Variations in Cyclotide Expression in *Viola* Species

Manuela Trabi,[†] Erika Svangård,[‡] Anders Herrmann,[‡] Ulf Göransson,[‡] Per Claeson,[‡] David J. Craik,*[,†] and Lars Bohlin[‡]

Institute for Molecular Bioscience, The University of Queensland, Brisbane QLD 4072, Australia, and Division of Pharmacognosy, Department of Medicinal Chemistry, Uppsala University, BMC, Box 574, 751 23 Uppsala, Sweden

Received December 11, 2003

Cyclotides, a family of approximately 50 mini-proteins isolated from various Violaceae and Rubiaceae plants, are characterized by their circular peptide backbone and six conserved cysteine residues arranged in a cystine knot motif. Cyclotides show a wide range of biological activities, making them interesting targets for both pharmaceutical and agrochemical research, but little is known about their natural function and the events that trigger their expression. An investigation of the geographical and seasonal variations of cyclotide profiles has been performed, using the native Australian violet, *Viola hederacea*, and the Swedish sweet violet, *Viola odorata*, as model plants. The results showed that in the Australian violet the relative peptide levels of some cyclotides remained almost constant throughout the year, while other cyclotides were present only at certain times of the year. Therefore, it appears that *V. hederacea* expresses a basic armory of cyclotides as well as special "add-ons" whose levels are influenced by external factors. In the Swedish violet, cyclotide levels were increased up to 14 times during the warmest period of the year. The larger variation in expression levels of the Swedish plants may be a reflection of a greater climatic variation.

In recent years a range of naturally occurring circular mini-proteins, i.e., proteins with their backbone cyclized through peptide bonds, has been discovered in various organisms, including bacteria, plants, and even a mammal.[1] The biggest group of circular mini-proteins is the cyclotides,[2] isolated from plants of the families Violaceae and Rubiaceae. Cyclotides generally contain between 29 and 37 residues, including six absolutely conserved cysteine residues that are arranged in a cystine knot motif: two disulfide bonds and the connecting backbone segments form a ring that is penetrated by the third disulfide bond. The so-called cyclic cystine knot (CCK),[3] the combination of this knotted cystine arrangement with the circular peptide backbone, renders the cyclotides exceptionally stable against both enzymatic digest and thermal degradation.

Ethnopharmacological observations led to the discovery of the first cyclotide, kalata B1, one of the active ingredients of a uterotonic decoction used by tribeswomen in the Congo.[4−6] In later studies it became apparent that cyclotides show diverse biological activities, including anti-HIV,[7−9] cytotoxic,[10] insecticidal,[11] and antimicrobial[12] activity. These properties, together with their extraordinary stability, make cyclotides prime targets for pharmaceutical and agrochemical research.[13]

Representative sequences from among the approximately 50 known cyclotides are shown in Table 1. Although found in Violaceae and Rubiaceae plants, cyclotides have a particularly high prevalence in plants from the Violaceae family. For example, six of the peptides listed in Table 1 have been discovered in Violaceae plants: varv peptide A (*Viola arvensis* Murray),[14] cycloviolacin H1 (*V. hederacea* Labill.),[2] cycloviolin A (*Leonia cymosa* Mart.),[9] cycloviolacin O6 (*V. odorata* L.),[2] vico A (*V. cotyledon* Ging.),[15] and hypa A (*Hybanthus parviflorus* (Mutis) Baill.).[17] Two further cyclotides, kalata B1[6,16] and kalata B2,[12,16] were originally reported from *Oldenlandia affinis* DC. (Rubiaceae), but were later also discovered in various Viola species.[2] Overall, approximately two-thirds of the published cyclotide sequences are derived from Violaceae plants.

The natural function of cyclotides seems to be as part of the plant's defense system.[11] However, little is known about whether cyclotides are produced continuously or whether their production is induced by external events. In an attempt to gain a better understanding of cyclotide expression in plants and the possible natural functions of these remarkable peptides, studies of both seasonal and geographical variations in cyclotide production have been performed. In these studies the native Australian violet, *Viola hederacea*, and the Swedish sweet violet, *Viola odorata*, were used as model plants. To study seasonal changes, different plant parts were extracted and the cyclotide profiles monitored by LC-MS. In the Australian part of the study, five different plant parts (roots, petioles, leaves, pedicels, and flowers) were extracted once a month over the course of 14 months. Due to climatic conditions, *V. odorata* is available in Sweden only from April to September. During this period, roots, leaves, and flowers were extracted every second month. Geographical differences in cyclotide production were also investigated for both species.

Results and Discussion

Employing established screening protocols for the isolation of cyclotides[2,15] and LC-MS analysis of the crude extracts, the seasonal changes and geographical variations in the cyclotide levels of *V. hederacea* (Australia) and *V. odorata* (Sweden) were investigated. The standard in-house protocols for cyclotide extraction differed slightly between the two participating laboratories. In short, *V. hederaceae* was extracted with methylene chloride/methanol (1:1), and the extract was subjected to partitioning against water. The aqueous phase was then lyophilized before it was redissolved and analyzed by LC-MS. *V. odorata* was extracted with ethanol/water (1:1), and the sample was dried in vacuo and then subjected to solid phase extraction on reverse-phase material. Thereafter, the sample was dried and

* To whom correspondence should be addressed. Tel: +61 7 3346-2019. Fax: +61 7 3346-2029. E-mail: d.craik@imb.uq.edu.au.
[†] The University of Queensland.
[‡] Uppsala University.

10.1021/np034068e CCC: $27.50
Published on Web 04/17/2004

Table 1. Representative Cyclotide Sequences and Masses[a]

cyclotide	amino acid sequence	mass
kalata B1	**C**GET**C**VGGT**C**...NTPG**C**T**C**.SWPV**C**TRNG.LPV	2892
kalata B2	**C**GET**C**FGGT**C**...NTPG**C**S**C**.TWPI**C**TRDG.LPV	2955
varv peptide A	**C**GET**C**VGGT**C**...NTPG**C**S**C**.SWPV**C**TRNG.LPV	2878
cycloviolacin H1	**C**GES**C**VYIP**C**L.TSAIG**C**S**C**KSK.V**C**YRNG.IP	3132
cyclopsychotride A	**C**GES**C**VFIP**C**V.TALLG**C**S**C**KSK.V**C**YKNS.IP.	3130
circulin A	**C**GES**C**VWIP**C**I.SAALG**C**S**C**KNK.V**C**YRNG.IP.	3152
cycloviolin A	**C**GES**C**VFIP**C**I.SAAIG**C**S**C**KNK.V**C**YRNGVIP.	3143
cycloviolacin O6	**C**GES**C**VWIP**C**I.SAAVG**C**S**C**KSK.V**C**YKNGTLP.	3184
vico A	**C**AES**C**VYIP**C**F.TGIAG**C**S**C**KNK.V**C**YYNGSIP.	3272
hypa A	**C**AES**C**VYIP**C**TITALLG**C**S**C**KNK.V**C**Y.NG.IP.	3143

[a] The cysteine residues are given in bold and boxed; the lines at the bottom of the table indicate the disulfide connectivities.

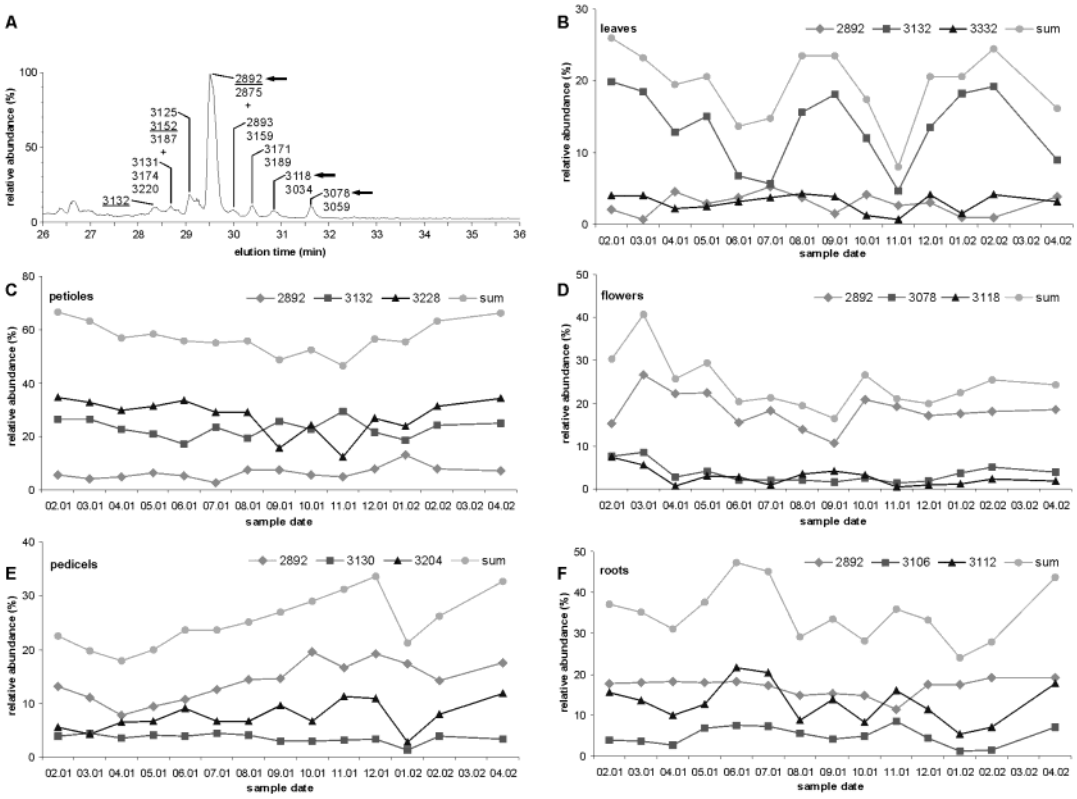

Figure 1. (A) Example of an LC-MS profile showing the cyclotides expressed in the flowers of *V. hederacea*. The cyclotide peaks are labeled with the masses of the peptides they contain; a plus (+) indicates the presence of small amounts of other cyclotides. The masses of these additional components are not given. Peaks that are not labeled do not contain peptides with masses corresponding to cyclotides. The arrows show the cyclotides represented in Figure 1D. Masses that match those of known cyclotides are underlined. Note that a known mass does not necessarily correspond to a known cyclotide, since one mass can correspond to different cyclotide sequences. (B−F) Expression of selected cyclotides in various plant parts of *V. hederacea*, monitored over a time period of 14 months. Kalata B1 (mass 2892 Da) is present in all plant parts and is shown as gray diamonds in the graphs. The relative amounts of two additional cyclotides, representative for the respective plant part, are also given for each plant part. The fourth trace in each graph, given as circles in light gray, shows the sum of the three cyclotides for each plant part. All cyclotides are labeled with their molecular masses. HPLC conditions: Phenomenex Jupiter column (150 × 2 mm (i.d.), 5 μm, 300 Å), flow rate 200 μL/min, 2%/min gradient (0.05% formic acid in water; 90% acetonitrile and 0.05% formic acid in water, v/v).

redissolved before analysis. Both protocols gave reproducible results and were efficient in extracting >80% of all cyclotides present in a single pass. Since we were interested more in relative changes in cyclotide expression in the individual species with time, rather than in absolute levels, in each protocol we adopted an approach involving a single extraction for each sample, rather than exhaustively re-extracting each sample to obtain quantitative yields of cyclotides. It should be stressed that all analyses performed on an individual plant species followed the same extraction protocol (dichlormethane/methanol for *V. hederacea* and ethanol/water for *V. odorata*, respectively), and therefore the relative cyclotide amounts can be accurately compared within each species, but cross-comparisons between the two

species may be less precise. However, in a control experiment, an explicit comparison of the two extraction methods for a single plant species (*V. odorata*) yielded essentially identical profiles and amounts of cyclotides. The cyclotide content was found to be approximately 0.02% of fresh plant weight, corresponding to 0.2% of plant dry weight, which is consistent with an earlier reported yield of ~2 g/kg dried plant weight.[4]

We analyzed the crude plant extracts by LC-MS, identifying cyclotides on the basis of their exceptional stability, late elution time on reversed-phase HPLC, and characteristic mass range. Cyclotides typically elute at retention times corresponding to 40−60% acetonitrile and have masses in the range 2700−3400 Da.[2,6−11,14,15,17−21] These criteria, together with the tendency not to fragment even under harsh MS conditions, were taken as strong evidence that the relevant LC-MS peaks were cyclotides.

To investigate seasonal effects, in the Australian study five plant parts (leaves, petioles, flowers, pedicels, and roots) of *V. hederacea* were examined individually. Kalata B1 was the main constituent in flowers and pedicels, but was also present in all other plant parts investigated. For each plant part, kalata B1 and two other cyclotides that constituted the main component of their respective chromatographic peaks were chosen for analysis. The MS response of these cyclotides was normalized to the total ion count of the respective sample. Figure 1 shows variations in the expression of the monitored proteins over the course of 14 months. In this figure and in the following discussion, cyclotides will be named with their mass; for example, a cyclotide with a mass of 3205 Da will be referred to as "cyclotide 3205".

From the total ion count of the LC-MS analysis, the overall amount of cyclotides expressed in petioles and pedicels showed only minor variation over the course of the study. In the other plant parts, the overall amount of cyclotides differed by a factor of up to 3, but with no obvious parallels to seasonal changes. These differences can be regarded as significant, although the total ion count is only a rough estimate of the overall quantity of cyclotides in the crude extract.

Figure 1 also shows that some cyclotides (e.g., cyclotide 3130 in pedicels) are expressed at a relatively constant level over a 14-month period. However, the relative amount of others does change significantly, as seen for cyclotide 3132 in the leaves or cyclotide 3228 in the petioles. In most plant parts there is no clear trend over the course of the year, such as, for example, one cyclotide being expressed mainly in summer, but not in winter, an indication that factors other than the seasons play an important role in cyclotide expression. Since the seasonal climatic variations in the subtropical region of Australia are comparatively minor, it was of interest to relate these results to a similar study conducted in a climatic zone with substantial climatic changes, in this case Sweden.

In the Swedish part of the study, three plant parts (leaves, flowers, and roots) of *V. odorata* were examined individually. For each plant part, varv A (2876 Da) and two other cyclotides, selected by their occurrence in all plant parts and by being the most abundant cyclotides, were chosen for analysis. Figure 2 shows variations in the expression of the monitored cyclotides over the period from April to September. The LC-MS analysis shows that cyclotide levels are increased during July, the warmest month of the study period, in all plant parts examined. In leaves the concentration of cyclotide 2876B was increased 14 times. In flowers the level of cyclotide 2922 was increased by a factor of 12, and finally in roots the level of cyclotide 2876 was increased 7 times over the basal level.

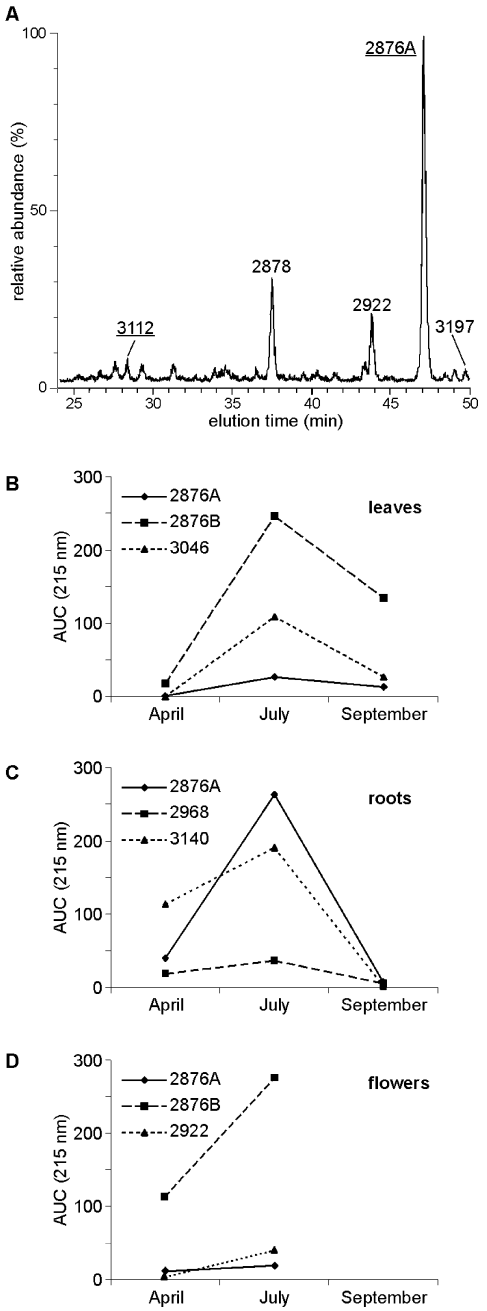

Figure 2. (A) Example of an LC-MS profile showing the cyclotides expressed in the leaves of *V. odorata*. The cyclotide peaks are labeled with the masses of the peptides they contain. Masses that match those of known cyclotides are underlined. Note that a known mass does not necessarily correspond to a known cyclotide, since one mass can correspond to different cyclotide sequences. (B−D) Expression of selected cyclotides by their AUC at 215 nm in various plant parts of *V. odorata*, monitored over a time period of 5 months. Varv A is present in all plant parts and is shown as cyclotide 2876A in the graphs. The relative amounts of two additional cyclotides, representative for the respective plant part, are also given for each plant part. All cyclotides are labeled with their monoisotopic molecular masses. HPLC conditions: Grom-Sil ODS-4 HE column (100 × 2 mm (i.d.), 3 μm, 200 Å), flow rate 300 μL/min with the following gradient: 0−8 min kept at 30% B; 30−65% B in 52 min; 65−100% B in 5 min; and 100% B for 2 min (A: 0.1% formic acid in water; B: 60% acetonitrile and 0.1% formic acid in water, v/v). Note that flowers were not available in September due to the cold climate in Sweden at this time of the year.

Further evidence for environmental modulation of the cyclotide expression was gained by investigating the influence of the location in which a plant grows (i.e., the

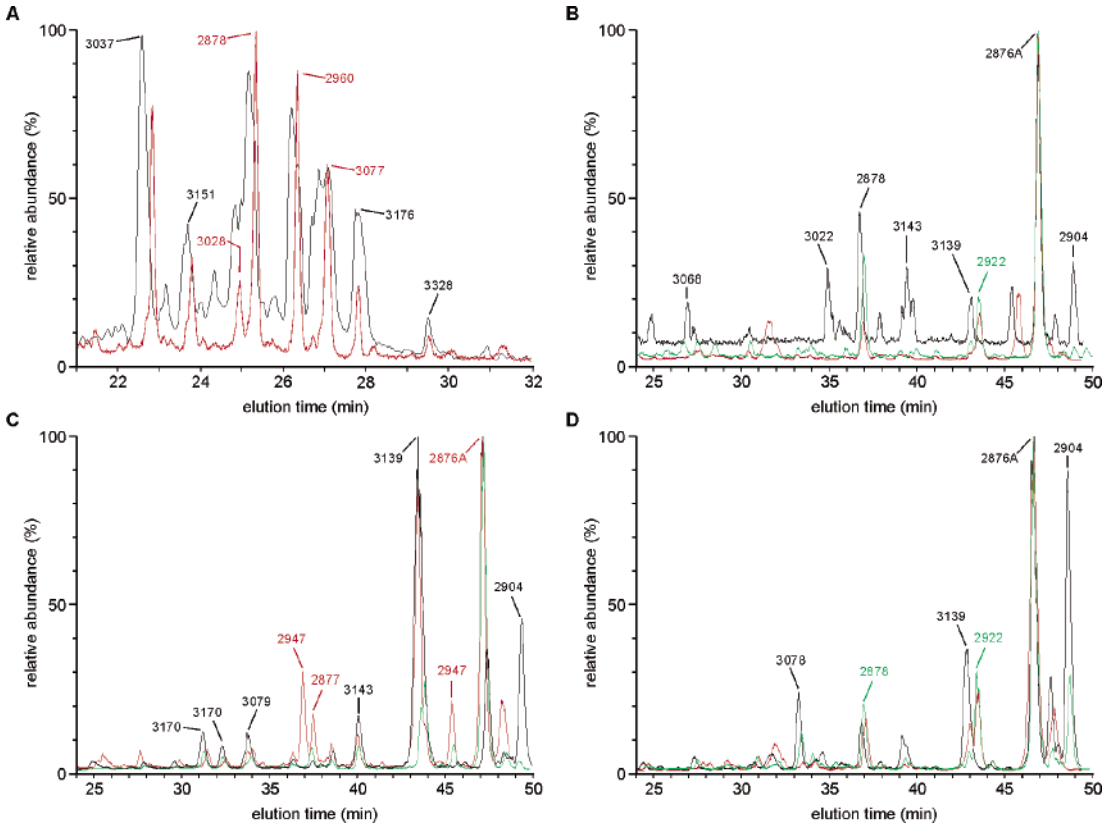

Figure 3. (A) LC-MS traces of different samples of *V. hederacea* collected on consecutive days at two different locations in the Carnarvon area, central Queensland, Australia (black: location 1, red: location 2). HPLC conditions: Phenomenex Jupiter column (50 × 2 mm (i.d.), 5 μm, 300 Å); solvents and gradient as described in the legend to Figure 1. (B−D) LC-MS traces of different samples of *V. odorata* collected on 1 day at three different locations in Uppsala, Sweden. The red trace indicates location A, the green trace location B, and the black trace location C. The LC-MS traces of leaves (B), roots (C), and flowers (D) are shown. HPLC conditions as described in the legend to Figure 2. Note that the largest peaks are normalized to 100% in all chromatograms; hence only relative differences are shown.

combination of light, water, soil pH, temperature, nutrients, and various other factors) on the cyclotide profile. Figure 3A shows the results obtained from two different samples of *V. hederacea*, harvested on consecutive days in two different gullies from the Carnarvon region in Queensland, Australia, approximately 2 km from each other. Despite the overall cyclotide profile of these two samples being rather similar, each trace shows the presence of some peaks that are missing in the other.

Figures 3B−D show corresponding results from Sweden. Here, three samples of *V. odorata* were harvested on the same day at three different locations in Uppsala, approximately 10 km from each other. Again, the overall cyclotide profiles of these three samples are similar, but there are some peaks that are missing in individual samples. The most abundant cyclotides are generally present in all samples (i.e., leaves and flowers: 2876, 2878, and 2922 Da; roots: 2876, 2904, and 3139 Da).

In summary, the Australian data did not show any apparent correlation between temperature and/or number of daylight hours (the main climatic seasonal changes if water supply is guaranteed) and the expression of cyclotides in *V. hederacea*. The overall amount of cyclotides varied by a factor of approximately 3, but these variations did not parallel the seasons. The biggest chromatographic peak derived from flowers and pedicels (LC-MS trace not shown) contained, among other minor components, kalata B1, the first cyclotide identified and the dominant peak in the cyclotide profile of *O. affinis*. In other tissues of *V. hederacea*, kalata B1 was less prominent. Some cyclotides, like 3112 in the roots or 3132 in the leaves, showed a rather irregular expression pattern. It appears that in Australia, *V. hederacea* has a "basic armory" of cyclotides that are expressed at a relatively constant level throughout the year with the additional production of "add-ons" whose levels are adjusted according to demand. In contrast, the Swedish data show an increase in cyclotide expression in *V. odorata* during the warmest part of the period investigated. It is conceivable that the comparatively large variations seen in *V. odorata* reflect the increased environmental pressure created by a short growth period (typically 5−7 months), while the pattern shown in *V. hederacea* corresponds to year-round growth.

Our knowledge of the natural function of cyclotides in plants is still very limited, making it difficult to identify cause and response. It is tempting to make the interpretation of a role of cyclotides in plant defense, as proposed by Jennings et al.,[11] and that the change in expression patterns is a response to specific environmental conditions, be it the lack of nutrients or the presence of a certain pest. Further studies and corroborative evidence will be necessary before this conclusion can be confirmed. However, there is no doubt that an interesting story will unfold around these fascinating natural products. The demonstration in this paper that variations in expression levels occur within a species over time is a first step to a better understanding of the biology of these plant-derived molecules.

Experimental Section

General Experimental Procedures. In the Australian part of the study, plant material to examine seasonal variations was sampled at the University of Queensland. The respective colony of *V. hederacea* was supplied with water in

regular intervals by an automatic sprinkler system. Plant material for the study of geographical variations was obtained in Carnarvon region (Queensland). Fresh plant material was extracted as described earlier.[2] Specifically, after grinding up the plant material with methanol and adding an equal amount of dichloromethane (at a ratio of 20 mL of solvent mixture per gram of plant material), the extraction was left without stirring at room temperature for 24 h. The extraction mixture was filtered and the filtrate was mixed with chloroform and small amounts of water until a phase separation was visible. The lower (organic) phase, containing all the chlorophyll, was discarded, and the upper (aqueous) phase, containing the cyclotides, was dried in vacuo, first on a rotary evaporator (Büchi), then on a lyophylizer (Dynavac FD12), yielding what will be further referred to as crude extract. This crude extract was redissolved in 45% acetonitrile and 0.05% TFA in water and analyzed by LC-MS as described below.

In the Swedish part of the study, the plant parts of *V. odorata* were sampled at the same location in Uppsala on the mornings of April 25, July 10, and September 3, 2002, respectively, to investigate seasonal variations. To study geographical variations, plant parts of *V. odorata* were sampled on July 10, 2003, at three different locations in Uppsala, approximately 10 km from each other. About 4 g of fresh material of each plant part was collected in 50% aqueous ethanol and extracted on a shaking table at room temperature. The extract was stored at −20 °C until analysis. The extracts were thawed and evaporated to dryness in vacuo. The dry extracts were redissolved in 10 mL of 10% CH_3CN containing 0.1% TFA (v/v) and desalted on a solid phase extraction column (500 mg, C_{18}EC, IST, Sorbent, Sweden). The captured substances were eluted with 40 mL of 60% CH_3CN containing 0.1% TFA (v/v).

Although the standard extraction procedures used in the two participating laboratories differ slightly, they are both highly reproducible and yield samples that are highly enriched in cyclotides and give very similar cyclotide profiles. This was confirmed by a comparative study performed on *V. odorata*, where each half of a sample of fresh plant material was extracted employing each of the procedures described above. Both extracts gave essentially identical cyclotide profiles.

Analysis by LC-MS. In the Australian part of the study, LC/MS analysis was used for qualitative and quantitative analysis. The redissolved crude extract was injected on a Phenomenex Jupiter column [150 × 2 mm (i.d.), 5 μm, 300 Å] with SecuryGuard guard column run at 200 μL/min. Cyclotides were eluted with a 2%/min gradient from 0.05% formic acid in water to 72% acetonitrile and 0.05% formic acid in water (v/v) and analyzed on a Micromass time-of-flight mass spectrometer controlled by a PC running MassLynx version 3.5 (Micromass Ltd., Manchester, UK). Mass spectra were obtained in positive ion mode over a range *m*/*z* 700−2000. The samples were analyzed directly after collection and again at the end of the 14 months time span, until which time they were stored at −20 °C. Except for slight broadening of peaks (probably due to column age), the LC-MS data derived from analysis of fresh and stored extracts were very similar. The relative quantity of the different collections was determined by the relative peak intensity of the respective cyclotides. The relationship between these parameters was linear for the injected amounts of cyclotides. For analysis, all peak intensities were normalized to the peak with highest intensity (at 100%).

For the Swedish part of the study, LC-MS analyses were done on a LCQ electrospray ion trap MS (Thermo Finnigan, San Jose, CA) operated in the positive mode and fed by an ÄKTA basic HPLC system (Amersham Biotech, Uppsala, Sweden) eluted at a flow rate of 0.3 mL/min with the following gradient: 0−8 min kept at 30% B; 30−65% B in 52 min; 65−100% B in 5 min; and 100% B for 2 min (A: 0.1% formic acid in water; B: 60% acetonitrile, 0.1% formic acid). A Grom-Sil ODS-4 HE (100 × 2 mm (i.d.), 3 μm, 200 Å) column was used for RP-HPLC. A 25 μL aliquot of each plant sample was injected into the LC-MS at a concentration of 5 mg/mL. Like in the Australian part of the study, the injected amounts were in the linear range of the mass detector; however, the calculations of relative amounts were based on the UV absorbance at 215 nm. Again, all peaks were given in relation to the strongest signal at 100%.

Acknowledgment. This work was supported by the Australian Research Council (ARC). D.J.C. is an ARC Professorial Fellow. The Institute for Molecular Bioscience is a Special Research Centre of the ARC. The authors are also grateful to Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning for financial support (FORMAS). We thank M. Wellard for helpful comments on the manuscript.

References and Notes

(1) Trabi, M.; Craik, D. J. *Trends Biochem. Sci.* **2002**, *27*, 132−138, and references therein.
(2) Craik, D. J.; Daly, N. L.; Bond, T.; Waine, C. *J. Mol. Biol.* **1999**, *294*, 1327−1336.
(3) Craik, D. J. *Toxicon* **2001**, *39*, 1809−1813.
(4) Gran, L. *Lloydia* **1973**, *36*, 174−178.
(5) Gran, L.; Sandberg, F.; Sletten, K. *J. Ethnopharmacol.* **2000**, *70*, 197−203.
(6) Saether, O.; Craik, D. J.; Campbell, I. D.; Sletten, K.; Juul, J.; Norman, D. G. *Biochemistry* **1995**, *34*, 4147−4158.
(7) Gustafson, K. R.; Sowder, R. C., II; Henderson, L. E.; Parsons, I. C.; Kashman, Y.; Cardellina, J. H., II; McMahon, J. B.; Buckheit, R. W., Jr.; Pannell, L. K.; Boyd, M. R. *J. Am. Chem. Soc.* **1994**, *116*, 9337−9338.
(8) Bokesch, H. R.; Pannell, L. K.; Cochran, P. K.; Sowder, R. C., II; McKee, T. C.; Boyd, M. R. *J. Nat. Prod.* **2001**, *64*, 249−250.
(9) Hallock, Y. F.; Sowder, R. C., II; Pannell L. K.; Hughes, C. B.; Johnson, D. G.; Gulakowski, R.; Cardellina, J. H., I; Boyd, M. R. *J. Org. Chem.* **2000**, *65*, 124−128.
(10) Lindholm, P.; Göransson, U.; Johansson, S.; Claeson, P.; Gullbo, J.; Larsson, R.; Bohlin, L.; Backlund, A. *Mol. Cancer Ther.* **2002**, *1*, 365−369.
(11) Jennings, C.; West, J.; Waine, C.; Craik, D. J.; Anderson, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10614−10619.
(12) Tam, J. P.; Lu, Y.-A.; Yang, J.-L.; Chiu, K.-W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8913−8918.
(13) Craik, D. J.; Simonsen, S.; Daly, N. L. *Curr. Opin. Drug Discovery Devel.* **2002**, *5*, 251−260.
(14) Claeson, P.; Göransson, U.; Johansson, S.; Luijendijk, T.; Bohlin, L. *J. Nat. Prod.* **1998**, *61*, 77−81.
(15) Göransson, U.; Broussalis, A. M.; Claeson, P. *Anal. Biochem.* **2003**, *318*, 107−117.
(16) Gran, L. *Lloydia* **1973**, *36*, 207−208.
(17) Broussalis, A. M.; Göransson, U.; Coussio, J. D.; Ferraro, G.; Martino, V.; Claeson, P. *Phytochemistry* **2001**, *58*, 47−51.
(18) Göransson, U.; Luijendijk, T.; Johansson, S.; Bohlin, L.; Claeson, P. *J. Nat. Prod.* **1999**, *62*, 283−286.
(19) Witherup, K. M.; Bogusky, M. J.; Anderson, P. S.; Ramjit, H.; Ransom, R. W.; Wood, T.; Sardana, M. *J. Nat. Prod.* **1994**, *57*, 1619−1625.
(20) Gustafson, K. R.; Walton, L. K.; Sowder, R. C. II; Johnson, D. G.; Pannell, L. K.; Cardellina, J. H., II; Boyd, M. R. *J. Nat. Prod.* **2000**, *63*, 176−178.
(21) Svangård, E.; Göransson, U.; Smith, D.; Verma, C.; Backlund, A.; Bohlin, L.; Claeson, P. *Phytochemistry* **2003**, *64*, 135−142.

NP034068E