIL).

The antimicrobial peptide and the protease inhibitor of amphibians are probably derived from a common ancestor

Several cDNAs (DQ672940-DQ672951) encoding the precursors of ORB were cloned from a skin cDNA library of O. grahami (Fig. 2). They are highly conserved and are found to be composed of 63 amino acid residues, including an N-terminal signal peptide, followed by an acidic spacer peptide and a C-terminal mature peptide. The mature peptide is similar to SFTI-1, the smallest serine protease inhibitor found in plants (8, 17) (Fig. 2). Aside from the mature peptide region, the overall structure of ORB precursors is quite similar to other precursors of antimicrobial peptides from this species, such as Nigrosin-OG1 (DQ672792; NOG1), and even those found from other amphibians, such as Brevinin-1E (18) (Fig. 2). It implies that ORB may have the same ancestor as amphibian antimicrobial peptides. Several reports have suggested that all amphibian AMPs originate from a common ancestor (1, 2). Based on the current study, we propose that amphibian AMPs and an amphibian serine protease inhibitor (i.e., ORB) share a common ancestor. Functionally, both antimicrobial peptides and some serine protease inhibitors are direct defensive molecules against microorganisms or pests. The functional similarity also implies their evolutionary connection. Apparently both protease inhibitors and AMPs from skin secretions of O. grahami are defensive molecules that serve as the first line of host defense against pests and pathogens, and have a similar overall structure, including the highly conserved signal peptides (Fig. 2). It is reasonable to assume that these two molecules might have a common ancestor, and AMPs likely are evolved from protease inhibitors, since the latter targets a wide spectrum of

**TABLE 1. The bioactivities of ORB and its homologues**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sequence</th>
<th>Trypsin inhibition (Kᵢ)</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>B. subtilis</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORB</td>
<td>AALKGCWTKSIPPKPCFGKR</td>
<td>3.06 × 10⁻⁴</td>
<td>3.20</td>
<td>5.83</td>
<td>1.85</td>
<td>2.40</td>
</tr>
<tr>
<td>ORB1</td>
<td>LKGWTKSIPPKPCF</td>
<td>4.00 × 10⁻³</td>
<td>2.34</td>
<td>1.76</td>
<td>2.34</td>
<td>4.69</td>
</tr>
<tr>
<td>ORB-CF</td>
<td>CWTKSIPPKPCF</td>
<td>2.20 × 10⁻³</td>
<td>8.90</td>
<td>5.96</td>
<td>10.50</td>
<td>19.4</td>
</tr>
<tr>
<td>ORB2-AAF</td>
<td>AALKGCWTKSIPPKPCFGF</td>
<td>2.70 × 10⁻³</td>
<td>2.84</td>
<td>2.12</td>
<td>2.44</td>
<td>4.76</td>
</tr>
<tr>
<td>ORB-CGF</td>
<td>GCWTKSIPPKPCF</td>
<td>7.50 × 10⁻⁴</td>
<td>ND</td>
<td>3.25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ORB2-K</td>
<td>LKGWTKSIPPKPCFGK</td>
<td>8.86 × 10⁻⁷</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ORB2-AK</td>
<td>-ALKGCWTKSIPPKPCFGK</td>
<td>7.58 × 10⁻⁷</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ORB2</td>
<td>LKGWTKSIPPKPCFG</td>
<td>6.85 × 10⁻⁷</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ORB2-F</td>
<td>LKGWTKSIPPKPCFGF</td>
<td>5.91 × 10⁻⁷</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ORB2-G</td>
<td>LKGWTKSIPPKPCFGG</td>
<td>6.10 × 10⁻⁷</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ORB-C</td>
<td>CWTKSIPPKPC</td>
<td>7.10 × 10⁻⁷</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ORB-O</td>
<td>CWTKSIPPKPG</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*MIC, minimal peptide concentration required for total inhibition of cell growth in liquid medium. ND, no detectable activity.*
pests and pathogens whereas AMP specifically acts on microorganisms. More proof, including functional analysis of synthetic peptides, for this hypothesis is discussed below.

**Functional analysis of synthetic peptides implies that trypsin inhibition is the primitive function**

A series of short peptides was designed and synthesized based on the structure of the ORB prototype. Their activities were also studied (Table 1). Five synthesized peptides contain both trypsin inhibitory and antimicrobial activities. They have significant activity for potential clinical therapeutic application, since they presumably resist the hydrolysis of trypsin-like proteases and have a much longer half-life. ORB-CGF, extending ORB-CF by a glycine at the C terminus, has a different antimicrobial spectrum than that of ORB-CF. ORB-CGF exerts antimicrobial capability only against *S. aureus*, which makes ORB-CGF a good template with which to design novel antimicrobial agents having a special bactericidal spectrum. Furthermore, it is apparent that all peptides except ORB with antimicrobial activity end with a phenylalanine following the disulfide-bridged hendecapeptide loop. The phenylalanine seems to be necessary for these peptides to exert the antimicrobial function.

Six other synthetic short peptides possessed only trypsin inhibitory activity (Table 1), which is much stronger than that of the five AMPs described above. ORB-C, composed of only 11 amino acid residues, has the strongest trypsin inhibitory ability, with a $K_i$ of $7.10 \times 10^{-7}$, which makes it the smallest peptidic serine proteinase inhibitor found so far. ORB-O without the disulfide-bridged hendecapeptide loop. The phenylalanine seems to be necessary for these peptides to exert the antimicrobial function.

The peptides synthesized in this study can all be divided into two families: one has both antimicrobial and trypsin inhibitory activities, and the other just has trypsin inhibitory activity. This phenomenon demonstrated that trypsin inhibition might be the basic or original function of this family of small peptides, which is consistent with the analysis of cDNA cloning: trypsin inhibition is the primitive function of the gene.

The factors inducing the tremendous function shift among these highly similar small peptides are still unclear. However, one possible explanation is the strong hydrophobicity of naked phenylalanine, which facilitates the aggregation of small peptides to form pore-like or channel-like structures on the cell membrane so as to kill microbes.

**Crystal structures of the complex ORB2K-trypsin suggest that ORB2K is a novel type of serine protease inhibitor from animals having the smallest molecular weight**

To reveal the mechanisms of ORB2 functions, the crystal structures of both complexes, ORB2-trypsin and ORB2K-trypsin, were solved. The two exhibit nearly the same structure except for one additional lysine observed at the C terminus of ORB2K. Because ORB2K-trypsin crystals diffracted at higher resolution, its structure will be described here (Fig. 3, PDB Accession No. 209Q). The 14 residues from $^{P5}$Gly to $^{P9}$Gly of the total 17 residues of ORB2K show clear electron density and adopt an antiparallel $\beta$-sheet conformation (Fig. 3A). All of the residues of ORB2K adopt reasonable conformations as determined by a Ramachandran plot. A disulfide bond between $^{P4}$Cys and $^{P7}$Cys forms an 11-residue reactive loop that binds to trypsin by an internal hydrogen network (Fig. 3A). The side chain of $^{P1}$Lys residue of ORB2K projects into the S1 pocket of trypsin and interacts directly with the carboxyl group of Asp189, the main chain carbonyl group of Ser-190, and two water molecules (Fig. 4). The two water molecules further form hydrogen bonds with the main chain carbonyl groups of Gly214, Trp221, and Val223. This $^{P1}$Lys determines the specificity of the inhibitor for trypsin (19).

The disulfide-mediated $\beta$-sheet structure and the $^{P1}$Lys-mediated binding mode described above demonstrate that ORB2K could be a new member of the
Bowman-Birk inhibitor family, but with novel characters. The consensus sequence of the reactive loop of the classical Bowman-Birk family is \( ^{P3}CTZXPPXC^{P6} \), where Z represents the P1 residue dictating the specificity (19). In ORB2K, the reactive loop is \( ^{P4}CWTKSIPPKPC^{P7} \), with two more residues of \( ^{P3}\text{Trp} \) and \( ^{P6}\text{Pro} \). The sequence and the conformation of residues \( ^{P2}\text{TKSIPPP}^{P4} \) are the same as those of SFTI-1 (20), the most potent naturally occurring plant Bowman-Birk inhibitor known so far (21) (Fig. 3B). The two prolines at \( ^{P5}Lys \) and \( ^{P7}Pro \) also adopt a cis-trans geometry to form a sharp turn as in SFTI-1, whereas two cysteines in ORB2K forming the disulfide bond are at \( ^{P3}Phe \) and \( ^{P6}Phe \) positions, different from the \( ^{P5}Lys \) and \( ^{P7}Pro \) positions in SFTI-1 (20). The disulfide-forming positions create a special 11-residue loop instead of the conserved 9-residue one in other Bowman-Birk inhibitors (21) (Fig. 3B). As far as we know, ORB2 and ORB2K are the only two Bowman-Birk inhibitors with an 11-residue reactive loop.

The disulfide bond of ORB2K is positioned close to trypsin instead of protruding out in SFTI-1 (Fig. 3B). As a result, the main chain of the \( ^{P6}Lys \) segment of ORB2K is more concave than SFTI-1 (Fig. 3B), which makes ORB2K closer to the enzyme surface. The structural differences may result from the insertion of \( ^{P5}\text{Trp} \) and \( ^{P6}\text{Pro} \) in ORB2K. Compared with the SFTI-1-trypsin complex (20), ORB2K binds trypsin with three more hydrogen bonds formed between the following: \( ^{P5}Lys \) and the carbonyl group of trypsin His58; the main chain ORB2K \( ^{P5}Gly \) and the main chain trypsin Gly214; and the main chain ORB2K \( ^{P8}Phe \) and the main chain trypsin Asn97 (Fig. 4). \( ^{P5}Lys \) is in the 11-residue reactive loop of ORB2K, whereas the corresponding residue is \( ^{P5}\text{Ile} \) in SFTI-1. Nonethe-

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**Figure 3.** A) Stereo view of the structure of ORB2K. The positions of each residue of ORB2K are labeled. B) Stereo view of the superimposition of the Co main chain of ORB2K and SFTI-1. P1, P3, and P6’ positions of ORB2K are labeled. For all pictures, trypsin is shown in green. ORB2K is colored blue; SFTI-1 is colored purple; disulfide bond is yellow. This figure was prepared using pyMol (http://pymol.sourceforge.net). The PDB Accession No. is 2O9Q.
less, two hydrogen bonds occurring in the SFTI-1-trypsin complex (20) are absent in the ORB2K-trypsin complex, which involves residues P9/Asp and P4/Arg of SFTI-1. The end conformation is another difference between ORB2K and SFTI-1, which is a cyclic peptide, whereas ORB2K is an acyclic one.

We also tried to cocrystallize ORB1 with trypsin, but failed. This is consistent with biological studies showing that ORB1 had very weak trypsin inhibitor activity. ORB1 is shorter than ORB2 by only a glycine at the C-terminal end (P9’ position). Although P9’ Gly is not directly involved in trypsin binding, the peptide bond between P8/Phe and P9’ Gly may help maintain the peptide conformation in order to be recognized by trypsin. In the trypsin-ORB2K complex, the side chain of P8/Phe is away from trypsin. Without P9’ Gly, the conformation of P8/Phe could be very flexible. The large side chain of ORB1 might collide with the trypsin surface, resulting in decreased trypsin binding ability.

ORB2K, a SFTI-1-like trypsin inhibitor, offers a new template for the design and improvement of the SFTI-1-based serine protease inhibitors, for its small size, the concave conformation of the main chain backbone, the new hydrogen bonds, and the preorganized rigid structure. In addition, the precursor and the mRNA of ORB2K reported here facilitate an understanding of the cyclization mechanism of SFTI-1, and hence help overcome the hurdle for industrial production, which is still unsolved for SFTI-1 because the repetition of Gly-Arg motif at either end of the mature sequence in its linear precursor provides three different cleavages (17) (Fig. 2).

Transmission electron microscopic analysis confirmed that ORB1’s antimicrobial capability is independent of its trypsin inhibitory capability

It has been observed that ORB1 exerted strong antimicrobial activities against all of the strains tested. To explain the possible mechanisms of ORB1 acting on Gram-positive Staphylococcus aureus ATCC 2592, transmission elec-
tron microscopy was performed according to the methods described by Friedrich et al. (16) on thin sections of bacteria treated with the peptide for 30 min. ORB1 kills bacteria by directly affecting its cell wall and membrane (Fig. 5). Large laminar mesosomes were seen to arise from the septa and cell wall of ORB1-treated bacteria, as indicated by the arrows in Fig. 5B, compared with no detectable mesosome structures in the control group of untreated bacteria (Fig. 5A), which may be indicative of cytoplasmic membrane alteration induced by the cationic peptides (16). Since the cytoplasmic membrane serves an important role in cell wall synthesis and turnover, perturbing it may also affect cell wall integrity and autolysin regulation. From the results of this study, the interface between the cell wall and membrane is not clear; in some regions, the interface even disappeared because of lysis of both or their separation. Moreover, it is clearly observed that fibers extend from the cell surface in the ORB1-treated bacteria, which together with the observations above suggests that ORB1 interacts with and disrupts the cytoplasmic membrane, leading to its dissolution and finally death of the cell itself. This process is similar to the putative mode of cationic antimicrobial peptides acting on Gram-positive bacterium. A condensed substance was found in the cytoplasm, which implies that an intracellular or alternative target probably exists in addition to important interactions with the cell walls. It could presumably be condensation of the DNA in this bacterium.

**SUMMARY**

ORB is the smallest serine protease inhibitor ever found in animals. This cDNA encoding the precursor of ORB has an overall structure similar to the known precursors of amphibian antimicrobial peptides. These discoveries suggest the possible evolutionary connection between antimicrobial peptides and serine protease inhibitors. cDNA and functional analysis of synthetic peptides suggest that antimicrobial peptides likely evolved from serine protease inhibitors and that both antimicrobial peptides and serine protease inhibitors have the same ancestor. The crystal structures of the complexes ORB2-trypsin and ORB2K-trypsin suggest that they are likely Bowman-Birk inhibitors, but the finding of an 11-residue reactive loop (Figs. 3, 4) that is different from the 9-residue reactive loop of other Bowman-Birk inhibitors renders the conclusion still tentative.

All antimicrobials contain multiple cationic residues that are easily destroyed by trypsin-like proteases. Thus, antimicrobial peptides with trypsin inhibitory capability should be excellent candidates for novel clinical antibiotics. A series of antimicrobial peptides with trypsin inhibitory activity and of small size are designed in this report. Their unique features give them high potential for both clinical and commercial applications (Table 1). Finally, the concept of the trypsin inhibitory loop has been introduced in this paper, so that different trypsin inhibitors or antimicrobial peptides can easily be designed on the basis of such a loop. This loop is also the smallest serine protease inhibitor known.

This work was supported by the Chinese National Natural Science Foundation (30570360, 30670456), Yunnan Natural Science Foundation (2005C0054M), and Jiangsu Natural Science Foundation (BK2005422). Professor Huw H. Rees provided friendly help with the writing for this paper.
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Received for publication December 17, 2006.
Accepted for publication February 15, 2007.