Isolation and Characterization of Bioactive Cyclotides from *Viola labridorica*

by **Jun Tang**[a])[c]), **Conan K. Wang**[b]), **Xulin Pan**[a]), **He Yan**[a])[c]), **Guangzhi Zeng**[a]), **Wenyan Xu**[a])[c]), **Wenjun He**[a])[c]), **Norelle L. Daly**[b]), **David J. Craik***[b]), and **Ninghua Tan***[a])

[a]) State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, P. R. China (phone: +86-871-5223800; fax: +86-871-5223800; e-mail: nhtan@mail.kib.ac.cn)

[b]) Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD 4072, Australia (phone: +61-7-3346-2019; fax: +61-7-3346-2029; e-mail: d.craik@imb.uq.edu.au)

[c]) Graduate School of the Chinese Academy of Sciences, Beijing 100049, P. R. China

Many Violaceae plants contain cyclotides, which are plant cyclopeptides distinguished by a cyclic cystine knot motif with 28–37 amino acid residues. In the current study, four new cyclotides, vila A–D (**1**–**4**, resp.), together with a known cyclotide, varv D (**5**), were isolated from *Viola labridorica* (Violaceae). A chromatography-based method was used to isolate the cyclotides, which were characterized using tandem mass spectrometry and 2D-NMR spectroscopy. Several of the cyclotides showed cytotoxic activities against five cancer cell lines, *i.e.*, U251, MDA-MB-231, A549, DU145, and BEL-7402, with vila A and B (**1** and **2**, resp.) being the most cytotoxic. The isolated cyclotides showed no antibacterial activity against *Staphyloccocus aureus* and *Candida albicans*. Homology modeling of the cyclotide structures was used to analyze structure–activity relationships.

Introduction. – Plant cyclopeptides are cyclic compounds comprising 2–37 amino acid residues [1]. The first plant cyclopeptide was structurally determined in 1959, and up to 2006 over 450 cyclopeptides have been isolated from higher plants belonging to 25 families, 65 genera, and 120 species. On the basis of their structural skeletons and distribution in plant species, cyclopeptides are structurally divided into eight types: cyclopeptide alkaloids, depsicyclopeptides, Solanaceae-type cyclopeptides, Urticaceae-type cyclopeptides, Compositae-type cyclopeptides, Caryophyllaceae-type cyclopeptides, Rubiaceae-type cyclopeptides, and cyclotides.

Cyclotides are disulfide-rich macrocyclic proteins of 28–37 amino acid residues, containing both a unique amide head-to-tail cyclized peptide backbone and a cyclic cystine knot (CCK) motif [2][3]. The CCK is a fascinating structural motif in which a small embedded ring formed by two disulfide bonds and their connecting backbone segments is threaded by a third disulfide bond. This motif produces a unique protein fold that is topologically complex and endows the protein with exceptional resistance to enzymatic breakdown and high chemical stability. In the early 1970s, the first cyclotide from the tropical African plant *Oldenlandia affinis* (Rubiaceae) was structurally determined [4], and was named kalata B1. The complete structural characterization of kalata B1 was not reported until some 20 years after the first report of its bioactivity [5]. The three-dimensional structure of kalata B1 is given in *Fig. 1*. Since the first bioactivity report, over 150 cyclotides have been isolated from *ca.* 30 plant species of the families

Violaceae, Rubiaceae, and Cucurbitaceae [6]. Cyclotides have a large range of bioactivities, including uterotonic [4], anti-HIV [7][8], anti-fungal [9], cytotoxic [10][11], antibacterial [9], nematocidal [12], molluscicidal [13], hemolytic [14], neurotensin antagonistic [15], insecticidal [16][17], and trypsin inhibitory [18] activities. The cyclotides are divided into two major subfamilies, *Möbius* and bracelet, depending on the presence or absence, respectively, of a *cis*-Pro peptide bond in loop 5 [2].

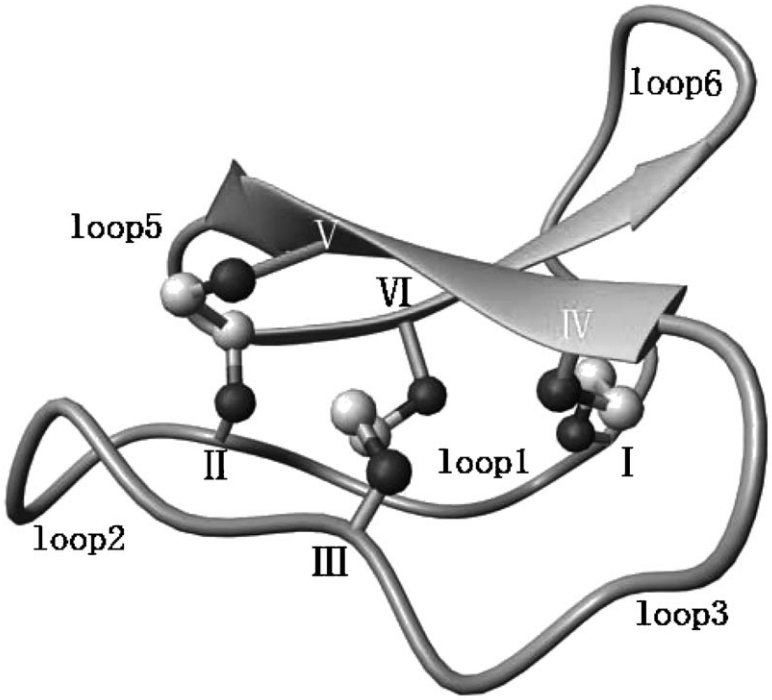

Fig. 1. *Structure of kalata B1* (PDB ID: 1NB1). Cyclotides have a well-defined three-dimensional structure stabilized by a cystine knot and a cyclic peptide backbone. Cyclotides have six loops and six Cys residues, labeled I – VI, and β-strands are indicated with arrows.

Viola labridorica SCHRANK., a common horticultural flower, is a member of the Violaceae family [19]. As cyclotide-containing plants often express a suite of cyclotides, it is of interest to study *V. labridorica* to identify novel cyclotides with therapeutic potential. In a preliminary study, three known cyclotides were reported: cycloviolacin O2 (cO2; **6**), varv A (**7**), and kalata B1 (**8**) [20]. In the current study, novel cyclotides from *V. labridorica* were isolated and characterized by MS/MS and two-dimensional (2D) NMR methods. The cyclotides were tested for cytotoxic and antibacterial activities, and their structure – activity relationships were analyzed by combining the assay data with homology modeling.

Results and Discussion. – The isolation of cyclotides from *V. labridorica* involved a modified version of previously published methods to extract and purify cyclotides from plants [21], and the TLC detection method that we have developed specifically for cyclotide detection from crude plant extracts was also used [22]. Briefly, plant materials were pre-extracted with $CHCl_3$ and subsequently extracted with 50% EtOH. The 50% EtOH extracts were then concentrated to an appropriate volume and subjected to polyamide chromatography to remove tannins. The tannin-free fraction was partitioned with AcOEt and BuOH. The BuOH fraction, which contained the cyclotides, was subjected to reversed-phase C_{18} and gel permeation *Sephadex LH-20* column chromatographies to remove carbohydrates, and other non-cyclotide constituents with low molecular weights. All of the separating steps were detected by silica-gel TLC plates with 0.2% ninhydrin EtOH solution after hydrolysis and improved *Coomassie* brilliant blue *G-250* solution [22][23]. Final purification was achieved by HPLC, and resulted in the isolation of four new cyclotides and one known cyclotide.

Compound **1** exhibited a $[M+2\,H]^{2+}$ ion peak at m/z 1584.93 in the ESI-MS (positive-ion mode), corresponding to a $[M+H]^{+}$ ion peak at m/z 3168.86. Reduction of compound **1** with tris(2-carboxyethyl)phosphine (TCEP) resulted in an increase in molecular weight of 6 Da, consistent with the presence of three disulfide bonds. Treatment of reduced **1** with trypsin/endoGlu-C produced six fragments with molecular weights of 2521, 2000, 1756, 1454, 1211, and 690 Da. The 2000- and 1211-Da fragments were analyzed by MS/MS, and their sequences were elucidated as SCVWIPCISSAIGCSCKDK and VCYRDGIPCGE, respectively. Analysis of the TOCSY and NOESY spectra of compound **1** indicated connectivities between spin systems consistent with the sequences CGESCVWI, SSAIGCSCK, and KVCYRDGI. The sequence of **1** was determined by overlapping the aforementioned peptides from 2D-NMR and MS/MS, and comparing these with the published cyclotides documented in Cybase [6]. The sequence was found to be cyclo-(GIP^{I}CGES^{II}CVWIP^{III}CISSAI-G^{IV}CS^{V}CKDKV^{VI}CYRD), a new cyclotide, named vila A (**1**).

A similar procedure was applied to determine the sequences of compounds **2**–**5**. Compound **2** exhibited a $[M+2\,H]^{2+}$ ion peak at m/z 1584.80 in the ESI-MS (positive-ion mode), corresponding to a $[M+H]^{+}$ ion peak at m/z 3168.60. After treatment with TCEP and trypsin/endoGlu-C or chymotrypsin/endoGlu-C, compound **2** provided seven fragments with 2365, 2521, 2000, 1210, 689, 540, and 846 Da. The 2365-, 2000-, 1210-, and 846-Da fragments were analyzed, and their sequences were elucidated as SCVWIPCISSAIGCSCRSKVCY, SCVWIPCISSAIGCSCRSK, VCYRDGIPCGE, and RDGIPCGE, respectively. Analysis of the TOCSY and NOESY spectra indicated the presence of the sequences CGESCVWI and SAIGCSCRSKVCYRDGI. The sequence of **2**, determined by the same methods as for sequence **1**, was cyclo-(GIP^{I}CGES^{II}CVWIP^{III}CISSAIG^{III}CS^{V}CRSKV^{VI}CYRD), a new cyclotide, named vila B (**2**).

Compound **3** exhibited a $[M+2\,H]^{2+}$ ion peak at m/z 1628.55 in the ESI-MS (positive-ion mode), corresponding to $[M+H]^{+}$ ion peak at m/z 3256.10. After treatment with TCEP and proteolytic enzymes as in **2**, **3** produced seven fragments with 2013, 1770, 1284, 2379, 1903, 1394, and 919 Da. The fragments of 2013, 1284, 2379, and 919 Da were analyzed by MS/MS, and their sequences were elucidated as SCVWIP-CISSVVGCSCKDK, VCYKDGTLPCGE, SCVWIPCISSVVGCSCKDKVCY, and

KDGTLPCGE, respectively. Analysis of the TOCSY and NOESY spectra indicated the presence of the sequences CGESCVWI and VGCSCKDKVCYKDGT. The sequence of **3** was determined, by the same means as **1** and **2**, as cyclo-(GTLP^{I}CGE-S^{II}CVWIP^{III}CISSVVG^{IV}CS^{V}CKDKV^{VI}CYKD), a new cyclotide, named vila C (**3**).

Compound **4** exhibited a $[M+2\,H]^{2+}$ ion peak at *m/z* 1475.17 in the ESI-MS (positive-ion mode), corresponding to $[M+H]^{+}$ ion peak at *m/z* 2949.34. After treatment with TCEP and proteolytic enzymes as in **2**, **4** produced three fragments with 2204, 788, and 2127 Da. The two larger fragments were analyzed by MS/MS, and their sequences were elucidated as TCAFGTCYTGGCSCSWPVCTR and TGGCSCSWPVCTRNGIPVCGE, respectively. Analysis of the TOCSY and NOESY spectra indicated the presence of the sequences VCGETCAFGTC, GGCSCS, and VCTRNGI. By overlapping of the above peptides from MS/MS and 2D-NMR, and comparison with cyclotides published in Cybase [6], the sequence of **4** was determined as cyclo-(GIPV^{I}CGET^{II}CAFGT^{III}CYTGG^{IV}CS^{V}CSWPV^{VI}CTRN), a new cyclotide, named vila D (**4**).

Compound **5** was confirmed to be varv D, which has been isolated previously from *V. arvensis* [11].

Based on the high similarities of cyclotide sequences, and the presence or absence of a *cis*-Pro peptide bond in loop 5 (the existence of a *cis*-Pro peptide bond in loop 5 in *Möbius* cyclotides was confirmed by several investigations [2][16][24][25]), the five aforementioned cyclotides, and three known cyclotides, cO2 (**6**), varv A (**7**), and kalata B1 (**8**), isolated from *V. labridorica* [20], can be classified in the *Möbius* and bracelet subfamilies. Specifically, vila D (**4**), varv D (**5**), varv A (**7**), and kalata B1 (**8**) belong to the *Möbius* subfamily, whereas vila A (**1**), B (**2**), C (**3**), and cO2 (**6**) are in the bracelet subfamily (*Fig. 2*). Several characteristics are common to the *Möbius* and bracelet subfamilies; namely, the presence of the six Cys residues that, with the cyclic backbone, form the CCK motif, the conserved Gly-Glu-Thr/Ser sequence in loop 1, and Ser/Thr in loop 4. In the remaining loops, the *Möbius* cyclotides have higher sequence homology

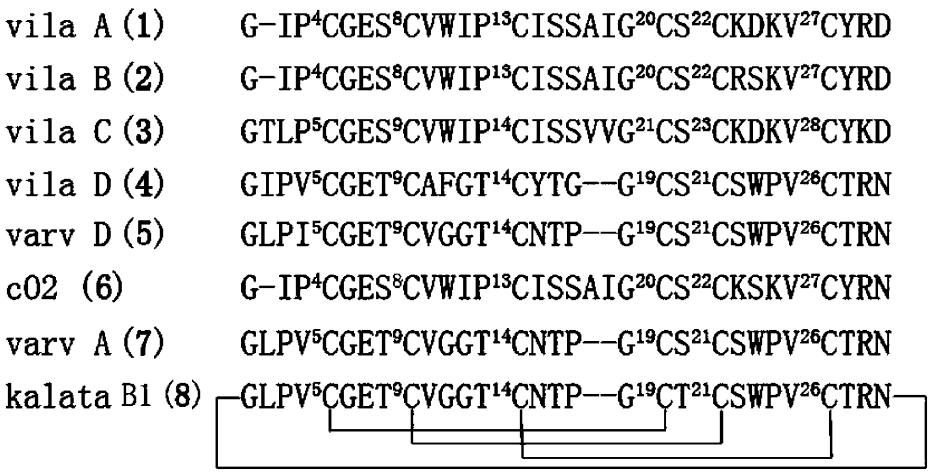

Fig. 2. *Sequences of cyclotides* **1**–**8** *isolated from* V. labridorica

than the bracelets. Compared with kalata B1 (**8**), the prototypic cyclotide vila D (**4**) differs only in six residues. The novel bracelet cyclotides vila A, B, and C (**1**–**3**, resp.) all have the Val-Trp-Ile-Pro sequence in loop 2, and the rich basic amino-acid residue sequence in loop 5, similar sequences in loop 6, and variable sequences in loop 3, which has been shown to form an α helix in cycloviolacin O1 [26].

The cyclotides **1**, **2**, and **4**–**6** were tested for toxicity against six human cancer cell lines: U251, MDA-MB-231, A549, DU145, BEL-7402, and BGC-823. The results are compiled in the *Table*, and the most potent cyclotides were found to be bracelet cyclotides vila A (**1**), B (**2**), and cO2 (**6**). cO2 has been shown to have cytotoxic activity [27] and was used as a control in the current study. Vila A (**1**) differs only in two residues from cO2 (**6**); in **1**, both a Ser-to-Asp substitution occurs in loop 5, as does a Asn-to-Asp substitution in loop 6. Interestingly, the introduction of the two negative charges into **1** does not influence the cytotoxicity significantly, whereas cationic peptides have been shown to exhibit stronger cytotoxic activity [28].

Table. *Cancer-Cell Toxicity of Cyclotides* **1**, **2**, *and* **4**–**6** *from* V. labridorica

Cyclotides	IC_{50} [μg/ml]					
	U251	MDA-MB-231	A549	DU145	BEL-7402	BGC-823
vila A (**1**)	7.08	5.13	> 10	5.08	5.80	> 10
vila B (**2**)	34.65	8.25	> 10	6.34	6.25	> 10
vila D (**4**)	49.59	> 10	> 10	> 10	> 10	> 10
varv D (**5**)	46.62	> 10	> 10	> 10	> 10	> 10
cO2 (**6**)	17.05	4.81	5.99	5.08	6.07	> 10

The five cyclotides, **1**, **2**, and **4**–**6**, were also tested for antibacterial activity against *Staphylococcus aureus* and *Candida albicans*. The results showed that the cyclotides were not active against them at 10 μg/ml.

The structures of cyclotides **1**, **2**, and **4**–**6** were constructed by Modellor 9v5, optimized by Amber 10, and analyzed by Sybyl 7.3 (*Fig. 3*). The results revealed that the most hydrophobic patches on the molecular surface are formed by different amino acid residues in the two subfamilies. In the *Möbius* cyclotides, the most hydrophobic patch is formed by Val/Phe in loop 2 and Trp and Pro in loop 5, whereas, in the bracelet cyclotides, this is formed by Val, Trp, Ile, and Pro in loop 2. Trp was found to be the center area of hydrophobic patches in both of these subfamilies. The results also showed that the hydrophobic patches are in closer proximity to one another on the surface of the bracelet cyclotides compared with the *Möbius* cyclotides; additionally, the bracelet cyclotides exhibit higher cytotoxicity against human cancer lines. From the analysis of the molecular surface, cytotoxicity seems to be related to the distribution of the hydrophobic areas on the structural surface.

In summary, we have improved techniques associated with the isolation and detection of cyclotides, and have used these methods to isolate and purify five cyclotides from *V. labridorica*, four of which are novel peptides, *i.e.*, vila A–D (**1**–**4**, resp.). Among them, together with one other known cyclotide cO2 (**6**), the bracelet cyclotides are more cytotoxic. In this study, no cyclotides were active against a bacteria or fungus. By analyzing molecular structures of cyclotides by homology modeling, it

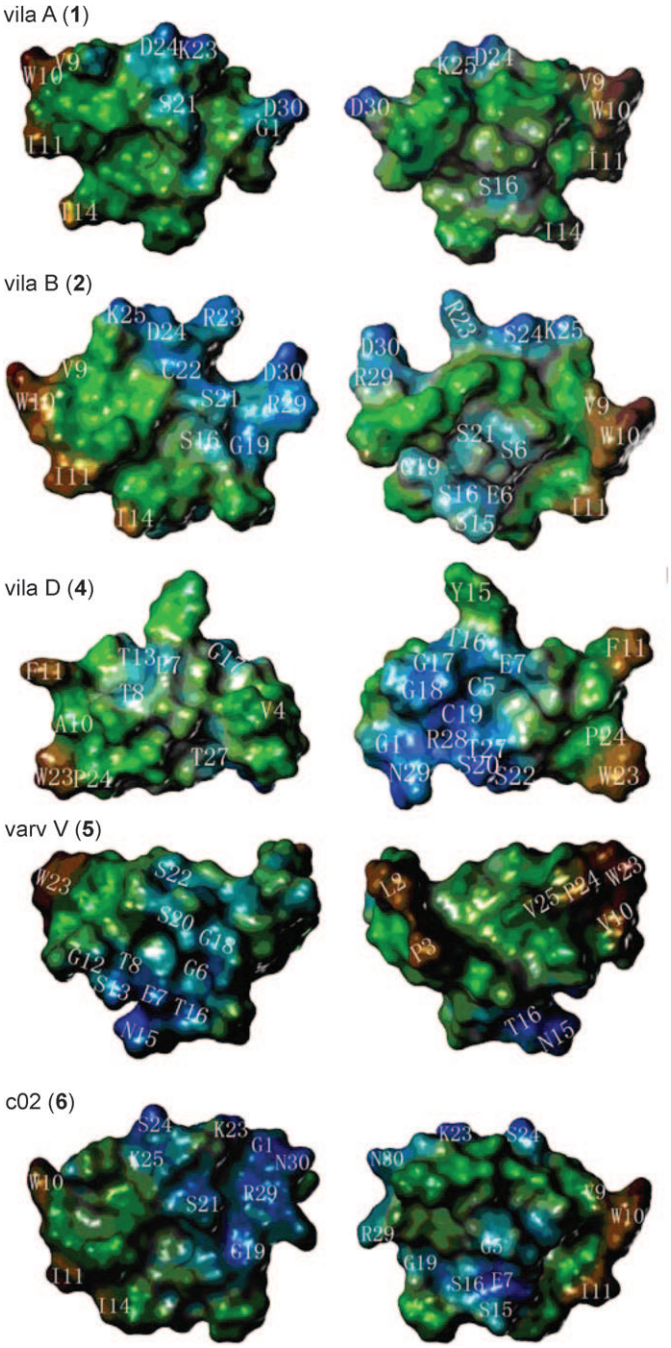

Fig. 3. *Surface representations of cyclotides* **1**, **2**, *and* **4**–**6** *from* V. labridorica. Most hydrophobic areas are shaded brown, moderately hydrophobic areas are shaded green, and hydrophilic areas are shaded blue.

appears that hydrophobicity of the molecular surface affects cytotoxicity. This finding will provide new insight into designing drugs based on cyclotides.

Experimental Part

General. HPLC: *Agilent 1100* series system with a UV detector at variable wavelengths of 206, 215, 225, 254, and 280 nm. ESI-MS: *Micromass LCT* mass spectrometer. MALDI-TOF-MS: *Voyager DE-STR* mass spectrometer (*Applied Biosystems*); 200 shots per spectrum were acquired in positive-ion reflector mode; the laser intensity was set to 2300, the accelerating voltage to 20000 V, and the grid voltage to 64% of the accelerating voltage; the delay time was 165 ns; the low-mass gate was set to 500 Da; data were collected between 500 and 5000 Da; calibration was undertaken using a peptide mixture obtained from *Sigma Aldrich* (MSCal1). Nanospray MS/MS analysis: *QStar* mass spectrometer; a cap. voltage of 900 V was applied, and the spectra were acquired at m/z 300–2000 for TOF spectra, and m/z 60–2000 for product-ion spectra; the collision energy for peptide fragmentation was varied between 10 and 50 V, depending on the size and charge of the ion; the analyst software program was used for data acquisition and processing.

Plant and Cell Materials. V. labridorica was collected from the Kunming Botanical Garden in Kunming, P. R. China, in November 2005. A voucher sample was deposited with the KUN herbarium, Kunming Institute of Botany, Chinese Academy of Sciences. Cancer cell lines, *i.e.*, U251, MDA-MB-231, A549, DU145, BEL-7402, and BGC-823, were purchased from the Cell Culture Center of the Institute of Basic Medical Science, Chinese Academy of Medical Sciences, Beijing, P. R. China. Bacteria *Staphylococcus aureus* and *Candida albicans* were purchased from China General Microbiological Culture Collection Center, Beijing, P. R. China.

Isolation and Purification of Cyclotides. Dried whole herbs of *V. labridorica* (10 kg) were pre-extracted by maceration for 24 h each time with $CHCl_3$ twice at r.t. The plant residue was extracted with EtOH/H_2O 1:1 (*v*/*v*) three times at r.t. After concentration of the EtOH/H_2O extracts to an appropriate volume, the liquid extract was subjected to polyamide chromatography to remove tannins. The tannin-free fraction was partitioned with AcOEt and BuOH. The BuOH fraction was separated by reversed-phase (RP) C_{18} chromatography eluted with 40% EtOH, 70% EtOH, and 95% EtOH, and finally 70% EtOH fraction by gel permeation on *Sephadex LH-20* eluted with 70% EtOH. All the separating steps were detected by TLC with 0.2% ninhydrin EtOH soln. after hydrolysis and improved *Coomassie* brilliant blue *G-250* soln. Final purification was achieved by HPLC. The yields of vila A–D, and varv D (**1**–**5**, resp.) were 2, 3, 2, 2, and 20 mg, resp.

Vila A (**1**): $C_{134}H_{207}N_{36}O_{41}S_6$; ESI: 3168.86 ($[M+H]^+$, 30 residues, +1 charge).

Vila B (**2**): $C_{133}H_{207}N_{38}O_{40}S_6$; ESI: 3168.60 ($[M+H]^+$, 30 residues, +1 charge).

Vila C (**3**): $C_{137}H_{212}N_{37}O_{43}S_6$; ESI: 3256.10 ($[M+H]^+$, 31 residues, +2 charge).

Vila D (**4**): $C_{123}H_{179}N_{34}O_{39}S_6$; ESI: 2949.34 ($[M+H]^+$, 29 residues, neutral charge).

Varv D (**5**): $C_{116}H_{178}N_{35}O_{39}S_6$; ESI: 2878.70 ($[M+H]^+$, 29 residues, neutral charge).

Reduction of Peptides and MALDI-MS Analysis. To 6 nmol of cyclotides in 20 µl of 0.1M NH_4HCO_3 (pH 8.0), 1 µl of 0.1 mM tris(2-carboxyethyl)phosphine (TCEP) was added, and the soln. was incubated at 55° for 30 min. The reduction was confirmed by MALDI-TOF-MS after desalting with *Ziptips* (*Millipore*), which involved several washing steps, before elution in 10 µl of 80% MeCN (0.5% HCO_2H). The desalted samples were mixed in a 1:1 ratio with a matrix consisting of a sat. soln. of α-cyano-4-hydroxcinnamic acid (CHCA) in 50% MeCN (0.5% HCO_2H).

Enzymatic Digestion and Nanospray MS/MS Sequencing. To the reduced cyclotides, a combination of endo-GluC and trypsin, or a combination of endo-GluC and chymotrypsin was added, to give a final cyclotide-to-enzyme ratio of 50:1, and the mixture was incubated for 3 h at 37°. The digestions were quenched by the addition of an equal volume of 0.5% HCO_2H and desalted using *Ziptips* (*Millipore*). Samples were stored at 4° prior to analysis. The fragments resulting from the digestions were first examined by MALDI-TOF-MS, followed by nanospray MS/MS analysis on a *QStar* mass spectrometer. MS/MS Data were examined and used to sequence the cyclotides based on *b*- and *y*-series of ions present (N- and C-terminal fragments).

NMR Sample Analysis. Samples of cyclotides were dissolved in H_2O/CD_3CN 9:1 to a concentration of 1 mM at pH 3. Spectra were recorded on a *Bruker ARX 600* or *DRX 500* spectrometer at a sample temp. of 298 K. For resonance assignment, a set of two-dimensional TOCSY, with a mixing period of 80 ms, and NOESY, with a mixing time of 200 ms, were recorded. All 2D spectra were collected over 4096 data points in the f2 dimension, and 512 increments in the f1 dimension and processed using TOPSIN (*Bruker*). Chemical shifts were referenced to H_2O at 4.75 ppm.

Homology Modeling. The sequences of cyclotides were determined by using mass spectra and 2D-NMR. Their structures were then modeled on the template structures, according to the results of searching *Brookhaven Protein Data Bank* (*PDB*) by the building method of Modeller 9v5 [29]. The homology modeling of these cyclotides contains three main steps:

1. *Homology Modeling.* All template sequences were chosen from the *PDB* by searching for homologous sequences to *V. labridorica* cyclotides using the Composer Package of Modeller 9v5. The structure of vila D (**4**) was aligned to kalata B2 (PDB ID: 1PT4); the structure of varv D (**5**) was aligned to kalata B1 (**8**; PDB ID: 1NB1); the structures of vila A, B, and cO2 (**1**, **2**, and **6**, resp.) were aligned to vhr 1(PDB ID: 1VB8). The 3D structures of these cyclotides were then built according to the alignment of sequences using the Build Package of Modeller 9v5.

2. *Optimizing for Structural Models.* The 3D structures of cyclotides were selected using the scoring function of Modeller, and further optimized by Amber10 using FF03 force field. The H-atoms were added by xLEaP module of Amber10, and each system was solvated by adding a spherical TIP3P H_2O grid of radius 12 Å. 2000 Cycle minimizations were performed, including 1000 of steepest descent and 1000 of conjugate gradient energy minimizations based on 10.0 kcal/mol Å restraints. Additional energy minimizations were performed, with restraints set to 0.0 kcal/mol $Å^2$ [30].

3. *Analysis for Structures.* Electrostatic potential and the hydrophobic surface of optimized structures of cyclotides were generated by MolCAD package of Sybyl 7.3 [31].

Cytotoxic Assays. The sulphorhodamine B (SRB) assay has been adopted for a quant. measurement of cell growth and viability [32]. Cells were cultured in *RPMI 1640* medium (*Sigma*). Aliquots of 90 µl were seeded in 96-well flat-bottomed microtiter plates (*Greiner*) with $3.3-7.7 \times 10^4$ cells/ml. In the following 24 h, 10 µl cyclotides, dissolved in DMSO and diluted with the medium, were added to the well with a final concentration of 10 µg/ml. After incubation at 37° and 5% CO_2 for 48 h, cells were fixed by the addition of 50% ice-cold CCl_3COOH and left at 4° for 1 h. After washing, air-drying, and staining for 15 min with 100 µl 0.4% SRB in 1% glacial AcOH, excessive dye was removed by washing with 1% glacial AcOH. After air-drying of the plates, SRB was resuspended in 100 µl of 10 mM *Tris* buffer, and the absorbance was recorded at 560 nm with a Plate Reader (*Molecular Devices*, *SPECTRA MAX 340*). Cell growth inhibition was expressed as IC_{50} values (50% inhibitory concentration), which were calculated by dose–response curves with serial five-fold cyclotides dilutions. Taxol was used for comparison.

Antibacterial Assays. The anti-microbial experiment was conducted according to the turbidimetric method on bacteria *Staphylococcus aureus* and *Candida albicans*. *S. aureus* and *C. albicans* were inoculated in *Mueller Hinton Broth* (*Oxiod*, *CM0405*, Hampshire, England) to *McFarland 0.5* and diluted with medium to 1×10^6 CFU/ml. Aliquots of 90 µl were filled in 96-well U-bottomed microplate. Cyclotides, dissolved in DMSO and diluted with the medium totally a volume of 10 µl, were dispended in the wells with the final concentration of 10 µg/ml. After culturing at 37° for 24 h, absorbance was recorded at 620 nm with the aforementioned microplate reader. The level of inhibition was calculated as the percentage of maximum absorbance (0.04% DMSO control) after adjusting for minimum absorbance (medium control). Ampicllin (*Keygen*, China) was used for comparison.

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