Full Papers

Cytotoxic Cyclotides from *Viola tricolor*[⊥]

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A crude fraction of *Viola tricolor* rich in small lipophilic proteins was prepared and subjected to fractionation guided by bioactivity, using RP-HPLC and a fluorometric cytotoxicity assay. Two human cancer cell lines, U-937 GTB (lymphoma) and RPMI-8226/s (myeloma), were used in this study. The most potent compounds isolated, that is, the compounds showing the lowest IC_{50} values, were shown to be three small proteins: vitri A ($IC_{50} = 0.6$ μM and $IC_{50} = 1$ μM, respectively), varv A ($IC_{50} = 6$ μM and $IC_{50} = 3$ μM, respectively), and varv E ($IC_{50} = 4$ μM in both cell lines). Their sequences, determined by automated Edman degradation, quantitative amino acid analysis, and mass spectrometry, were cyclo-GESCVWIPCITSAIGCSCKSKVCYRNGIPC (vitri A), cyclo-GETCVGGTCNTPGCSCSWPVCTRNGLPVC (varv A), and cyclo-GETCVGGTCNTPGCSCSWPVCTRNGLPIC (varv E), of which vitri A is described for the first time. Each forms a head-to-tail cyclic backbone, with six cysteine residues being involved in three disulfide bonds, characteristic of the family of small proteins called the cyclotides. This is the first report on cyclotides from the species *V. tricolor* and the first report on the sequence of the cytotoxic cyclotide vitri A.

Within a research project aimed at finding polypeptides (from plants) with potential in drug discovery, a fractionation protocol was developed to provide fractions rich in polypeptides.[1] As a result, the complete sequence of the first head-to-tail cyclic polypeptides from *Viola arvensis* Murray (Violaceae) were reported,[1,2] later shown to be part of the family of small proteins found so far only in the Rubiaceae, Violaceae, and Cucurbitaceae plant families, called the cyclotides.[3,4] Cyclotides are about 30 amino acids in size with a head-to-tail cyclic backbone and three disulfide bonds arranged in a cysteine knot.[3] The well-defined three-dimensional structures and the fact that they are gene-encoded products make it relevant to refer to the cyclotides as topologically complex small proteins.[5] The cysteine knot and the N- and C-termini joined in a peptide bond constitute an extremely stable and rigid structure that enables them to coexist with various proteases, suggesting that they represent perfect host defense proteins.[4] They also show antibacterial, antifungal,[6] and insecticidal[5] properties, supporting the host defense theory.[7]

Recently, our group reported potent cytotoxic activity for three cyclotides from two *Viola* species: varv A and varv F from *V. arvensis* and cycloviolacin O2 from *V. odorata* L.[8] This previous study concentrated on these three already purified compounds, but there is a possibility that more potent compounds are present in the complex mixture of cyclotides that *Viola* species contain. To address this possibility, plant material was subjected, in this study, to isolation guided by bioactivity. Moreover, additional data regarding the cytotoxic effects of these small lipophilic proteins were gathered, which might result in a deeper understanding of their structural properties in relation to the observed activity.

Results and Discussion

Isolation and Sequence Determination. Using established fractionation protocols,[1,2,9] a crude fraction of *V. tricolor* rich in small lipophilic proteins was obtained and subjected to preparative RP-HPLC. The most potent cytotoxic fraction was further separated by means of analytical RP-HPLC and subsequently investigated for cytotoxic activity.

The three most potent cytotoxic compounds, that is, the compounds showing the lowest IC_{50} values, isolated from *V. tricolor* were shown to be three head-to-tail, small cyclic proteins. Their amino acid sequences and molecular masses, determined by automated Edman degradation, quantitative amino acid analysis, and mass spectrometry, are shown in Tables 1 and 2.

They all form a head-to-tail cyclic backbone, and the six cysteine residues are involved in three disulfide bonds, a characteristic feature of the cyclotide family of small proteins from plants.[3] Reduction and alkylation of the cyclotides (with iodoacetamide) generated derivatives with an increased molecular mass (348 Da) that is consistent with the presence of six disulfide-linked cysteines (58 Da for each reduced and carbamidomethylated cysteine). Digestion of the reduced and alkylated cyclotides with endoproteinase GluC resulted in singular linear products,

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Table 1. Amino Acid Composition and Molecular Mass of Vitri A, Varv A, and Varv E (Residues from Amino Acid Analyses Are Listed to the Left (aa anal.) and the Residues from Sequencing (seq.), to the Right (n.d. = not determined))

	vitri A		varv A		varv E	
amino acid	aa anal.	seq.	aa anal.	seq.	aa anal.	seq.
Asp/Asn (D/N)	1.3	1 N	2.0	2 N	2.0	2 N
Thr (T)	1.1	1	3.8	4	3.5	4
Ser (S)	4.3	4	2.4	2	2.6	2
Glu/Gln(E/Q)	1.2	1 E	1.2	1 E	1.2	1 E
Pro (P)	1.8	2	2.9	3	2.8	3
Gly (G)	3.4	3	5.0	5	4.9	5
Ala (A)	1.0	1				
Cys (C)	5.7[a]	6[b]	6.0[a]	6[b]	5.8[a]	6[b]
Val (V)	2.4	2	2.7	3	2.3	2
Ile (I)	3.8	4			1.2	1
Leu (L)			0.9	1	0.9	1
Tyr (Y)	0.9	1				
Lys (K)	1.7	2				
Arg (R)	1.0	1	1.0	1	0.9	1
Trp (W)	n.d.	1	n.d.	1	n.d.	1
no amino acids		30		29		29
mass, native (measd)[c]		3152.8		2877.9		2892.2
mass, native (calcd)[d]		3152.4		2878.4		2892.4
mass, alkylated and digested (measd)[c]		3518.5		3243.2		3259.2
mass, alkylated and digested (calcd)[e]		3519.2		3244.7		3258.7

[a] Half-cystine was determined to be cysteic acid following oxidation with performic acid. [b] Cysteine alkylated with iodacetamide. [c] Determined by nanospray MS. [d] Calculated using average masses with the total sum from amino acid composition adjusted to the head-to-tail cyclic structure (−18 Da) and three disulfide bridges (−6 Da). [e] Calculated using average masses with the total sum from amino acid composition adjusted to the digested (+18 Da) and alkylated structure (+348 Da).

each with an observed molecular mass that was 18 Da higher than that of the reduced and alkylated cyclotide. This observation is explained by the head-to-tail cyclic nature of these small proteins, which accounts for the uptake of water (18 Da) when the cyclic backbone is digested. Thus, they show both the head-to-tail cyclic backbone and the involvement of the six cysteine residues in three disulfide bonds that are characteristic of the cyclotide family.[3] Although the disulfide bonding patterns of the cyclotides isolated in this work have not been experimentally determined, the very high sequence homology with structurally known cyclotides makes it reasonable to assume that the CysI−CysIV, CysII−CysV, and CysIII−VI cyclic cysteine knot motif is conserved.[3,10]

This is the first report on cyclotides from the species *V. tricolor*, but the sequences of two of the cyclotides match those of the previously isolated cyclotides from *V. arvensis* (i.e., varv A and varv E).[1,2] We use the same names, varv A and varv E, for these two cyclotides from *V. tricolor*, in accordance with the principles proposed for naming the growing family of cyclotides.[9] The third sequence, which did not match any previous isolated cyclotide, was hereby named vitri A, using the principles cited above for this novel cyclotide from *V. tricolor*.

Human Cancer Cell Line Assay. The crude fraction of *V. tricolor* was subjected to fractionation guided by bioactivity, using the fluorometric microculture cytotoxicity assay (FMCA)[11] for the human lymphoma cell line U-937 GTB and the human myeloma cell line RPMI-8226/s. The most potent cytotoxic compounds isolated were the three cyclotides described above. After 72 h of treatment, all three showed cytotoxicity in a dose-dependent manner (Figure 1), and their IC_{50} values were in the micromolar range (Table 2). The potencies of the three cyclotides are in the range of the clinically used anticancer drug doxorubicin (Table 2).[12]

In the myeloma cell line, vitri A was approximately 3 and 4 times more potent than varv A and varv E, respectively, and in the lymphoma cell line, vitri A turned out to be approximately 10 and 7 times more potent than varv A and varv E, respectively. Hence, their structural properties are discussed in relation to the observed biological activity.

Structural Properties in Relation to Cytotoxic Activity. All cyclotides contain at least one cationic amino acid (Arg or Lys). This conserved feature may favor interactions with cell membranes, and indeed their antibacterial activity has been reported to be salt dependent, indicating that the initial interaction is electrostatic.[6] These cationic residues are exposed on the peripheral edges of the molecule, as revealed by both modeled and experimentally determined 3-D structures of cyclotides.[10] In this study, vitri A, with a net charge of +2 at physiological pH, was more potent than varv A and varv E, with a net charge of ±0. These results are in line with the previous study by our group; a similar trend was shown when testing the cyclotide cycloviolacin O2 (from *V. odorata*), with a net charge of +2, and the two noncharged cyclotides varv A and varv F (from *V. arvensis*) (Table 2).[8] Taken together, these results further strengthen the hypothesis that these cationic residues are of importance for the exhibition of

Table 2. IC_{50} Values for Isolated Cyclotides and a Clinically Used Anticancer Drug, Using Human Cancer Cell Lines (concentration in μM)[a]

Name	Sequence alignment	Net charge	IC_{50} U-937 GTB	IC_{50} RPMI-8226/s	Ref.
vitri A	cyclo-(G**E**SCVWIPCITSAIGCSCKSKVCYRNGIP-C)	+2	0.6 μM	1 μM	This paper
varv A	cyclo-(G**E**TCVGGTC--NTPGCSCSWPVCTRNGLPVC)	±0	6 μM	3 μM	This paper
varv E	cyclo-(G**E**TCVGGTC--NTPGCSCSWPVCTRNGLPIC)	±0	4 μM	4 μM	This paper
c. O2[b]	cyclo-(G**E**SCVWIPCISSAIGCSCKSKVCYRNGIP-C)	+2	0.3 μM	0.1 μM	8
varv A	cyclo-(G**E**TCVGGTC--NTPGCSCSWPVCTRNGLPVC)	±0	6 μM	3 μM	8
varv F	cyclo-(G**E**TCVGGTC--NTPGCSCSWPVCTRNGLPIC)	±0	7 μM	6 μM	8
doxorubicin			0.1 μM	0.1 μM	12

[a] The aligned amino acid sequences of the cyclotides are presented using the one-letter amino acid code. The residues of glutamic acid are bolded, the cationic amino acids are underlined, and cysteines involved in disulfide bonds are shaded. [b] c. O2 = cycloviolacin O2.

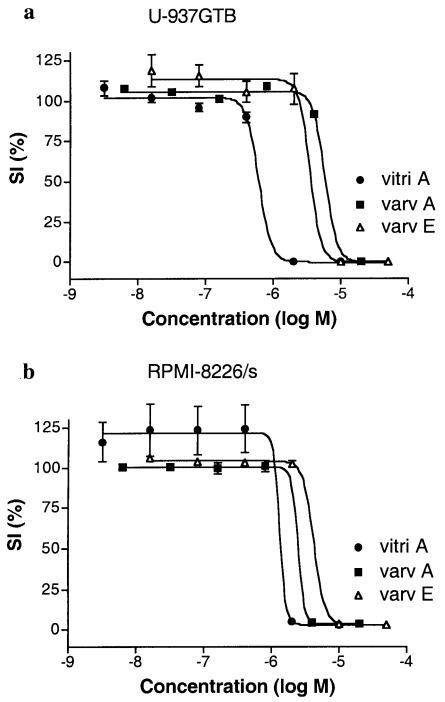

Figure 1. Survival index curves of vitri A, varv A, and varv E at six concentrations on the human cancer cell lines U-937 GTB (a) and RPMI-8226/s (b). Each point represents the mean ± SEM, calculated using nonlinear regression in GraphPad Prism (GraphPad Software, Inc., San Diego).

cytotoxic activity. All three cyclotides tested in the present study also showed an apparently sharp profile in their dose−response curves. The 10−90% response interval in the log dose−response curves takes place within the narrow concentration range of less than 0.3 μM. This conspicuous phenomenon was also seen in the previous study on cyclotide cytotoxicity.[8]

These observations, that is, the importance of the cationic residues and the sharp dose−response profile, are common to another family of peptides structurally related to the cyclotides, the defensins. These defense peptides are known to disrupt cell membranes by formation of pores; a well-studied example is the human neutrophil defensin HP-1.[13−15] This peptide also shows cytotoxicity with a similar sharp dose−response curve at concentrations (1−8 μM)[16] comparable to that found for the cyclotides in this study. Human neutrophil defensins have been shown to fuse and lyse negatively charged membranes, but display reduced activity to neutral membranes, which suggests that fusion and lysis are primarily mediated by electrostatic forces.[17] Hence, it is tempting to conclude that the observed higher potency of vitri A is indeed related to the discussed differences in charges and that the cyclotides act as pore-forming agents like the defensins. However, corroborating evidence is needed.

In conclusion, the bioactivity-guided fractionation of the crude fraction of *V. tricolor* (rich in small lipophilic proteins) resulted in the isolation of three cyclotides, of which the novel vitri A, with a net charge of +2 at physiological pH, showed the most potent effects. Moreover, this study expanded the knowledge of structure−activity relationships, in support of the hypothesis that cationic amino acid residues play a crucial role in the cytotoxic effects of cyclotides.

Experimental Section

General Experimental Procedures. HPLC was done using an Amersham Pharmacia Biotech ÄKTA basic, equipped with a UV detector collecting data at the wavelengths 215, 254, and 280 nm. A Shimadzu LC10 system was also used, equipped with a photodiode array detector (SPD-M10AVP, Shimadzu) collecting data within the wavelength interval 200−300 nm. For MS, a Finnigan LCQ equipped with a Protana nanospray source (Protana, Odense, Denmark) was used. Proteolytic enzymes were of sequencing grade and purchased from Promega Co., WI, whereas CH_3CN was of HPLC gradient grade and purchased from Merck, along with TFA, which was of spectroscopy grade. All chemicals used in the cancer cell line assay were obtained from Sigma Chemical Co., St. Louis, MO.

Plant Material. Commercial grade aerial parts of *Viola tricolor* L. (Violaceae) were obtained from Alfred Galke GmbH (Gittelde, Germany). A voucher sample of the plant material used for extraction has been deposited at the Division of Pharmacognosy, Department of Medicinal Chemistry, Uppsala University, Biomedical Centre, Uppsala, Sweden, with collection number VM183. Analyzing the purchased plant material by means of LC-MS, the cyclotide pattern was found to be in agreement with that of the previous collection of *V. tricolor* from the surroundings of Uppsala, Sweden. Vitri A, varv A, and varv E were identified in both commercial and authentical plant material.

Extraction and Isolation. The dried and powdered plant material (250 g) was extracted by maceration for 24 h with 2600 mL of CH_2Cl_2. This procedure was repeated seven times, and the CH_2Cl_2 extract was discarded. The plant residue was dried at room temperature, and the main extraction was then carried out in an analogous manner, with 8 × 2400 mL of 50% EtOH in H_2O. This extract was evaporated in vacuo to a 1200 mL volume and then acidified by addition of AcOH to a final concentration of 2% AcOH. Tannins were removed by passing the acidified extract through a column containing 25 g of polyamide 6S (Riedel-de Haen, Seelze, Germany), as described previously.[1] The tannin-free extract was evaporated in vacuo to a 280 mL volume and then partitioned three times with *n*-BuOH of equal amount. The *n*-BuOH phases were evaporated to dryness in vacuo, redissolved in 70 mL of 10% CH_3CN containing 0.1% TFA (v/v), and desalted on a 10 g C_{18} end-capped Isolute solid phase extraction column (IST, Mid Glamorgan, U.K.). The bound substances were eluted with 140 mL of 60% CH_3CN containing 0.1% TFA (v/v).

Further fractionation was achieved by means of preparative HPLC (LC10, Shimadzu), using a 250 × 10 (i.d) mm Rainin Dynamax column (C_{18}, 5 μm, 300 Å) eluted (over 25 min at a flow rate of 4 mL/min) with a linear gradient of CH_3CN (10−60%) containing 0.1% TFA (v/v). Aliquots of 10 mL were collected, pooled, and subjected to cytotoxic testing. Final fractionation of the most potent fraction was done by means of analytical HPLC using a 250 × 4.6 (i.d.) mm Rainin Dynamax column (C_{18}, 5 μm, 300 Å) eluted (over 20 min at a flow rate of 0.8 mL/min) with a linear gradient of CH_3CN (55−70%) containing 0.1% TFA (v/v). This resulted in the isolation of varv A (0.5 mg), varv E (0.5 mg), and vitri A (0.3 mg). The proteins were collected and subjected to sequence determination and cytotoxic testing.

Sequence Determination. The amino acid content of the cyclotides was quantitatively determined at the Amino Acid Analysis Centre, Department of Biochemistry, Uppsala University, as reported previously.[1]

For the Edman degradation, the cyclotides were reduced, alkylated, and cleaved as described previously.[10] Desalting and isolation of alkylated and linear cyclotides were accomplished by means of RP-HPLC using a 100 × 2 (i.d.) mm Grom-Sil ODS-4 HE column (3 μm, 200 Å), which was eluted with a linear CH_3CN gradient (10−60%) containing 0.1% TFA at a flow rate of 0.3 mL/min. The linear and alkylated product was subjected to Edman degradation performed at the Protein Analysis Center (PAC), Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden.

Human Cancer Cell Line Assay. The human cancer cell lines used were RPMI-8226/s (myeloma) and U-937 GTB (lymphoma), procured and maintained as described earlier.[18] Cell-growth medium was prepared from RPMI-1640 stock,

supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin.

Fractions of *V. tricolor* and purified cyclotides were dissolved in 10% EtOH (equal to a final concentration of EtOH of 1% in the assay). V-shaped 96-well microtiter plates (Nunc, Roskilde, Denmark) were prepared with 20 μL per well of test solution in triplicate for each concentration. Also, six solvent-control wells (20 μL per well of 10% EtOH), six blank wells (200 μL per well of cell-growth medium), six positive-control wells (20 μL per well of 10% Triton X-100), and six negative-control wells (20 μL per well of phosphate-buffered saline solution, PBS) were prepared on each microtiter plate. All experiments were repeated once.

Initial cell viability was assessed by the trypan blue dye exclusion test. The tumor cells suspended in cell-growth medium were dispensed on the prepared microtiter plates (20 000 cells/180 μL per well) and incubated at 37 °C and 5% CO_2. After 72 h of incubation, the cells were washed with PBS, and 100 μL of fluorescein diacetate (10 μg/mL in a physiological buffer) was added to each well. The plates were incubated at 37 °C and 5% CO_2 for 40 min, and the generated fluorescence was measured in a 96-well scanning fluorometer, exciting fluorescence at 485 nm and sequentially reading at 538 nm.[10] The fluorescence (as measured) is proportional to the number of living cells, and thus cell survival can be quantified as a survival index (SI), defined (in units of percent) as the fluorescence of the test wells relative to the average fluorescence for control wells, with average for the blank wells subtracted. IC_{50} values, which correspond to the 50% survival index (Figure 1), were calculated using nonlinear regression in GraphPad Prism (GraphPad Software, Inc., San Diego).

Quality-assessment criteria for a successful experiment included a fluorescence signal in control wells of more than 10 times the average value for blank wells, with an average value for the coefficient of variation in blank and control wells being less than 30%.[18]

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References and Notes

(1) Claeson, P.; Göransson, U.; Johansson, S.; Luijendijk, T.; Bohlin, L. *J. Nat. Prod.* **1998**, *61*, 77−81.
(2) Göransson, U.; Luijendijk, T.; Johansson, S.; Bohlin, L.; Claeson, P. *J. Nat. Prod.* **1999**, *62*, 283−286.
(3) Craik, D. J.; Daly, N. L.; Bond, T.; Waine, C. *J. Mol. Biol.* **1999**, *294*, 1327−1336.
(4) Trabi, M.; Craik, D. J. *Trends Biochem. Sci.* **2002**, *27*, 132−138.
(5) Jennings, C.; West, J.; Waine, C.; Craik, D. J.; Anderson, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10614−10619.
(6) Tam, J. P.; Lu, Y. A.; Yang, J. L.; Chiu, K. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8913−8918.
(7) Craik, D. J. *Toxicon* **2001**, *39*, 1809−1813.
(8) Lindholm, P.; Göransson, U.; Johansson, S.; Claeson, P.; Gullbo, J.; Larsson, R.; Bohlin, L.; Backlund, A. *Mol. Cancer Therap.* **2002**, *1*, 365−369.
(9) Broussalis, A. M.; Göransson, U.; Coussio, J. D.; Ferraro, G.; Martino, V.; Claeson, P. *Phytochemistry* **2001**, *58*, 47−51.
(10) Svangård, E.; Göransson, U.; Smith, D.; Verma, C.; Backlund, A.; Bohlin, L.; Claeson, P. *Phytochemistry* **2003**, *64*, 135−142.
(11) Larsson, R.; Nygren, P. *Anticancer Res.* **1989**, *9*, 1111−1119.
(12) Gullbo, J.; Arsenau, D.; Grundmark, B.; Alvfors, C.; Larsson, R.; Lindhagen, E. *Anticancer Res.* **2002**, *6C*, 4191−4198.
(13) Kagan, B. L.; Selsted, M. E.; Ganz, T.; Lehrer, R. I. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 210−214.
(14) Ganz, T.; Lehrer, R. I. *Mol. Med. Today* **1999**, *5*, 292−297.
(15) Heller, W. T.; Waring, A. J.; Lehrer, R. I.; Harroun, T. A.; Weiss, T. M.; Yang, L.; Huang, H. W. *Biochemistry* **2000**, *39*, 139−145.
(16) Bateman, A.; Singh, A.; Jothy, S.; Fraser, R.; Esch, F.; Solomon, S. *Peptides* **1992**, *13*, 133−139.
(17) Fujii, G.; Selstedt, M. E.; Eisenberg, D. *Protein Sci.* **1993**, *8*, 1301−1312.
(18) Dhar, S.; Nygren, P.; Csoka, K.; Botling, J.; Nilsson, K.; Larsson, R.; Huang, H. W. *Br. J. Cancer* **1996**, *74*, 888−896.

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