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Primary and 3-D modelled structures of two cyclotides from *Viola odorata*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

Two polypeptides named vodo M and vodo N, both of 29 amino acids, have been isolated from *Viola odorata* L. (Violaceae) using ion exchange chromatography and reversed phase HPLC. The sequences were determined by automated Edman degradation, quantitative amino acid analysis, and mass spectrometry (MS). Using MS, it was established that vodo M (cyclo-SWPVCTRNGAPICGESCFTGKCYTVQCSC) and vodo N (cyclo-SWPVCYRNGLPVCGETCTLGKCYTAGCSC) form a head-to-tail cyclic backbone and that six cysteine residues are involved in three disulphide bonds. Their origin, sequences, and cyclic nature suggest that these peptides belong to the family of cyclic plant peptides, called cyclotides. The three-dimensional structures of vodo M and vodo N were modelled by homology, using the experimentally determined structure of the cyclotide kalata B1 as the template. The images of vodo M and vodo N show amphipathic structures with considerable surface hydrophobicity for a protein modelled in a polar environment.

Keywords: *Viola odorata* L.; Violaceae; Sweet violet; Isolation; Primary structure; Homology modelling; Cyclotide; Macrocyclic polypeptide; Vodo M; Vodo N

1. Introduction

Cyclotides are plant peptides of about 30 amino acids with a head-to-tail cyclic backbone and six cysteine residues involved in three disulphide bonds. They also show structural features such as a triple-stranded beta-sheet and a cysteine knot arrangement of the three disulphide bonds (Craik et al., 1999). Several macrocyclic polypeptides, considered members of the cyclotide family, have been isolated from the plant families Cucurbitaceae, Rubiaceae and Violaceae (Craik et al., 2002). They display a range of biological activities; for example, uterotonic (Gran, 1973), haemolytic (Schöpke et al., 1993), antibacterial, antifungal (Tam et al., 1999), anti-HIV (Bokesch et al., 2001; Gustafson et al., 1994; Hallock et al., 2000), insecticidal (Jennings et al., 2001), and cytotoxic activities (Lindholm et al., 2002). The antibacterial, antifungal, and insecticidal properties of the cyclotides suggest that they are involved in plant host defence (Craik, 2001). It has been proposed that the cyclotides represent the perfect host-defence proteins, since 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 (Trabi and Craik, 2002). A recent finding reports that cyclotides are gene products that within each plant species are found in several isoforms (Jennings et al., 2001). The production of several isoforms within a single species further supports the hypothesis that these peptides are involved in defence. To make possible the establishment of their structure-function relationship, it is necessary to carefully characterise the three-dimensional (3-D) structure of cyclotide isoforms.

In this study, two cyclotides from *Viola odorata* L. (Violaceae) have been isolated and characterized using

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experimental and modelling techniques. For the latter, which is now routinely being applied towards the construction of 3-D models (Cheetham et al., 1999; Tung et al., 2002), high sequence similarity among the cyclotides makes it possible to construct 3-D models of good quality, using cyclotides with experimentally determined structures as templates. We named these new cyclotides vodo M (**1**) and vodo N (**2**), in compliance with recently proposed principles (Broussalis et al., 2001).

2. Results and discussion

2.1. Isolation and structure determination

By following an established fractionation protocol (Claeson et al., 1998; Göransson et al., 1999), with some modifications, the two cyclotides vodo M (**1**) and vodo N (**2**) were isolated. Extraction with *n*-BuOH has been shown to capture the cyclotides and facilitate the isolation process (Broussalis et al., 2001). Also, cation exchange chromatography was used, since at least one positively charged amino acid (i.e. Arg or Lys) is a conserved feature of the cyclotides. The molecular masses of the native vodo M and vodo N were determined by electrospray ionisation (ESI) MS (LCQ, Finnigan) and found to be 3077.1 Da [M] and 3048.1 Da [M], respectively.

Reduction and alkylation of vodo M (**1**) and vodo N (**2**) (with iodoacetamide) generated derivatives with an increased molecular mass of 348 Da. This is consistent with the presence of six disulphide-linked cysteines (58 Da for each acetamide group). Digestion of the reduced and alkylated vodo M (**1**) and vodo N (**2**) with endoproteinase GluC resulted in singular products, each with an observed molecular mass that was 18 Da higher than the reduced and alkylated peptides. This indicates that the peptides are macrocyclic, and accounts for the loss of water (18 Da) due to cyclisation. Although the disulphide bonding pattern of vodo M (**1**) and vodo N (**2**) has not been experimentally established, it is probable that the CysI–CysIV, CysII–CysV, and CysIII–VI cyclic cysteine knot motif is conserved (Craik et al., 1999).

Quantitative amino acid analysis of the hydrolysate of the cyclotides revealed the amino acid composition shown in Table 1. From the amino acid analysis, the total amount of vodo M (**1**) and vodo N (**2**) isolated could be estimated to be 64 μg and 466 μg, respectively. The amino acid sequences obtained for the linear products via automated Edman degradation (verified by MS^2 analysis) are shown in Table 2. The sequences determined by automated Edman degradation and quantitative amino acid analyses were in agreement with the molecular masses determined experimentally by MS (Table 1).

Table 1
The amino acid composition and molecular masses of vodo M (**1**) and vodo N (**2**)

Amino acid	vodo M (**1**)		vodo N (**2**)	
	aa anal.	seq.	aa anal.	seq.
Asp/Asn (D/N)	1.3	1 N	1.2	1 N
Thr (T)	2.9	3	3.0	3
Ser (S)	2.8	3	2.3	2
Glu/Gln (E/Q)	2.2	1 E/1Q	1.2	1 E
Pro (P)	1.8	2	2.0	2
Gly (G)	3.3	3	4.3	4
Ala (A)	1.1	1	1.0	1
Cys (C)	5.1[a]	6[b]	5.4[a]	6[b]
Val (V)	2.3	2	2.2	2
Ile (I)	1.7	1	–	–
Leu (L)	–	–	2.0	2
Tyr (Y)	1.1	1	1.8	2
Phe (F)	1.2	1	–	–
Lys (K)	1.4	1	1.1	1
Arg (R)	0.9	1	1.0	1
Trp (W)		1		1
No amino acids		29		29
Mass, native (measured)[c]	3077.1			3048.1
Mass, native (calculated)[d]	3077.5			3048.5
Mass, alkylated and digested (measured)[e]	3443.4			3414.5
Mass, alkylated and digested (calculated)[f]	3443.5			3414.5

Residues from amino acid analyses are listed to the left (aa anal.); and the residues from sequencing (seq.), to the right.

[a] Half-cystine was determined as cysteic acid with a separate sample following oxidation with performic acid.

[b] Cysteine alkylated with iodacetamide.

[c] Determined by nanospray MS (LCQ, Finnigan).

[d] Calculated using average masses with the total sum from amino acid composition adjusted to the macrocyclic structure (−18 Da) and three disulfide bridges (−6 Da).

[e] Determined by nanospray MS (QTOF, Micromass).

[f] Calculated using average masses with the total sum from amino acid composition adjusted to the digested (+18 Da) and alkylated structure (+348 Da).

2.2. Homology modelling

A search of the Protein Data Bank (PDB) revealed the sequence of kalata B1 [PDB entry 1kal (Saether et al., 1995)] to be most similar to both vodo M (**1**) and vodo N (**2**), with sequence identities of 65 and 76%, respectively. The aligned amino acid sequences are shown in Table 2. Since the structure of kalata B1 [PDB entry 1kal (Saether et al., 1995)] has been resolved solely by NMR, we used the program OLDERADO (Kelley and Sutcliffe, 1997) to identify the most representative structure, which was used as the single template for the model building; this was NMR structure number 9 of the 10 NMR structures of kalata B1. The 3-D structures of vodo M (**1**) and vodo N (**2**) generated by MODELLER (Sali and Blundell, 1993) and the experimentally determined structure of kalata B1 (Saether et al.,

Table 2
The sequences of the two cyclotides, vodo M (**1**) and vodo N, aligned with the template sequence kalata B1

Cyclotide	Sequence alignment	References
kalata B1	cyclo-SWPVCTRNGLPVCGETCVGGTCNTPGCTC	Saether et al., 1995
vodo M	cyclo-SWPVCTRNGAPICGESCFTGKCYTVQCSC	this paper
vodo N	cyclo-SWPVCYRNGLPVCGETCTLGKCYTAGCSC	this paper

The alignment is produced in QUANTA (Accelrys Inc, San Diego), using the one-letter code for amino acids. The sites of the conserved cysteines involved in disulphide bonds are shaded.

1995) are shown in Fig. 1. The structures of vodo M (**1**) and vodo N (**2**) superimposed on kalata B1 using the programs QUANTA (Accelrys Inc, San Diego) and SQUID (Oldfield, 1992) show root mean square deviations (RMSD) of 0.64 and 0.65 Å, respectively (Fig. 2), which is indicative of only minor deviations in backbone structures of the models and the template; most of the changes are localized to the turn regions.

2.3. Evaluation of models

The stereochemical quality of the models was analysed using PROCHECK (Laskowski et al., 1993). The covalent geometry of the modelled structures was in agreement with the template structure, kalata B1, as judged by the examination of the Ramachandran plots (not shown). In fact, in contrast to six residues that are in strained conformations in the template, the structures of vodo M (**1**) and vodo N (**2**) have only one residue (Cys22) violating the Ramachandran plot.

To further check the quality of the homology models with reference to known rules of protein folding, energy profiles were calculated using PROSAII (Sippl, 1993). The main outcome of this comparison was that the models are overall correctly folded as indicated by low energy profiles (Fig. 3). The energy profiles of residues number 17–27 of both models deviated to some extent from the template. These deviations where, however, not considered to incriminate the general impression of the model accuracy.

Upon refining the modelled structures using molecular dynamics (MD; in Fig. 3, referred to as vodo M-MD and vodo N-MD) structures were observed with essentially similar structural violations as the originally obtained models, hence the approach of MD was disregarded in subsequent analyses.

In order to examine the validity of the side-chain rotamers of the models, the program SCWRL (Bower et al., 1997) was used. The RMSD differences between our models and those generated by SCWRL for all non-hydrogen atoms were 1.5 Å both for vodo M and vodo N. This could be compared to the average difference of 1.2 Å between the 10 NMR structures of kalata B1, and the average of 1.5 Å for SCWRL applied to each of the 10 NMR structures of kalata B1. The similarities of modelled and experimental variations in coordinates further reinforces our models.

The secondary structures of the models were analysed using QUANTA (Accelrys Inc, San Diego) and an antiparallel β-sheet separated by tight turns was found to be the main elements of the secondary structures in both models and template, which is in agreement with previously reported results (Saether et al., 1995).

2.4. 3-D Structure characteristics

The 3-D structure of the template kalata B1, also reflected in the models of vodo M (**1**) and vodo N (**2**), reveal a compact structure (Fig. 1). As illustrated in the figure (with the peptides characterised by dimensions of $\sim 10 \times 13 \times 18$ Å), a cyclic backbone constrained by three disulphide bridges at its core gives the molecules a globular shape with flattened sides, making it relevant to refer to cyclotides as topologically complex microproteins. The conceivable rigidity imposed by the cyclic cysteine knot motif permits the cyclotides to present hydrophobic amino acids on the surface even in a polar environment; this, although not yet characterised experimentally, is probably biologically significant.

The 3-D modelled structures of vodo M (**1**) and vodo N (**2**) show clear clusters of hydrophobic and hydrophilic residues across opposite faces that lead to an amphipathic spatial distribution of amino acids (Fig. 1). These amino acids could possibly be involved in either the formation of functional self-aggregates or in non-polar interactions with some target receptors. Additionally, residues that might contribute to strong polar interactions with a target are located at similar positions in the molecules. While the anionic residue (Glu15) is located on the flatter surface, the cationic residues (Arg7 and Lys21) are located on the peripheral edges.

The conserved structural features of vodo M (**1**) and vodo N (**2**) represent a scaffold with extreme stability and with a well defined pattern of surface exposed residues. Due to the disulphide bonds, the intercysteine loops form tight turns (Fig. 2), outwards presenting defined amino acids, which have the possibility of interacting with a hypothetical target. Although the functional significance of this fascinating distribution of charged and hydrophobic residues is currently

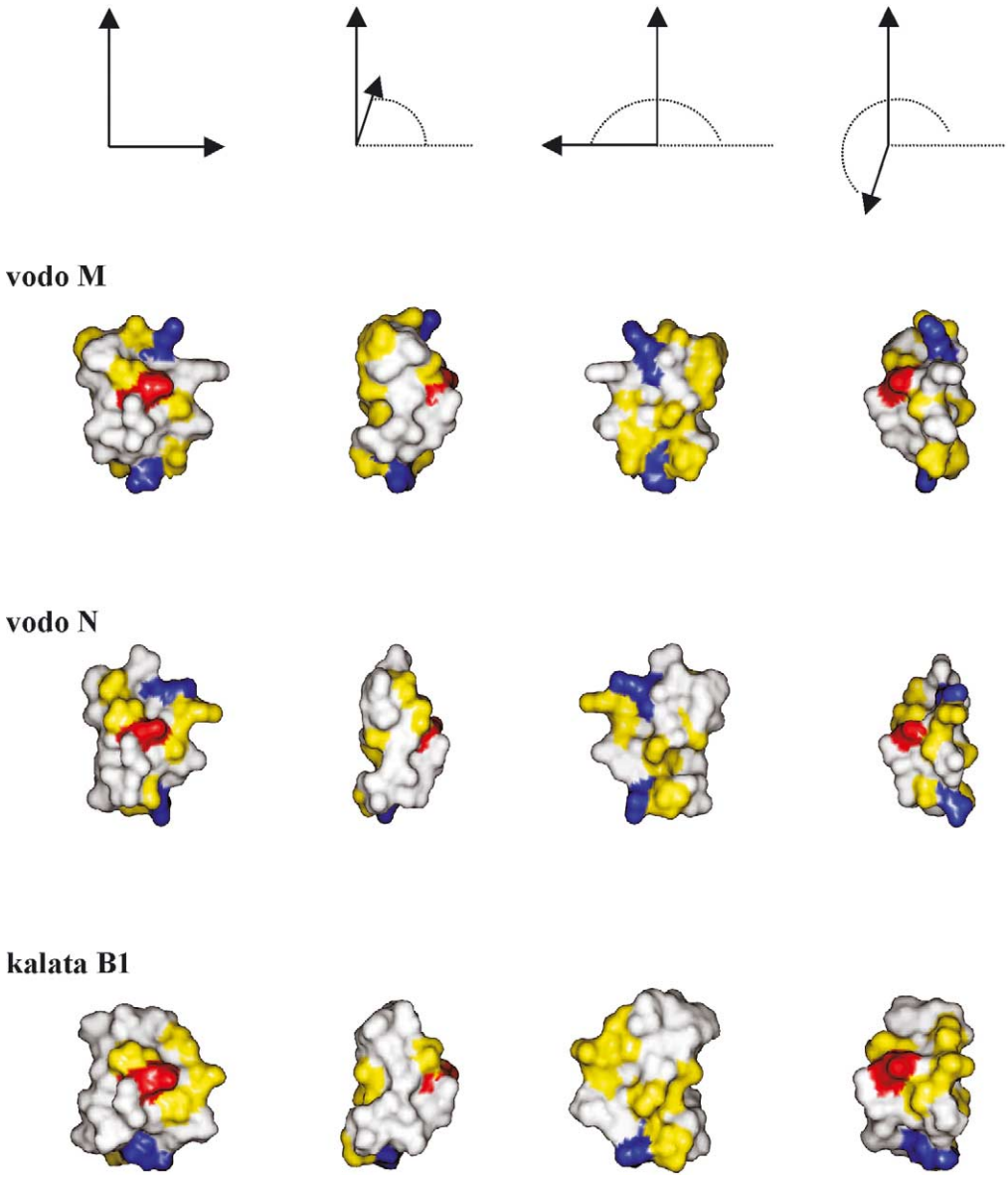

Fig. 1. A molecular surface plot of vodo M (**1**), vodo N (**2**) and kalata B1, coloured by polarity (red indicating negatively charged residues, blue indicating positively charged residues, white indicating hydrophobic residues, and yellow indicating hydrophilic residues). The molecular surfaces were calculated in QUANTA (Accelrys Inc, San Diego). Each peptide is presented in four molecular surface plots, representing rotations of 90° around the vertical axis, as indicated at the top of the figure. The modelled structures of vodo M and vodo N show amphipathic structures with hydrophobic amino acids presented on the surface (even in a polar environment).

unknown, it appears likely that they form the basis for a distinct structure–function relationship.

3. Experimental

3.1. General experimental procedures

HPLC was done using an Amersham Pharmacia Biotech ÄKTA system, equipped with a UV detector collecting data at the wavelengths 215, 254, and 280 nm. Also, a Shimadzu LC10 system was used, equipped with a photodiode array detector (SPD-M10AVP, Shimadzu) collecting data in the wavelength interval 200-300 nm. For MS, a Finnigan LCQ and a Micromass ESI-QTOF were used. Edman degradation was done using Applied Biosystems ABI 477A equipment. Calculations for homology modelling were made on a Silicon Graphics O2. Endoproteinase Glu-C sequencing grade was purchased from Promega Co., WI, USA. HPLC gradient grade CH_3CN and *iso*-PrOH, and spectroscopy grade TFA, were from Merck.

3.2. Plant material

The aerial parts of Sweet violet, *Viola odorata* L. (Violaceae), were collected in Uppsala, Sweden. A voucher

Fig. 2. The backbone superpositions in stereo view for the modelled structures of vodo M (**1**) (blue colour) and vodo N (**2**) (red colour) over the template kalata B1 (green colour). The disulphide bonds are also displayed in the pictures. Backbone RMSD of vodo M and vodo N to the template was 0.64 and 0.65 Å, respectively. Structures and calculations were performed in QUANTA (Accelrys Inc, San Diego).

specimen is deposited at the Uppsala University herbaria (UPS) with number V-128404. In this work, large scale quantities of commercial grade aerial parts of *V. odorata* were obtained from Alfred Galke GmbH (Gittelde, Germany). A voucher sample of the plant material used for extraction is deposited at the Department of Medicinal Chemistry, Division of Pharmacognosy, Uppsala University, Biomedical Centre, Uppsala, Sweden with collection number VM184. Analysing the plant material (VM184) by LC-MS, the cyclotide pattern was found to be in agreement with the previous collection (V-128404).

3.3. Extraction and isolation

The dried and powdered plant material (260 g) was extracted by maceration for 24 h with CH_2Cl_2 (7×1500 ml), 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 50% aq EtOH (7×1500 ml). This extract was evaporated in vacuo to a circa 200 ml volume and then acidified by addition of HOAc to a final concentration of 2% HOAc. 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 (Claeson et al., 1998). The tannin free extract was evaporated in vacuo and then redissolved in H_2O (250 ml). The aqueous solution was then partitioned thrice with *n*-BuOH of equal amount. The *n*-BuOH phases were evaporated to dryness *in vacuo,* and cation exchange chromatography was performed, using two 50×7.5 (i.d.) mm Vydac SCX 400 VHP575 columns in series, which were eluted over 30 min at a flow rate of 1 ml/min with a linear NaCl gradient (0–1 M) containing 25% CH_3CN and 0.1% TFA (v/v). Further purification 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), and eluted over 20 min at a flow rate of 4 ml/min with a linear CH_3CN:*iso*-PrOH (3:2) gradient (30–50%) containing 0.1% TFA (v/v). HPLC peaks were screened for cyclotides, using a photodiode array detector and ESI-MS (LCQ, Finnigan). Final purification was done by means of analytical HPLC using a 250×4.6 (i.d.) mm Rainin Dynamax (C_{18}, 5 μm, 300 Å), and eluted isocratically at 32.5% CH_3CN with 0.1% TFA for vodo N and 34% CH_3CN with 0.1%TFA for vodo M, at a flow rate of 0.8 ml/min.

3.4. Determination of primary structure

The amino acid content of the peptides was quantitatively determined at the Amino Acid Analysis Centre, Department of Biochemistry, Uppsala University, as reported previously (Claeson et al., 1998).

For the amino acid sequence analysis, the peptide was reduced with dithioerytrothiol in 0.25 M Tris–HCl containing 1 mM EDTA and 6 M guanidine–HCl (pH 8.5, 24 °C, 2 h) and subsequently *S*-alkylated by addition of iodoacetamide to the same solution (37 °C, 1 h). The reduced and alkylated peptide was cleaved with endoproteinase Glu-C in 50 mM ammonium bicarbonate buffer (pH 7.8, 37 °C, 4 h). Desalting and isolation of alkylated and linear peptides were accomplished by means of HPLC using a 30×2.1 (i.d.) mm Brownlee Aquapore column (C_{18}, 7 μm, 300 Å), which was eluted with a linear CH_3CN gradient (6–36%) containing 0.1% TFA at a flow rate of 0.2 ml/min. The linear and alkylated product was subjected to automated Edman degradation and MS^2 (ESI-QTOF, Micromass) to verify the C-terminal sequence.

3.5. Homology modelling

All calculations were performed on a Silicon Graphics O2 using a R10000 200 MHz processor. Structures were graphically displayed using the software QUANTA (Accelrys Inc, San Diego). Experimentally determined cyclotide structures available in the Protein Data Bank (PDB) were compared to vodo M (**1**) and vodo N (**2**) amino acid sequences, using BLASTp (Altschul et al., 1997) in the default settings. The sequence showing the highest similarity was used as the template structure. The program OLDERADO (Kelley and Sutcliffe, 1997) was used to calculate the most representative structure within the ensemble of NMR structures of the template. By entering the PDB file of the template in OLDERADO, the program clustered the NMR structures, and the one closest to the centroid of the largest cluster was selected as the most representative structure.

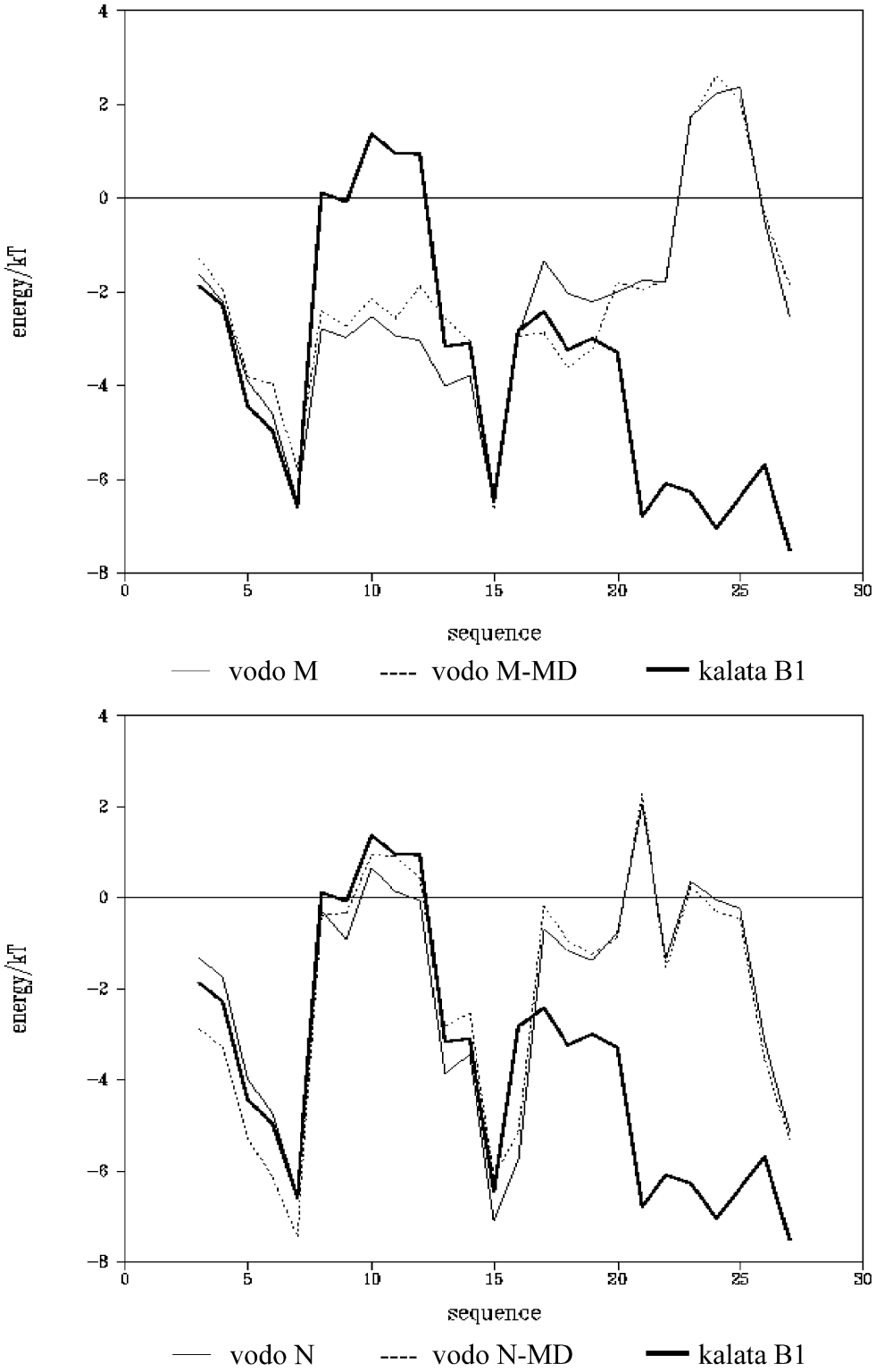

Fig. 3. The sequence-structure compatibilities for the structures generated by MODELLER (vodo M (**1**) and vodo N (**2**)), the structures obtained from molecular dynamics simulation (vodo M-MD and vodo N-MD), and the template (kalata B1), assessed using the PROSAII program. The two axes display the energetic architecture of the protein fold, with high energies corresponding to strained regions of the protein chain.

The template and target sequences were superimposed on the basis of alignments in QUANTA (Accelrys Inc, San Diego). The program MODELLER (Sali and Blundell, 1993) was used to calculate ten homology models of each peptide by optimising the objective function from ten different initial conformations. For each calculation the model with the lowest value of the objective function was selected as the best model. The

program MODELLER needed a special patch for the definition of cyclic structure. This was kindly provided by Dr. David Norman, School of Life Sciences, Division of Biological Chemistry and Molecular Microbiology, University of Dundee.

The root mean square deviations (RMSD) of the modelled structures from the template were calculated in QUANTA (Accelrys Inc, San Diego).

As a complement to homology modelling, molecular dynamics (MD) simulations were performed on the modelled structures of vodo M and vodo N to relax the structure and to improve the stereochemical quality. The initial models were placed in a 35 Å sphere of water, and vodo M (**1**) and vodo N (**2**) were solvated in 996 and 1005 water molecules, respectively. The peptides were modelled with CHARMM potential (MacKerell et al., 1998) while the water molecules were modelled using the TIP3 potential (Jorgensen et al., 1983). The molecular dynamics protocol for vodo M (**1**) and vodo N (**2**) consisted of an initial minimisation of the models to relax the structures and to remove bad contact with water, a heating phase to reach 300 K, and subsequently, the simulation was performed for 200 ps at 300 K. The time step in the MD simulation was 1 fs. Trajectories were saved every 1 ps.

3.6. Evaluation of models

The 3-D structures of vodo M (**1**) and vodo N (**2**) created by MODELLER and the structures generated by MD simulation were analysed interactively on a graphics workstation. The stereochemistry of the representative models was further analysed using PROCHECK (Laskowski et al., 1993), and sequence-structure compatibility was assessed using the PROSAII program (Sippl, 1993). In order to examine the validity of the side-chain rotamers of the models, the program SCWRL (Bower et al., 1997) was used.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Bokesch, H.R., Pannell, L.K., Cochran, P.K., Sowder, R.C., McKee, T.C., Boyd, M.R., 2001. A novel anti-HIV macrocyclic peptide from *Palicourea condensate*. J. Nat. Prod. 64, 249–250.

Bower, M.J., Cohen, F.E., Dunbrack Jr, R.L., 1997. Prediction of protein side-chain rotamers from a backbone-dependent rotamer library: a new homology modeling tool. J. Mol. Biol. 267, 1268–1282.

Broussalis, A.M., Göransson, U., Coussio, J.D., Ferraro, G., Martino, V., Claeson, P., 2001. First cyclotide from *Hybanthus* (Violaceae). Phytochemistry 58, 47–51.

Cheetham, G.M.T., Jeruzalmi, D., Steitz, T.A., 1999. Structural basis for initiation of transcription from an RNA polymerase-promoter complex. Nature 399, 80–83.

Claeson, P., Göransson, U., Johansson, S., Luijendijk, T., Bohlin, L., 1998. Fractionation protocol for the isolation of polypeptides from plant biomass. J. Nat. Prod. 61, 77–81.

Craik, D.J., 2001. Plant cyclotides: circular, knotted peptide toxins. Toxicon. 39, 1809–1813.

Craik, D.J., Daly, N.L., Bond, T., Waine, C., 1999. Plant cyclotides: A unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. J. Mol. Biol. 294, 1327–1336.

Craik, D.J., Simonsen, S., Daly, N.L., 2002. The cyclotides: novel macrocyclic peptides as scaffolds in drug design. Curr. Opin. Drug Discovery Dev. 5, 251–260.

Gran, L., 1973. Isolation of oxytocic peptides from *Oldenlandia affininis* by solvent extraction of tetraphenylborate complexes and chromatography on Sephadex LH-20. Lloydia 36, 207–208.

Gustafson, K.R., Sowder II, R.C., Henderson, L.E., Parsons, I.C., Kashman, Y., Cardellina II, J.H., McMahon, J.B., Buckheit Jr, R.W., Pannell, L.K., Boyd, M.R., 1994. Circulins A and B: novel HIV-inhibitory macrocyclic peptides from the tropical tree *Chassalia parvifolia*. J. Am. Chem. Soc. 116, 9337–9338.

Göransson, U., Luijendijk, T., Johansson, S., Bohlin, L., Claeson, P., 1999. Seven novel macrocyclic polypeptides from *Viola arvensis*. J. Nat. Prod. 62, 283–286.

Hallock, Y.F., Sowder, R.C., Pannell, L.K., Hughes, C.B., Johnson, D.G., Gulakowski, R., Cardellina, J.H., Boyd, M.R., 2000. Cycloviolins A-D, anti-HIV macrocyclic peptides from *Leonia cymosa*. J. Org. Chem. 65, 124–128.

Jennings, C., West, J., Waine, C., Craik, D.J., Anderson, M., 2001. Biosynthesis and insecticidal properties of plant cyclotides: the cyclic knotted proteins from *Oldenlandia affinis*. Proc. Natl. Acad. Sci. USA 98, 10614–10619.

Jorgensen, W.L., Chandrasekhar, J., Madura, J.D., Impey, R.W., Klein, M.L., 1983. Comparison of simple potential functions for simulating liquid water. J. Chem. Phys. 79, 926–935.

Kelley, L.A., Sutcliffe, M.J., 1997. OLDERADO: on-line database of ensemble representatives and domains. Protein Sci. 6, 2628–2630.

Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Crystallogr. 26, 283–291.

Lindholm, P., Göransson, U., Johansson, S., Claeson, P., Gullbo, J., Larsson, R., Bohlin, L., Backlund, A., 2002. Cyclotides—a novel type of cytotoxic agents. Mol. Cancer Therap. 1, 365–369.

MacKerell, A.D., Bashford, D., Bellott, M., Dunbrack, R.L., Evanseck, J.D., Field, M.J., Fischer, S., Gao, J., Guo, H., Ha, S., Joseph-McCarthy, D., Kuchnir, L., Kuczera, K., Lau, F.T.K., Mattos, C., Michnick, S., Ngo, T., Nguyen, D.T., Prodhom, B., Reiher, W.E., Roux, B., Schlenkrich, M., Smith, J.C., Stote, R., Straub, J., Watanabe, M., Wiorkiewicz-Kuczera, J., Yin, D., Karplus, M., 1998. All-atom empirical potential for molecular modeling and dynamics studies of proteins. J. Phys. Chem. B 102, 3586–3616.

Oldfield, T.J., 1992. SQUID: a program for the analysis and display of data from crystallography and molecular dynamics. J. Mol. Graphics 10, 247–252.

Saether, O., Craik, D.J., Campbell, I.D., Sletten, K., Juul, J., Norman,

D.G., 1995. Elucidation of the primary and three-dimensional structure of the uterotonic polypeptide kalata B1. Biochemistry 34, 4147–4158.
Sali, A., Blundell, T.L., 1993. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815.
Schöpke, T., Hasan Agha, M.I., Kraft, R., Otto, A., Hiller, K., 1993. Hämolytisch aktive Komponenten aus *Viola tricolor* L. und *Viola arvensis*. Murray. Sci. Pharm 61, 145–153.
Sippl, M.J., 1993. Recognition of errors in three-dimensional structures of proteins. Proteins 17, 355–362.
Tam, J.P., Lu, Y.A., Yang, J.L., Chiu, K.W., 1999. An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. Proc. Natl. Acad. Sci. USA 96, 8913–8918.
Trabi, M., Craik, D.J., 2002. Circular proteins-no end in sight. Trends Biochem. Sci. 27, 132–138.
Tung, C.S., Joseph, S., Sanbonmatsu, K.Y., 2002. All-atom homology model of the *Eschericia coli* 30S ribosomal subunit. Nat. Struct. Biol. 9, 750–755.