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Isolation and characterization of cytotoxic cyclotides from *Viola philippica*

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ABSTRACT

Cyclotides are a large family of plant peptides characterized by a macrocyclic backbone and knotted arrangement of three disulfide bonds. This unique structure renders cyclotides exceptionally stable to thermal, chemical and enzymatic treatments. They exhibit a variety of bioactivities, including uterotonic, anti-HIV, cytotoxic and hemolytic activity and it is these properties that make cyclotides an interesting peptide scaffold for drug design. In this study, eight new cyclotides (Viphi A–H), along with eight known cyclotides, were isolated from *Viola philippica*, a plant from the Violaceae family. In addition, Viba 17 and Mram 8 were isolated for the first time as peptides. The sequences of these cyclotides were elucidated primarily by using a strategy involving reduction, enzymatic digestion and tandem mass spectroscopy sequencing. Several of the cyclotides showed cytotoxic activities against the cancer cell lines MM96L, HeLa and BGC-823. The novel cyclotides reported here: (1) enhance the known sequence variation observed for cyclotides; (2) extend the number of species known to contain cyclotides; (3) provide interesting structure–activity relationships that delineate residues important for cytotoxic activity. In addition, this study provides insights into the potential active ingredients of traditional Chinese medicines.

1. Introduction

Cyclotides are a family of small disulfide-rich and plant-derived peptides made up of ~30 amino acids. They contain a unique structural motif that consists of a head-to-tail cyclic backbone and knotted arrangement of three disulfide bonds, together referred to as the cyclic cystine knot (CCK) [4]. This motif engenders cyclotides with exceptional resistance to thermal, chemical and enzymatic degradation [2], and they are therefore an attractive scaffold for the design of peptide-based therapeutics [1,9,15].

The six backbone loops between the conserved cysteine residues display a variety of sequences and provide cyclotide-containing plants with a natural combinatorial library of bioactive peptides. The sequence variation occurs mainly in loops 2, 3, 5 and 6, with loops 1 and 4 being more highly conserved [42]. The cyclotide family is divided into two major subfamilies, Möbius and bracelet, depending on the presence or absence, respectively, of a *cis*-Pro peptide bond in loop 5 [4].

It is estimated that the total number of cyclotides in the Violaceae plant family alone exceeds 9000 [29]. So far, more than 200 cyclotides have been isolated from approximately 30 plants in the Violaceae, Rubiaceae, Cucurbitaceae and Fabaceae families [22,42]. Cyclotides exhibit a range of interesting bioactivities, including uterotonic [26,31], anti-HIV [10], antimicrobial [23,33], cytotoxic [12,18,32,36], hemolytic [28,36], neurotensin antagonistic [43], insecticidal [16], and trypsin inhibitory activities [11].

Like other cyclotide-containing plants (for example *Oldenlandia affinis*), *Viola philippica*, from the Violaceae plant family, has been used in traditional Chinese medicines. The ornamental flower is native to China, North Korea and Japan and is used for heat-clearing, detoxification, anti-inflammation and pain relief. Additionally, the tender leaves are an edible wild vegetable [20]. To date, there has been limited research on the bioactive constituents of *V. philippica* with the only previous study reporting the isolation of flavone constituents [5]. As part of our investigation of bioactive cyclotides [34–37,44], eight new cyclotides and eight known cyclotides with cytotoxicity were characterized from *V. philippica*.

2. Materials and methods

2.1. General experimental procedures

For MALDI-TOF MS analysis, a Voyager DE-STR mass spectrometer (Applied Biosystems) was used and data were collected between 300 and 4000 Da. Nanospray MS/MS experiments were conducted using the QStar Pulsar mass spectrometer (Applied

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Biosystems) and ion spray voltage was applied between 900 and 1100 V. To analyze fractions using electrospray ionization (ESI), a mass range of 200–2000 was used; the multiply charged ions were then deconvoluted to yield neutral masses within the cyclotide mass range (2500–4000 Da). The collision energy for cyclotide fragmentation was varied between 15 and 90 V. Semipreparative RP-HPLC was performed on a HP 1100 Series HPLC (Agilent Technologies) equipped with a UV detector, Phenomenex Jupiter C18 (10 mm × 250 mm, 5 μm, 300 Å) columns at a flow rate of 3 mL/min and a Phenomenex Jupiter C18 (4.6 mm × 250 mm, 5 μm, 300 Å) column at a flow rate of 1 mL/min. Analytical RP-HPLC was performed using a Phenomenex Jupiter C18 (2 mm × 150 mm, 5 μm, 300 Å) column at a flow rate of 0.3 mL/min (solvent A: Milli-Q water with 0.05% TFA; solvent B: 90% acetonitrile in Milli-Q water with 0.045% TFA). LCMS was carried out on a HP 1100 Series HPLC (Agilent Technologies) that was connected to a ESI-TOF mass spectrometer (Applied Biosystems) at a flow rate of either 0.3 or 1 mL/min (solvent A: Milli-Q water with 0.1% formic acid; solvent B: 90% acetonitrile in Milli-Q water with 0.1% formic acid).

2.2. Plant material

Plant material from *V. philippica* was collected from Kunming Botanical Garden, Kunming, Yunnan Province, China, in May 2010. The plant was identified by Prof. Heng Li, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (No. 0917566) was deposited at the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation of cyclotides

Air-dried and powdered plant material of *V. philippica* (874 g) was extracted with 1:1 CH_2Cl_2/MeOH at room-temperature and filtered through a cotton wool plug [41]. The filtrate was transferred into a separating funnel, and Milli-Q water was added to separate the mixture into organic and aqueous layers. The aqueous layer was collected and evaporated to remove the solvent using a rotary evaporator and a freeze-drier. The lyophilized crude extract (23 g) was loaded onto a C18 flash column and purified using MeOH/H_2O. Fractions were analyzed by ESI MS and purified by RP HPLC to afford Viphi A and Mram 8 (12 mg), Viphi B (5 mg), Viphi C (2 mg), Viphi D and E (5 mg), Viphi F and G (8 mg), Viphi H (2 mg), Viba 17, Viba 15, Varv A and Kalata B1 (88 mg), Viba 11 (15 mg), cycloviolacin O2 (cO2; 20 mg) and cycloviolacin O12 (cO12; 42 mg).

2.4. Reduction and enzymatic digestion of cyclotides

A sample of 20 μg of native cyclotide in 20 μL of NH_4HCO_3 (0.1 M) was added to 1 μL of freshly prepared TCEP (0.1 M), and the solution was incubated under nitrogen at 55 °C for 30 min.

To the resulting reduced cyclotides, 5 μL trypsin (40 μg/mL) and 5 μL endoGlu-C (40 μg/mL) or 5 μL chymotrypsin (40 μg/mL) and 5 μL endoGlu-C (40 μg/mL) were added and incubated at 37 °C for 3 h. The samples were desalted using Ziptips (Millipore, C18) and stored at −20 °C prior to analysis. The fragments resulting from the digestion were first examined by MALDI-TOF MS, after which nanospray MS/MS analysis was conducted. The MS/MS spectra were examined and the peptides sequenced on the basis of the presence of both b- and y-series of ions (N- and C-terminal fragments) [3,41].

2.5. Cytotoxicity assay

Cytotoxicity of cyclotides on melanoma cells (MM96L), cervical cancer cells (HeLa) and foreskin fibroblast cells (HFF-1) were measured with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded in a 96 well flat-bottomed plate at a density of 5.0×10^4 cells/cm^2 in RPMI (Gibco BRL) in 10% FBS, DMEM in 10% FBS, DMEM in 15% FBS, respectively, and maintained at 37 °C in an atmosphere of 5% CO_2. Twenty-four hours later, cyclotides were added in duplicate at concentrations ranging from 0.1 μM to 100 μM to a final volume of 100 μL in each well. After being incubated for another 5 h, 10 μL of MTT (Sigma) solution in PBS (5 mg/mL) was added to each well before a further 1 h incubation. Media was removed, and formazan crystals were resuspended in 100 μL of DMSO. Absorbance was then read at 600 nm. Five percent DMSO and cO2, and cells with vehicle (H_2O) were used as the positive and negative controls, respectively. A sigmoidal dose–response curve was plotted using GraphPad Prism for statistical analysis.

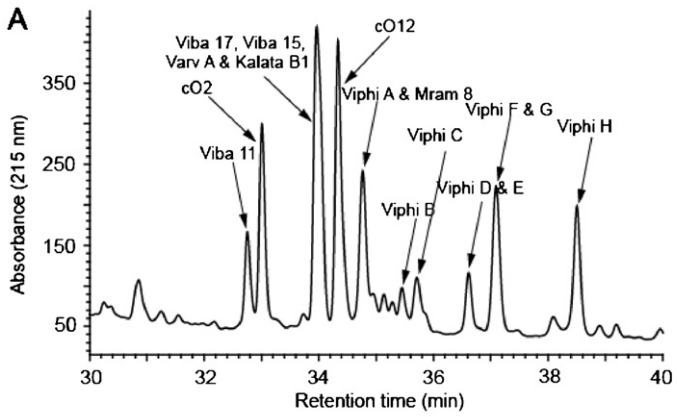
The cytotoxicity of cyclotides on human gastric cancer cells (BGC-823) was measured using sulforhodamine B (SRB), a method described in our previous publication [36]. Cells were cultured in RPMI-1640 in 10% FBS. Cyclotides, dissolved in DMSO and diluted with medium, were added in triplicate at concentrations ranging from 0.08 to 10 μg/mL to a final volume of 100 μL per well. Taxol and medium containing 0.4% DMSO were used as the positive and negative controls, respectively.

3. Results

To gain a better understanding of the sequence diversity of cyclotides, we isolated and characterized cyclotides from *V. philippica* using an established extraction, purification, reduction and digestion protocol [41]. Large-scale purification of the cyclotide-containing fractions of a CH_2Cl_2/MeOH extract of air-dried and powdered plant material provided eight new cyclotides, Viphi A–H, named according to their order of elution. The separation profile obtained by RP-HPLC is shown in Fig. 1. The cyclotide sequences were determined by a combination of enzymatic digests and tandem MS sequencing. In addition to the new cyclotides (Viphi A–H), eight known cyclotides, Viba 17 [45], Viba 15 [45], Varv A [32], Kalata B1 [26], Mram 8 [38], cO2 [35], cO12 [13] and Viba 11 [45] were identified by a combination of enzymatic digests, tandem MS sequencing, and comparison to the sequences of cyclic proteins in CyBase, a database containing the sequences and structures of backbone-cyclized proteins [17,42]. Viba 17 and Mram 8 were previously predicted from precursor sequences [38,45] but this is the first time they have been isolated as peptides.

Tandem MS sequencing after reduction and digestion of peptide fractions is an efficient method for the identification and elucidation of the primary structures of new and known cyclotides [3,6]. The molecular weight of Viba B was determined by LCMS to be 2985.18 Da. Reduction of this cyclotide with tris(2-carboxyethyl)phosphine (TCEP) resulted in an increase in molecular weight of 6 Da, indicating the presence of three disulfide bonds. The reduced cyclotides were digested with trypsin and endoGluC. The major fragments that resulted from this digestion had monoisotopic masses of 2239.86 Da and 787.31 Da. These fragments were sequenced using nanospray MS and correspond to the sequences TCTIGTCYTAGCTCSWPICTR and NGLPVCGE (Supporting Information Table 1). The combination of these fragments yielded the complete sequence of Viba B. All fragments mentioned above were confirmed with chymotrypsin and endoGluC digestions (see Supporting Information).

Another advantage of tandem MS sequencing is that it is possible to unambiguously define amino acid sequences for a mixture of cyclotides [3]. In the case of Viba 17, Viba 15, Varv A and kalata B1, the four cyclotides co-eluted in a single HPLC peak. The molecular weights of these peptides were determined by LCMS to be 2846.02,



B

	loop 1 I	loop 2 II	loop 3 III	loop 4 IV–V	loop 5 VI	loop 6	Expt. *m/z*	Theory
Viphi A	CGESC	VFIPC	ISSVI-G	CACK	SKVC	YKNGSIP	3170.28	3170.47
Viphi B	CGETC	TIGTC	YTAG---C	TCS	WPIC	TRNGLPV	2985.18	2985.25
Viphi C	CGESC	VYIPC	ITSVI-G	CSCS	SKVC	YING-VP	3059.18	3059.35
Viphi D	CGESC	VFIPC	ISSVI-G	CSCS	SKVC	YRNG-IP	3086.30	3086.37
Viphi E	CGESC	VFIPC	ISAVI-G	CSCS	NKVC	YKNGSIP	3156.24	3156.41
Viphi F	CGESC	VFIPC	ISAII-G	CSCS	SKVC	YKNGSIP	3143.30	3143.42
Viphi G	CGESC	VFIPC	ISAII-G	CSCS	NKVC	YKNGSIP	3170.36	3170.43
Viphi H	CAESC	VWIPC	TVTAIVG	CSCS	WGVC	Y-NG-IP	3096.24	3096.32
Viba 17	CGETC	VGGTC	NTPG---C	GCS	WPVC	TRNGLPV	2846.02	2846.16
Viba 15	CGETC	VGGTC	NTPG---C	ACS	WPVC	TRNGLPV	2860.04	2860.18
Varv A	CGETC	VGGTC	NTPG---C	SCS	WPVC	TRNGLPV	2876.04	2876.17
Kalata B1	CGETC	VGGTC	NTPG---C	TCS	WPVC	TRNGLPV	2890.06	2890.19
Mram 8	CGESC	VFIPC	LTSAI-G	CSCK	SKVC	YRNG-IP	3113.26	3113.42
cO2	CGESC	VWIPC	ISSAI-G	CSCK	SKVC	YRNG-IP		
cO12	CGETC	VGGTC	NTPG---C	SCS	WPVC	TRNGLPI		
Viba 11	CGESC	VWIPC	ISGAI-G	CSCK	SKVC	YRNG-IP		

Fig. 1. (A) RP-HPLC trace of a crude cyclotide extract from *V. philippica* showing the retention times and relative intensities of the different cyclotides. The gradient was 2% solvent B starting at 5% solvent B at 0.3 mL/min. (B) Alignment of the sequences of the new and known cyclotides. The cysteine residues (numbered I–VI) are highlighted in bold and the backbone loops are numbered 1–6. Ile and Leu were assigned by using sequence homology and based on the preferential cleavage by chymotrypsin after Leu-residues.

2860.04, 2876.04 and 2890.06 Da, respectively. The peptides were reduced using TCEP and each gained six mass units, indicating the presence of six Cys residues involved in three disulfide bonds. The reduced cyclotides were digested with trypsin and endoGluC. The major fragments that resulted from this digestion had monoisotopic masses of 2101.68 Da (Viba 17), 2115.69 Da (Viba 15), 2131.69 Da (Varv A) and 2145.70 Da (kalata B1) (Fig. 2A). These fragments were sequenced using nanospray MS and correspond to the sequences TCVGGTCNTPGCGCSWPVCTR, TCVGGTCNTPGCACSWPVCTR, TCVGGTCNTPGCSCSWPVCTR, and TCVGGTCNTPGCTCSWPVCTR, respectively (Supporting Information Table 1). Additional fragments were sequenced as outlined in the Supplementary data. The combination of these fragments yielded the complete sequences of Viba 17, Viba 15, Varv A and kalata B1. All fragments mentioned above were confirmed with chymotrypsin and endoGluC digestions (see Supporting Information). The differences between all four known cyclotides occur at one position in the sequence (marked with dashed circle in the upper-left corner of Fig. 2A) in loop 4.

The other cyclotides were sequenced using a similar approach. A list of the cyclotide fragments, sequences, masses of product ions, experimental masses, theoretical masses, and enzymes that aided the deduction of the sequences, is provided in the Supporting Information. Based on the presence or absence of a *cis*-Pro peptide bond in loop 5 [4], the characterization of the cyclotides in *V. philippica* resulted in the characterization of one new Möbius cyclotide (Viphi B) and seven new bracelet cyclotides (Viphi A, Viphi C to H).

Viphi A, Mram 8, Viphi D–G, Viba 17, Viba 15, Varv A, Kalata B1 and cO2 were evaluated for cytotoxic activity against MM96L, HeLa, BGC-823 cancer cell lines and HFF-1, a non-cancer cell line. Viphi B, C and H were not tested because of an insufficient quantity of material. The tested cyclotides showed cytotoxicity against cancer cell lines (Table 1).

4. Discussion

An analysis of the conserved and variable residues in the six loops within the cyclotide sequences from *V. philippica*, shown in Fig. 1, emphasizes the diversity of cyclotide sequences as well as the high conservation of key residues within the stable cyclotide framework (*i.e.* the placement and spacing of cysteine residues within the cyclotide sequence). The six Cys residues, the Glu in loop 1 and the Asn in loop 6 are the most conserved residues. As mentioned above, the CCK motif, formed by the conserved Cys residues, makes the structure of cyclotides exceptionally stable [2,4]. The Glu residue has been shown to have a key role in cyclotide bioactivity [12,30] and, structurally, is thought to provide additional stability via involvement in a hydrogen bond network [7,25,40]. The Asn residue in loop 6 has been demonstrated to be an essential residue for cyclization in the biosynthetic pathway of the cyclotides [8,14,16,19,24,27,39].

In addition to having key conserved residues, cyclotides exhibit variations in the compositions of their loops [13,21] and in this study, the eight new sequences again provided new variations. It is interesting to note that Viba 17, Viba 15, Varv A and kalata B1, which co-eluted in RP-HPLC, differ only by a single amino acid residue in loop 4; *e.g.*, Gly/-Ala/-Ser/-Thr. The co-elution in the RP-HPLC and similarity in the sequences of these four cyclotides is suggestive of similarity in their three-dimensional structures. The novel sequences from the current study provide new insights into the tolerance of the cystine knot framework of cyclotides to amino acid substitutions and will facilitate cyclotide engineering applications. In particular, they provide some insight into possible structural variations in the design of artificial cyclotides with pharmaceutical applications.

Table 1
Cytotoxic activities IC_{50} (μM) of selected cyclotides from *V. philippica*.

Cyclotides	Cell lines			
	HFF-1	MM96L	HeLa	BGC-823
Viphi A and Mram 8	3.19 ± 0.01	4.91 ± 0.04	15.5 ± 0.06	1.75 ± 0.05
Viphi D–E	1.55 ± 0.09	2.51 ± 0.03	5.24 ± 0.40	Not active
Viphi F–G	1.76 ± 0.12	1.03 ± 0.03	6.35 ± 0.31	2.91 ± 0.06
Viba 17, Viba 15, Varv A and Kalata B1	2.38 ± 0.09	3.10 ± 0.06	10.21 ± 0.43	1.32 ± 0.15
cO2	1.29 ± 0.07	0.65 ± 0.06	3.43 ± 0.15	4.81 ± 0.48
Taxol				0.0025 ± 0.0030

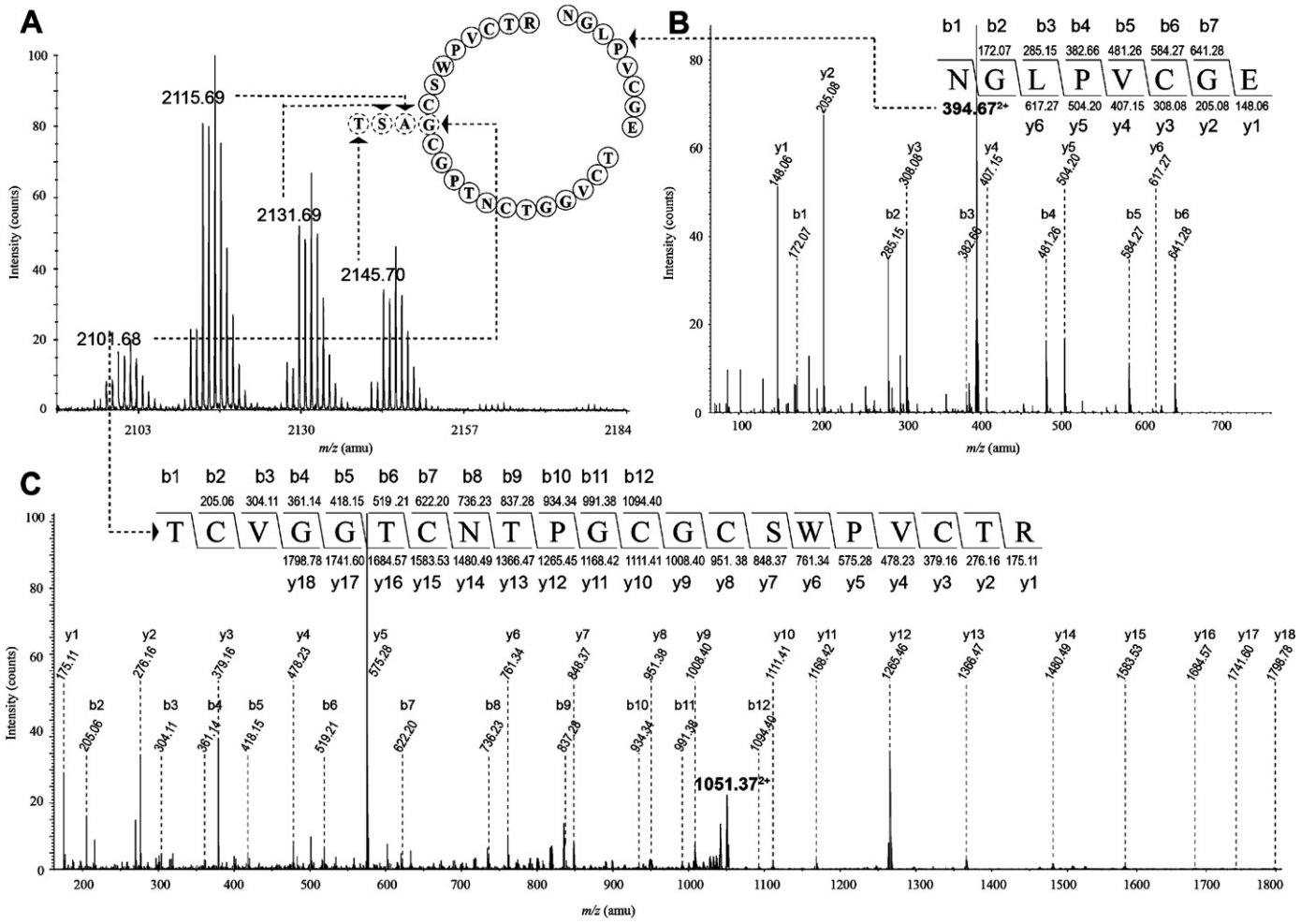


Fig. 2. (A) MALDI spectrum of Viba 17, Viba 15, Varv A and kalata B1. The fragments that lead to the sequences of these cyclotides are shown in the upper-right corner. The residue that differs within the group is marked with dashed circle in loop 4, shown in upper-right corner. (B) MS/MS spectrum of the doubly charged ion *m*/*z* 394.67 of Viba 17. The fragment sequence of Viba 17, the detected b- and y-series of ions are shown. "b" designates ions having the charge retained on the N-terminal fragment, and the "y" ions having the charge retained on the C-terminal fragment. (C) MS/MS spectrum of the doubly charged ion *m*/*z* 1051.37 of Viba 17. The fragment sequence of Viba 17, the detected b- and y-series of ions are shown.

Given that cyclotides have been shown to have cytotoxic activity [12,18,32,36], we tested several of the isolated cyclotides from *V. philippica* for toxicity against three cancer cell lines, MM96L, HeLa and BGC-823, and one non-cancer cell line HFF-1. The results in Table 1 indicate minimal differences across all four cell lines that were tested. Hydrophobicity and the glutamic acid residue in loop 1 of cyclotides have been shown to be important for cytotoxic activity [12,36]. All of the tested cyclotides contain eight to eleven hydrophobic amino acid residues (Ala, Val, Leu, Ile, Pro, Phe and Trp) and have a similar ratio of each of these residues in the sequences. All cyclotides contain the Glu residue in loop 1 in the sequences. Interestingly, cyclotides Viphi D/E do not show activity against the human gastric cancer cell line, in contrast to the other cyclotides tested. These peptides have similar sequences to Viphi F/G, indicating that even minimal sequence changes can have significant influences on bioactivity. Consistent with this observation, individual point mutations have previously been shown to remove hemolytic and insecticidal activity [30].

In summary, we have isolated and characterized a suite of new and known cyclotides from *V. philippica*, which enhances the known sequence variation observed for cyclotides and extends the number of species known to contain cyclotides. The novel cyclotides show cytotoxic activity against several cancer cell lines and the new data adds to the information regarding the structure–activity relationships of cyclotides.

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Appendix A. Supplementary data

Sequence fragments and sequences from the enzymatic digestion of the native cyclotides from *V. philippica*. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2011.06.016.

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