JOURNAL OF NATURAL PRODUCTS

ARTICLE

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Cyclotides from an Extreme Habitat: Characterization of Cyclic Peptides from *Viola abyssinica* of the Ethiopian Highlands

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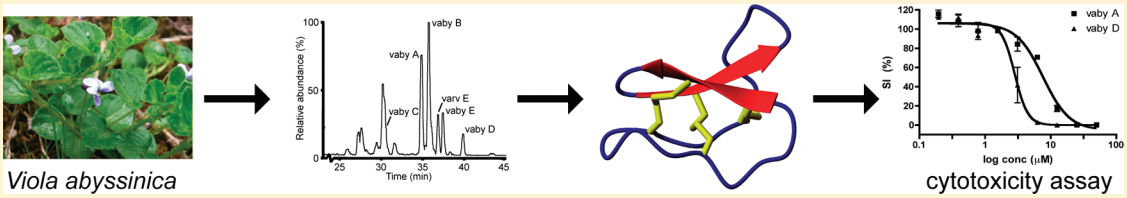
Supporting Information

ABSTRACT:



As part of ongoing explorations of the structural diversity of cyclotides, the cyclotide content of a native violet of the East African highlands, *Viola abyssinica* (which grows at altitudes up to 3400 m), was studied. Six new cyclotides, vaby A−E (**1**−**5**) and varv E (**6**), were isolated and characterized by employing HPLC and MS techniques and quantitative amino acid analysis. Cyclotides **1**−**5** were found to have new sequences, and **1**−**3** have a further novel feature in their sequences, an alanine moiety in loop 2. Two of the cyclotides (**1** and **4**) also exhibited cytotoxic properties in a flourometric microculture cytotoxicity assay. The findings corroborate the hypothesis that investigating the cyclotide contents of violets growing in diverse environments is a promising approach for extending our knowledge of both the structural and biological diversity of cyclotides.

Cyclotides comprise a family of circular mini-proteins that have been isolated from various plants and have a wide range of bioactivities. They have a characteristic head-to-tail cyclized backbone generally composed of 28−37 amino acids, and a knotted disulfide topology involving six conserved cysteine residues.[1−3] The cyclic backbone and conserved cysteine residues form a unique structure termed the cyclic cystine knot (CCK), in which two of the disulfide bonds and their connecting backbone segments form an embedded ring that is penetrated by the third disulfide bond.[2] Apart from being a characteristic structural feature of the cyclotide family, the CCK motif makes these mini-proteins exceptionally resistant to chemical, enzymatic, and thermal degradation.[4−6]

The discovery of cyclotides dates back to the 1960s, when the folkloric use of a decoction of the plant *Oldenlandia affinis* DC (Rubiaceae) by women to facilitate childbirth in the Congo (then Zaire) and the Central African Republic was reported.[6,7] The prototypic member of the cyclotide family was subsequently isolated as the main uterotonic principle in the decoction and designated kalata B1 (from the name of the decoction, kalata-kalata, used by the local people in Luluaburg, Congo). However, the full sequence and three-dimensional structure of kalata B1 were not elucidated for another 25 years.[8]

A number of other plant-derived peptides with similar circular backbones and cystine knot structures were discovered during the following years.[9−11] This family of peptides with homologous structures became known as the cyclotides in the late 1990s.[12] The cyclotides have been further classified into two main subfamilies based on topological differences. The Möbius subfamily contains a *cis*-Pro amide bond that creates a twist in the circular peptide backbone; a graphic representation of the backbone then resembles a Möbius strip. Cyclotides that lack the *cis*-Pro bond form the bracelet subfamily.[12]

In addition to the *cis*-Pro bond, Möbius and bracelet cyclotides differ in size, amino acid composition, and the nature of their intercysteine loops. A typical bracelet cyclotide contains one or two more residues than Möbius counterparts. Moreover, the bracelets contain a higher number of cationic residues. Figure 1 presents a schematic drawing of cyclotides of the Möbius subfamily, to which the cyclotides isolated in this study belong. Cyclic trypsin inhibitors may be regarded as a third cyclotide subfamily; these proteins share the CCK motif with Möbius and bracelet cyclotides, but otherwise their sequences have little in common with other cyclotides.[13]

The cyclotides have considerable pharmaceutical, and hence medicinal, significance arising from their exceptionally stable framework, which makes them potential scaffolds in drug design.[14−17] Cyclotides also have a wide range of inherent biological and pharmacological activities, including anti-HIV,[9,18,19] antihelmintic,[20]

Received: November 1, 2010
Published: March 24, 2011

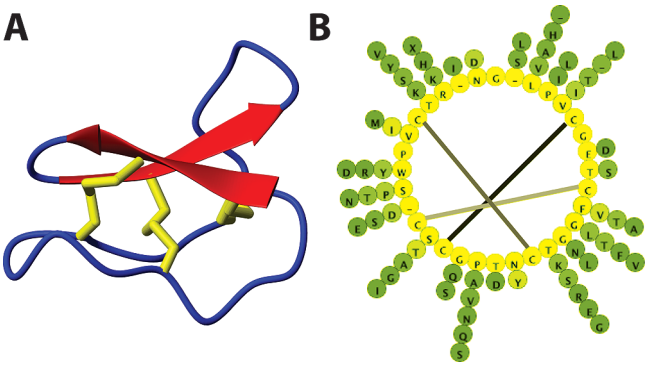


Figure 1. Cyclotide backbone structure depicting the CCK motif (A) and diversity wheel of the Möbius cyclotides, with the one-letter codes of amino acids (B). The occurrence of an amino acid at a given position decreases as one moves from the inner circle of the wheel toward the branches.

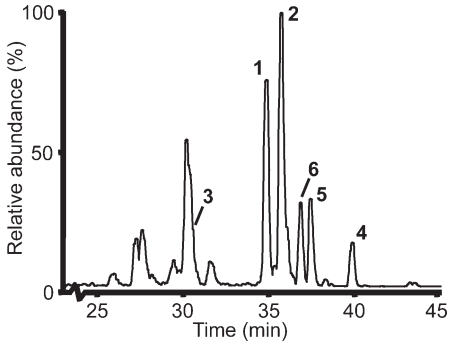


Figure 2. LC-MS profile, base peak ion chromatogram, of *V. abyssinica* depicting the five new cyclotides, vaby A−E (**1**−**5**), and varv E (**6**).

insecticidal,[21] antimicrobial,[22] uterotonic,[23] cytotoxic,[24,25] neurotensin inhibitory,[11] hemolytic,[10] trypsin inhibitory,[26] molluscicidal,[27] and antifouling[28] activities. Hence, there is increasing interest in exploring the plant kingdom to identify further cyclotides.

More than 140 cyclotides have been characterized at the protein or RNA (cDNA) levels,[19] but the search to chart the structural diversity of the cyclotides has just begun. Their occurrence seems to be concentrated within the Violaceae and Rubiaceae plant families, although a recent report indicates that cyclotides are also present in the Apocynaceae.[29] Members of a closely related class of peptides with a CCK motif that are homologues to squash trypsin inhibitors have been isolated from the Cucurbitaceae.[13]

Of these families Violaceae is of particular interest, since all the members thus far investigated have been found to contain cyclotides, and Violaceae species are also the sources of most plant cyclotides currently known. In efforts to extend knowledge of the structural diversity of cyclotides, we are focusing on members of the family of various genera growing under extreme conditions. Here we report an examination of the cyclotide content of *Viola abyssinica* Steud. ex Oliv., a violet that grows at altitudes up to 3400 m in the Ethiopian Highlands.

RESULTS AND DISCUSSION

Cyclotide Isolation and Sequencing. A preliminary small-scale screening using 10 g of dry plant material was first carried out, using LC-MS, to investigate the possible content of cyclotides in *V. abyssinica*. The LC-MS survey (Figure 2) detected a group of late-eluting compounds with masses ranging from 2.8 to 3 kDa, characteristic of cyclotides. A large-scale extraction (400 g of dried plant material), based on a protocol described by Herrmann et al.,[30] was then performed to isolate and sequence individual cyclotides, as follows. The plant material was first exhaustively extracted with 60% MeOH in H_2O, followed by liquid−liquid extraction with CH_2Cl_2 to remove highly hydrophobic substances such as pigments.[31] The MeOH fraction was then diluted with H_2O and applied to a C-18 column. A cyclotide-rich fraction was isolated by eluting the moderately hydrophobic compounds (including cyclotides) with 100% MeOH.

Six cyclotides (**1**−**6**), which were the most abundant compounds in the eluate, were purified using preparative HPLC. Before proceeding to tandem mass spectrometry (MS-MS) for sequence analysis, the cyclotides were chemically and enzymatically treated. They were first reduced to break the disulfide bonds and subsequently *S*-carbamidomethylated, a procedure that increased the mass of each cyclotide by 348 Da, confirming the presence of six cysteine moieties. Either trypsin or endoproteinase Glu C was then used to prepare linear fragments of the modified proteins, yielding fragments cleaved at the C-terminus of cationic residues or Glu, respectively. The fragments were fully sequenced by MS-MS analyses in combination with the results of quantitative amino acid analyses (see Supporting Information). Of the six (**1**−**6**) identified cyclotides, five (**1**−**5**) were found to have novel sequences and **1**−**3** had a further novel feature, an alanine moiety in loop 2. The term "loop" as used in cyclotides is the amino acid sequence between any two of the six conserved cysteine residues. The sixth (**6**) had an identical sequence to that of a known cyclotide, varv E.[32] The novel cyclotides were named vaby A−E, following the naming system suggested by Broussalis et al.,[33] i.e., a construction of a pronounceable acronym of the botanical name. The percentage yield of one of the abundant cyclotides, **1**, was about 0.04% by dry weight of extract. Table 1 shows the complete sequences and molecular masses of the six cyclotides. Sequence similarity to previously identified cyclotides was used to determine the positions of isobaric Ile and Leu residues within the sequences.

While determining the sequences of the cyclotides, it was observed that the mass of **3** increased by 2 units after reduction and alkylation, and MS-MS analyses of enzymatic digests of the peptide revealed that the change of mass occurred in the fragments containing Asn residues. Nonenzymatic deamidation of Asn to Asp in peptides often occurs,[34,35] promoted by alkaline conditions, particularly when an Asn is followed by either a Gly or, to a lesser extent, Thr.[35] The reduction and alkylation reactions in this study were conducted at a pH of 8.5, and either Gly or Thr follows the Asn residues in **3**. Hence, the gain of 2 mass units can be best explained by deamidation of the two Asn residues (residual mass 114.1), yielding either Asp (115.0) or *iso*-Asp (115.0). Further studies are required to determine why deamidation occurred only in **3**, although the Asn residues of **1**, **2**, **4**, **5**, and **6** are at similar positions in each peptide and all the peptides were subjected to similar experimental treatments.

All the new cyclotides isolated in this study belong to the Möbius subfamily of cyclotides. However, **1**−**3** contain an Ala residue in loop 2, which has not been previously reported. The role of cyclotides as defense molecules and accordingly variations in cyclotide expressions have been accounted for.[36] The

dx.doi.org/10.1021/np100790f |*J. Nat. Prod.* 2011, 74, 727–731

Table 1. Sequence Alignment of Vaby A−E (1−5) and Varv E (6) Isolated from *V. abyssinica*

Cyclotide	Sequence alignment[1]	Monoisotopic mass (Da)		Net charge
Loop	1 2 3 4 5 6	measured	calculated	
1	GETCAGGTCNTPGCSCS-WPICTRNGLPVC	2861.86	2862.11	0
2	GETCAGGTCNTPGCSCT-WPICTRNGLPVC	2875.88	2876.13	0
3	GETCAGGRCNTPGCSCS-WPVCTRNGLPVC	2903.02	2903.13	+1
4	GETCFGGTCNTPGCTCDPWPVCTRNGLPVC	3063.12	3063.21	-1
5	GETCFGGTCNTPGCSCDPWPVCTRNGLPVC	3049.14	3049.20	-1
6	GETCVGGTCNTPGCSCS-WPVCTRNGLPIC	2889.96	2890.14	0

[1] The amino acids are represented using the IUPAC one-letter amino acid code, charged residues are emboldened, and cysteines involved in disulfide bonds are shaded.

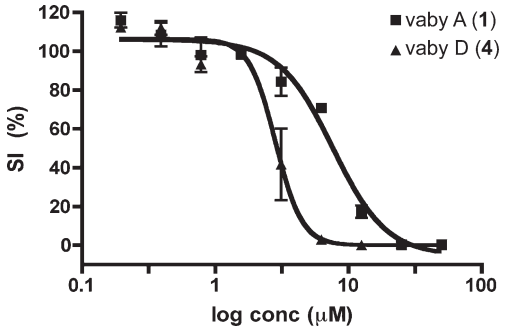


Figure 3. Concentration versus survival index (SI) curves of vaby A (**1**) and vaby D (**4**) obtained from experiments with the human lymphoma cell line U-937 GTB. The IC_{50} of **1** (7.6 μM) is 3-fold higher than that of **4** (2.8 μM).

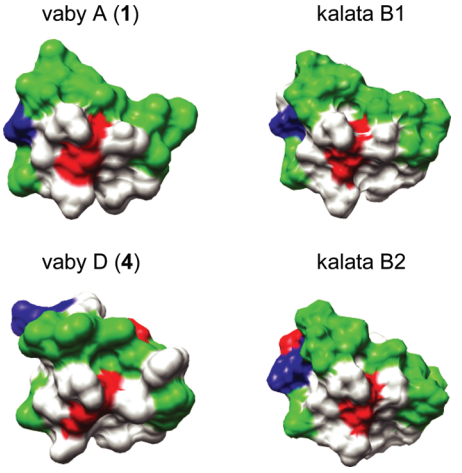


Figure 4. Comparison of 3D-model structures of of vaby A (**1**) with kalata B1 and vaby D (**4**) with kalata B2. The color scheme of the models is green for hydrophobic residues (Ala, Leu, Ile, Pro, Trp, and Val), blue for cationic residues (Arg and Lys), red for anionic residues (Glu), and white for the other amino acids.

unusual content of Ala in vaby cyclotides requires further study, in order to deduce whether the plant has evolved the ability to biosynthesise these cyclotides in response to selective pressures in its environment. *V. abyssinica* is a highland violet, growing at altitudes between 1750 and 3400 m. It is widely distributed in Ethiopia, where samples for this study were collected, as well as in other parts of Africa.[37]

Cytotoxicity of 1 and 4. A fluorometric microculture cytotoxicity assay was employed to screen the possible cytotoxic activity of two of the vaby cyclotides, **1** and **4**. Both cyclotides exhibited dose-dependent cytotoxicity against lymphoma cells (Figure 3). The activity of **4** (IC_{50}, 2.6 μM) was almost 3 times that of **1** (IC_{50}, 7.6 μM). The IC_{50} value of doxorubicin, a known anticancer drug, in a similar assay was 0.16 μM.[38] Although both **1** and **4** contain charged residues within their sequences, the net charge of **1** is neutral, while **4** is a negatively charged peptide. This difference in net charge may account for the significant differences between the activities of these two cyclotides, as it has been reported that the cytotoxic activity of cyclotides is correlated with their net charge content and content of charged residues.[39] Comparison of the cytotoxic potencies of **1** and **4** with those of other cyclotides by our research group revealed that the potency of **1** is similar to that of kalata B1, while the activity of **4** is close to that of kalata B2 (manuscript in preparation). Furthermore, there are similarities between the sequences and, more importantly, the 3D structures of **1** and kalata B1; the same holds true for **4** and kalata B2 (Figure 4). This fact is in line with, and further strengthens, the idea that the cytotoxic potency of cyclotides is a function of their surface properties as well.[39,40]

The findings corroborate the hypothesis that investigating the cyclotide contents of violets growing in diverse environments is a

dx.doi.org/10.1021/np100790f |*J. Nat. Prod.* 2011, 74, 727–731

promising approach for extending our knowledge of both the structural and biological diversity of cyclotides.

EXPERIMENTAL SECTION

Plant Material. The aerial parts of *Viola abyssinica* were collected from Mount Entoto, Addis Ababa, Ethiopia, at an altitude of 3400 m. The authenticity of the collected material was confirmed by Mr. Melaku Wondafrash, of the National Herbarium, Department of Biology, Addis Ababa University. A voucher specimen (#V-239889) was deposited at the Uppsala University Herbarium.

Extraction and Isolation. Powdered, air-dried aerial parts of *V. abyssinica* (400 g) were extracted using aqueous 60% MeOH in H_2O (4 L). The resulting mixture was filtered, and strongly hydrophobic constituents were removed from the filtrate by liquid−liquid extraction with CH_2Cl_2 (3 × 500 mL). The aqueous fraction was diluted with milli-Q H_2O (1:1 v/v) and loaded onto C-18 silica gel that was initially washed with 30% MeOH in H_2O and subsequently eluted with MeOH. The MeOH eluate was dried in vacuo, redissolved in 10% CH_3CN, and fractionated by preparative HPLC. Fractions were collected every minute for 1 h, yielding 60 fractions. The cyclotide contents in each fraction were analyzed by electrospray ionization (ESI) MS, and individual cyclotides were isolated and purified by analytical RPC 18-HPLC, as described below.

HPLC. Preparative RPC 18-HPLC runs were carried out on a Shimadzu LC 10 HPLC system equipped with a photodiodide array detector. A ReproSil-Pur C 18-AQ column (250 × 20 mm i.d., 10 μm, 300 Å) was eluted at a linear gradient from 10% CH_3CN in 0.05% TFA (buffer A) to 60% CH_3CN in 0.05% TFA (buffer B) over a period of 60 min at a flow rate of 5 mL/min. Analytical RPC 18-HPLC experiments were performed on an Amersham Pharmacia Biotech ÄKTA (Uppsala, Sweden) system, equipped with a UV detector collecting data at 215, 254, and 280 nm. A Vydac C 18 column (250 × 4.6 mm i.d., 5 μm, 300 Å) and a linear gradient from buffer A to buffer B over 30 min at a flow rate of 1 mL/min were used.

Mass Spectrometry. ESIMS was performed using a Finnigan LCQ ion trap mass spectrometer in positive ion mode (Thermo Electron Co., Waltham, MA, USA), with the capillary temperature maintained at 220 °C and the spray voltage at 4 kV. Nanospray mass spectrometry was carried out using the same instrument, after installing a Protana NanoES source (Proxeon, Odense, Denmark), with the capillary temperature set at 150 °C and the spray voltage at 0.5 kV. All samples were injected in 60% CH_3CN in 0.1% HCOOH. CID values were set for each experiment in MS-MS sequencing, common values ranging between 25% and 40%. Monoisotopic masses were measured using a Q-Tof Micro MS (Waters, Milford, MA), with the capillary voltage maintained at 3 kV.

Quantitative Amino Acid Analysis. The amino acid composition of each cyclotide was determined at the Amino Acid Analysis Centre, Department of Biochemistry and Organic Chemistry, Uppsala University. Cyclotides were completely hydrolyzed by incubation with 6 N HCl containing 2 mg/mL PhOH (100 °C, 24 h); their amino acid contents were then determined using ninhydrin and an LKB model 4151 Alpha Plus amino acid analyzer.

Reduction, Alkylation, and Enzymatic Cleavage of the Cyclotides. For sequence analysis, peptides were reduced with dithiothreitol in 0.25 M Tris-HCl (pH 8.5) containing 4 mM EDTA and 8 M guanidine-HCl, incubated at 37 °C in the dark and under N_2 for 2 h. Iodoacetamide (50 mg, in 0.5 M Tris-HCl, 2 mM EDTA) was added to alkylate free thiols. The S-alkylation was quenched after 1 h by adding 250 μL of 0.5 M citric acid. The reduced and alkylated peptides were purified by analytical HPLC and cleaved by incubation with either trypsin or Glu C (37 °C, 4 h) in 50 mM NH_4HCO_3 buffer (pH 7.8).

Determination of Cytotoxic Activity. The possible cytotoxic properties of **1** and **4** were determined using an FMC assay[41] with the human lymphoma cell line U-937 GTB.[42] The peptides were dissolved in 10% aqueous EtOH, a dilution series was prepared, and 20 μL portions of each dilution were added to wells of V-shaped, 96-well microtiter plates (Nunc, Roskilde, Denmark). In addition, six blank wells (with 200 μL per well of cell-growth medium) and six solvent-control wells (with 20 μL per well of 10% aqueous EtOH) were included on each plate. A 180 μL portion of a suspension of U-937 GTB cells in cell-growth medium containing ca. 20 000 cells was then added to each well apart from the solvent controls, and the plates were incubated for 72 h at 37 °C and 5% CO_2. Numbers of viable cells in the wells were estimated by adding fluorescein diacetate (which is converted to fluorescein by cells with intact plasma membranes) into each well and incubating the plates for a further 40 min under the same conditions. The resulting fluorescence was measured with a Fluoroscan II at 538 nm, following excitation at 485 nm. The fluorescence in each well was considered to be proportional to the number of living cells and was expressed as a survival index (SI), defined as the fluorescence in experimental wells, as a percentage of that in control wells, after subtracting blank values. IC_{50} values, which correspond to the concentration at an SI of 50%, were calculated by nonlinear regression analysis using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). The experiments were carried out in duplicate, and each was performed three times.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

Mr. M. Wondafrash is thanked for his assistance in the collection and identification of the plant material. M.Y.Y. is grateful to the Swedish International Development Cooperation Agency for financial support provided through its SAREC wing (ref #7500723501); U.G. is supported by the Swedish Research Council (621-2007-5167) and The Swedish Foundation for Strategic Research (F06-0058).

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