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Research Article

Key role of glutamic acid for the cytotoxic activity of the cyclotide cycloviolacin O2

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Abstract. Cyclotides are cyclic plant proteins with potent cytotoxic effects. Here we systematically probed the importance of surface-exposed charged amino acid residues of the cyclotide cycloviolacin O2, using a strategy involving chemical modifications. We show that the single glutamic acid plays a key role for the cytotoxicity: methylation of this residue produced a 48-fold decrease in potency. Virtually no change in potency was observed when masking the single arginine residue using 1,2-cyclohexanedione, while acetylation of the two lysine residues reduced the potency 3-fold. The derivative with modifications at both arginine and lysine residues showed a 7-fold loss of potency. In addition, we show that the activity is dependent on an intact disulfide network and that the short sequences between the six cysteine residues, that is, the backbone loops, are devoid of cytotoxic activity.

Key words. Anti-cancer; cyclotide; structure-activity; chemical modification; cytotoxic; cyclic cystine knot (CCK); human lymphoma cell line.

Plant cyclotides are small head-to-tail cyclic proteins that contain approximately 30 amino acid residues, including six conserved cysteine residues that form three disulfide bonds. The disulfides are arranged in a cystine knot: two disulfide bonds and their connecting backbone segments form a ring that is penetrated by the third disulfide bond. Together, these properties define the so-called cyclic cystine knot (CCK) motif [1]. This motif gives the cyclotides a compact and well-defined three-dimensional structure with 'inside-out' properties: the interior of the protein is occupied by disulfides that force hydrophobic amino acid residues to be exposed on the surface of the molecule. The CCK motif also confers an extraordinary stability against enzymatic digestion and thermal degradation [2, 3], which is a rare feature among proteins.

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The family name 'cyclotides' (*cyclo*-pep*tides*) was introduced by Craik et al. in 1999 [1], but the first cyclotide and the founding member of the family, kalata B1, had already been isolated in the 1970s from the tropical African plant *Oldenlandia affinis* DC. (Rubiaceae) [4]. Interest in the chemistry and pharmacology of this plant had initially followed observations that women of the Lulua tribe in Zaire used a decoction of it to accelerate childbirth [4]. The first report was followed by detailed studies of the isolation, uterotonic activity, and primary structure of kalata B1 [5, 6], but it took another 22 years before the complete sequence and structure of the protein were determined [7]. At that time, a few additional cyclotides had been discovered, that is the HIV-inhibitory circulins A and B [8], the hemolytic violapeptide I [9], and the neurotensin-binding inhibitor cyclopsychotride A [10]. These reports were followed by the discovery of a series of cyclotides from the plant family Violaceae, including the varv

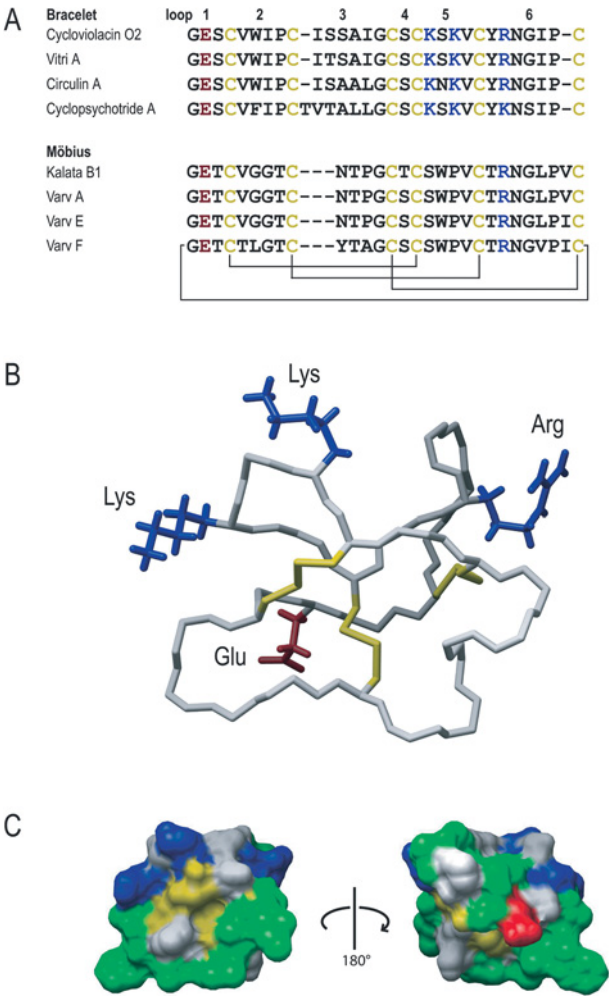

Figure 1. Cyclotide sequence and structure. (*A*) Representative examples of bracelet and Möbius cyclotide sequences with the subject of this study, cycloviolacin O2, at the top, together with the numbering of the loops. The brackets below the sequences illustrate the arrangement of the disulfide bonds and the cyclic backbone. Note the conserved features common to the whole family and the ones distinguishing each sub-family. For example, the anionic Glu residue in loop 1, the occurrence of a basic residue in loop 6, and the cystine knot motif are conserved across the sub-families, while the *cis*-Pro residue and the cluster of positively charged residues in loop 5 are found only in the Möbius and the bracelet sub-families, respectively. (*B*) The modeled protein backbone of cycloviolacin O2. The white ribbon represents the amino acid backbone and the yellow ribbons the disulfide bonds. The side chains of the anionic (Glu) and cationic (Lys and Arg) amino acid residues are represented in red and blue, respectively. These residues were chemically modified in this work. (*C*) The single negatively charged Glu residue (in red) and the positively charged Arg and Lys residues (in blue) are all exposed on the surface of the molecule. The two surface plots represent rotation of 180º around the vertical axis. Hydrophobic amino acid residues are shown in green, and surface-exposed Cys residues in yellow. See Materials and methods for details about modeling.

proteins A–F isolated from *Viola arvensis* Murr. [11, 12] and the cycloviolacins O1–O12 from *V. odorata* L. [1]. To date, over 50 cyclotides have been isolated and characterized from the Rubiaceae, Violaceae, and Cucurbitaceae plant families [13–16]; these cyclotides have been subjected to detailed structural studies [17–20], and have been reported to exhibit insecticidal [21], anti-microbial [22], anti-fouling [23], and cytotoxic effects [24, 25].

The cyclotides can be divided into two main sub-families, as shown in figure 1. Approximately one-third of the cyclotides contain a single cis-Pro protein bond, resulting in a conceptual twist of the protein backbone [1]. In combination with the circular backbone, a ribbon presentation of the protein then displays the characteristics of a Möbius strip. Hence, this subgroup is referred to as the Möbius cyclotides, while cyclotides lacking the cis-Pro peptide bond are referred to as bracelet cyclotides [1]. The division into Möbius and bracelet cyclotides also appears to coincide with other differences in amino acid sequence, as shown in figure 1. For example, cyclotides in the bracelet sub-family tend to contain a higher number of positively charged amino acids (Lys and Arg). The figure also highlights the similarities between the sub-families. For example, beside the conserved pattern of disulfide bonds, all cyclotides contain one Glu residue located at the same position in the sequence. In addition, all known cyclotides contain at least one Lys or Arg residue. Both experimental and modeled structures show that these cationic residues are exposed on the peripheral edges of the molecule.

Using a panel of ten cancer cell lines representing defined types of drug resistance, we recently showed that cyclotides have a potent cytotoxic activity and that they have unique properties that warrant further study [24, 25]. First, all of the five tested cyclotides exhibited concentration-dependant activities with IC_{50} values in the low micromolar range (comparable to clinically used anticancer drugs [25]). Second, the cyclotides showed a unique activity profile in the cell line panel, which strongly suggests a different mechanism of action from that of the cytotoxic anti-cancer drugs currently in clinical use. Third, selective and potent activity was observed in primary cultures of cancer cells directly obtained from patients: for example, the IC_{50} value of the cyclotides when tested against chronic lymphocytic leukemia cells was eight times lower than that against healthy lymphocytes, suggesting a potentially favorable selective effect [24]. Cytotoxic activity was also observed in solid tumors, for which most established chemotherapeutic drugs are less effective [24]. Lastly, and most important, potency and selectivity seemed to be dependent on the primary structure of the cyclotide. As shown in figure 1, the tested cyclotides (cycloviolacin O2, varv A, E, F, and vitri A) represent both sub-families, and the observed activity showed that bracelet cyclotides with a net charge of +2 were one order of magnitude more potent than the Möbius ones with a neutral net charge [24, 25].

In this study we systematically examined the importance of the charged residues for the most potent of the tested

cytotoxic cyclotides, cycloviolacin O2 (cyO2). As shown in figure 1, this cyclotide contains three positively charged residues (one Arg, two Lys) and one negatively charged residue (one Glu). The functional groups of these residues were then masked, solely or in combination, by chemical modifications. In addition, we tested reduced and alkylated cyO2, that is the macrocyclic protein with broken disulfide bonds, to determine the importance of the cystine knot. Furthermore, a set of short peptides corresponding to the individual loop sequences were tested to evaluate the possibility that the activity is due to an intrinsic toxicity of any of the loops. Our investigations show that both the charged residues and the network of disulfides are crucial for the activity.

Materials and methods

Materials. Acetic acid, acetic acid anhydride, acetyl chloride, boric acid, 1,2-cyclohexanedione (CHD), dithioerythriol (DTE), iodacetamide (IAM), bromoethylamine (BEA), MeOH, trypsin, and endoproteinase GluC were all obtained from Sigma (St. Louis, Mo). Acetonitrile of gradient grade (Merck, Darmstadt, Germany) was used for chromatography. Water was of Millipore grade. Aerial and dried parts of *V. odorata* were obtained from Galke (Gittelde, Germany; lot 6922, harvested 11 June 2004). The backbone loops were obtained from Thermo Electron (Waltham, Mass.) and were of >95% purity. The N and C terminals of these short peptides were acetylated and amidated, respectively. The chemicals for the assay, i.e. glutamine, penicillin, streptomycin, fluorescein diacetate, phosphate-buffered saline solution (PBS), and heat-inactivated fetal calf serum were all obtained from Sigma.

RP-HPLC. An ÄKTA basic RP-HPLC system (Amersham Biosciences, Uppsala, Sweden) equipped with a Monitor UV-900 UV detector was used for all RP-HPLC experiments. Cyclotides were detected at 215, 254, and 280 nm, and peaks were integrated at 280 nm. A Repro-Sil-Pur C18-AQ column (250×20 mm i.d., 10 µm, 300 Å) was used for preparative RP-HPLC, using a linear gradient from 10% acetonitrile in 0.05% trifluoracetic acid (buffer A) to 60% acetonitrile in 0.045% trifluoracetic acid (buffer B) over 45 min operated at a flow rate of 5 ml/min. A Vydac column (250×4.6 mm i.d., 5 µm, 300 Å) was used for the purification and analysis of native and modified cyclotides, using a linear gradient from buffer A to buffer B over 30 min operated at a flow rate of 1 ml/min.

Mass spectrometry. For ESI-MS, a Finnigan LCQ ion trap mass spectrometer was used in positive-ion mode (Thermo Electron). The capillary temperature was set at 220 °C and the spray voltage at 4 kV. For nanospray mass spectrometry, a Protana NanoES source (Proxeon Engineering, Odense, Denmark) was mounted on the same instrument. The capillary temperature was set at 150 °C, and the spray voltage at 0.5 kV. All samples were sprayed in 60% acetonitrile in 0.1% formic acid. For MS/MS sequencing, the CID was set individually for the different experiments; typical values ranged between 25 and 45%. Average isotopic masses were used for all calculated molecular weights.

Isolation of cyO2. CyO2 was isolated as previously described [11, 26] with some minor modifications. Briefly, the dried plant material was subjected to repetitive extractions with 50% aqueous EtOH. After filtration and concentration in vacuo, chlorophyll and other highly hydrophobic substances were removed by liquid-liquid extractions with dichloromethane (1:1). The aqueous phase was then taken to dryness, redissolved in water, and then extracted three times against n-butanol (1:1). The butanol phase was dried in vacuo and then subjected to preparative RP-HPLC using a linear gradient from buffer A to B. Collected fractions were analyzed using ESI-MS, and the fractions containing cyO2, detected by means of its molecular mass (3140 Da), were further purified with RP-HPLC. After freeze-drying, cyO2 was obtained as a white powder and the purity as judged by analytical RP-HPLC was >96%.

Chemical modification, analyses, and stability test of proteins. For esterification of the Glu residue, 0.34 ml (5 mmol) acetyl chloride was slowly added to 2 ml dry MeOH [27]. The mixture was stirred at room temperature for 5 min. Of this solution, 700 µl (1.5 mmol, 15,000 equivalents) was added to 300 µg (100 nmol) dry protein. The reaction was carried out at room temperature for 1 h. The reaction mixture was then diluted with 1.4 ml water and directly purified by RP-HPLC. For acetylation of the two Lys residues, 600 µg (200 nmol) dry protein was dissolved in 1 ml 50 mM NH_4HCO_3 in water. Then, 10 µl acetic acid anhydride (120 µmol, 600 equivalents) in 150 µl dry MeOH was added to the dissolved protein [28]. The reaction was carried out at room temperature for 2 min, diluted with 2 ml water, and immediately purified by RP-HPLC. For the modification of the Arg residue, 9.4 mg CHD (84 mmol) was dissolved in 1 ml 0.2 M H_3BO_3 containing 1 M NaCl [29]. Then, 500 µl (40 µmol, 400 equivalents) of the solution was added to 300 µg (100 nmol) dry protein. The reaction was kept for 18 h at 37 °C under N_2 atmosphere. The reaction was stopped by the addition of 500 µl of 30% acetic acid followed by incubation at room temperature for 10 min and then purification by RP-HPLC.

To confirm the selectivity of the modifications, the modified proteins were reduced with DTE in 0.25 M Tris-HCl

containing 1 mM EDTA and 6 M guanidine-HCl (pH 8.5) for 2 h at 37 °C under darkness and N_2 atmosphere. The free thiols of the protein were subsequently alkylated either by adding IAM or BEA to the solution followed by incubation for 60 min at room temperature (IAM) or 4 h at 37 °C (BEA) in two separate experiments [30]. The alkylated proteins were purified using RP-HPLC, and then digested with trypsin or endoproteinase GluC in 50 mM NH_4HCO_3 for 4 h at 37 °C. The proteins were dissolved in 60% acetonitrile in 0.1% formic acid before the molecular weight and the sequences of the proteins were determined by nanospray MS.

Protein alkylated with IAM was also tested in the human cancer cell line assay. In this case, native cyO2 was reduced and alkylated.

For stability tests of the modified proteins, 7.5 µg of each protein derivative was dissolved in 10% EtOH, or in the case of ([Lys(Ac)]$_2$[Arg(CHD)])cyO2, in 10% DMSO at a concentration of 300 µM, heated at 37 °C for 24 h, and analyzed using RP-HPLC and ESI-MS as described above.

Native and modified cyO2 were quantified using a Shimadzu UV-160A spectrophotometer using a calculated and experimentally determined molar absorption coefficient of 7420 mol^{-1} cm^{-1} at 280 nm.

Homology modeling and graphical display. The model of cyO2 was generated using the Modeller 7v7 package from the Departments of Biopharmaceutical Sciences and Pharmaceutical Chemistry, and California Institute for Quantitative Biomedical Research, University of California, San Francisco, Calif. [31] as described in Svangård et al. [32], using the structure of circulin A (PDB entry 1bh4 [33]) as the template. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, Calif. (supported by NIH P41 RR-01081) [34, 35].

Human cancer cell line assay. The human lymphoma cell line U-937 GTB [36] was used in the fluorometric microculture cytotoxicity assay (FMCA) as described by Larsson and Nygren [37]. The cell line was procured and maintained as described earlier [38].

For the assay, the proteins were dissolved in the following solvents at the following concentrations: CyO2, [Glu(Me)]cyO2, [Lys(Ac)]$_2$cyO2, and [Cys(CAM)]$_6$cyO2 were dissolved in water, [Arg(CHD)]cyO2 in 10% EtOH, and ([Lys(Ac)]$_2$[Arg(CHD)])cyO2 in 10% DMSO (yielding final EtOH or DMSO concentrations of 1% in the assay) to concentrations of 40 µM {for cyO2 and [Arg(CHD)]cyO2} and 800 µM (for other protein derivatives). The loop peptides were dissolved in 10% EtOH at a concentration of 1 mM. Dilution series were then made at a 1:1 ratio from these stock solutions. The purity of the tested peptides was >95% as determined by RP-HPLC.

V-shaped, 96-well microtiter plates (Nunc, Roskilde, Denmark) were prepared using 20 µl per well of protein test solution in duplicates for each concentration. In addition, six blank wells (200 µl per well of cell growth medium), six negative-control wells (20 µl per well of PBS), and six solvent control wells (20 µl per well of 10% EtOH or DMSO) were prepared on each microtiter plate. None of the used solvents affected cell growth at their final concentrations (1% EtOH or DMSO). All experiments were repeated three times.

Initial cell viability (>90%) was assessed using 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 previously filled with 20 µl of test solution at ten times the desired concentration) and incubated at 37 °C and 5% CO_2. After 24 h of incubation, the cells were washed with PBS, and 100 µl fluorescein diacetate (10 µg/ml) was added to each well. The plates were incubated at 37 °C and 5% CO_2 for 40 min, and fluorescence was then measured in a 96-well scanning fluorometer at 538 nm, following excitation at 485 nm [37]. The fluorescence is proportional to the number of living cells and cell survival is quantified as a survival index (SI) expressed in percent. The SI is defined as the fluorescence of the test wells relative to the average fluorescence of control wells (PBS), with the average for the blank wells subtracted. IC_{50} values, which correspond to the concentration at an SI of 50%, were calculated using non-linear regression in GraphPad Prism 4 (GraphPad Software, San Diego, Calif.).

Quality-assessment criteria for a successful experiment included a fluorescence signal in control wells of more than ten 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% [38].

Results

The relationship between charged amino acid residues and the cytotoxicity of cyO2 was determined by testing the activity of the cyclotide after chemical modification of the side chains of Glu, Lys, and Arg. This strategy requires specific chemistry: established methods to target these residues selectively were therefore optimized for the tested cyclotide. CyO2 was thus modified by esterification of the Glu side chain with acetyl chloride in MeOH, acetylation of the two Lys residues with acetic acid anhydride, and reaction of the guanidino group of Arg with CHD, as shown in figure 2. Reduced and S-carbamidomethylated (CAM) cyO2 was also tested to assess the importance of the cystine knot and a maintained three-dimensional structure. Native cyO2, isolated from

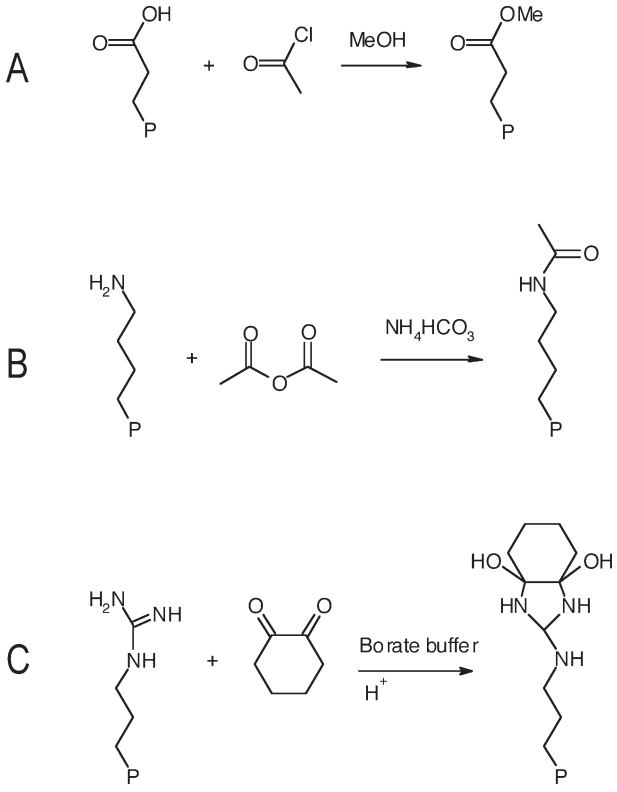

Figure 2. Reaction schemes for chemical modifications of charged side groups. The Glu residue was modified with acetyl chloride in MeOH (*A*), the two Lys residues were acetylated with acetic acid anhydride (*B*), and the Arg residue was reacted with 1,2-cyclohexanedione (*C*). P represents the protein backbone.

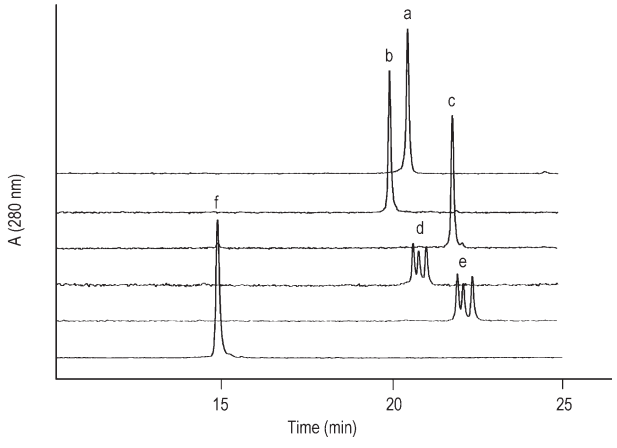

Figure 3. RP-HPLC analyses of native and modified proteins. The chromatograms show, from the top, native cyO2 (a) and the modified proteins: [Glu(Me)]cyO2 (b), ($[Lys(Ac)]_2$)cyO2 (c), [Arg(CHD)]cyO2 (d), ($[Lys(Ac)]_2$[Arg(CHD)])cyO2 (e), and $[Cys(CAM)]_6$cyO2 (f). As shown by traces (d) and (e), the Arg modification resulted in three peaks, due to the formation of isomeric condensation products. Interestingly, the increase in retention of the doubly modified protein (e) is similar to the sum of the increase of (c) and (d) alone, as compared to the native protein (a). In addition, the UV traces indicate the purity of the tested proteins.

Table 1. The yield of $[Lys(Ac)]_2$cyO2 at different equivalents and reaction times.

Equivalents of reagent	Reaction time (min)	Yield (%)
18,000	15	20
2,000	5	23
600	15	32
600	2	79
600	1	59
300	2	53

V. odorata, was used as the starting material for all chemical modifications. In addition, we tested the activity levels of the synthetic peptides corresponding to the surface-exposed loops between the cysteines (i.e. GES, VWIP, ISSAIG, KSKV, and YRNGIP). The single amino acid serine of loop 4 was not tested. The N and C termini of these short peptides were acetylated and amidated, respectively, to mimic their appearance in native cyO2.

Chemical modification of cyO2. The side chain of the Glu residue was esterified using acetyl chloride in dry MeOH [27], as shown in figure 2. This reagent has been used to modify carboxylic acids, thus masking the negative charge of anionic amino acid residues (i.e. Glu and Asp) and C terminals [27]. In the current work, the reagent could be used specifically to eliminate the charge of the Glu residue, as this is the only carboxylic acid of the cyclic protein cyO2. The reaction was straightforward, and after a reaction time of 1 h at room temperature, the modified protein was purified by RP-HPLC with a yield of ~90%. The isolated protein derivative showed an increase in molecular weight of 14 Da, which corresponds to the addition of one methyl group. As shown in figure 3, [Glu(Me)]cyO2 eluted slightly in front of native cyO2, indicating that the hydrophobicity of the protein was maintained after this modification.

To eliminate the positive charges at the two Lys residues, their amine functions were acetylated using acetic acid anhydride in an ammonium bicarbonate buffer, as shown in figure 2 [28]. At first, the reaction gave several side products, of which the major ones showed masses corresponding to proteins in which only one of the Lys residues was acetylated (i.e. the MW of cyO2 +42 Da), and only a low yield of the desired doubly acetylated protein, $[Lys(Ac)]_2$cyO2 (+84 Da), was obtained. The yield of the doubly acetylated derivative was found to vary with reaction time and equivalents of acetic acid anhydride, as summarized in table 1. A maximum yield of 79% was obtained when the protein was reacted with 600 equivalents of acetic acid anhydride for 2 min at room temperature. As for [Glu(Me)]cyO2, the retention time on RP-HPLC after acetylation of the Lys residues differed only slightly from that of the native protein, as shown in figure 3.

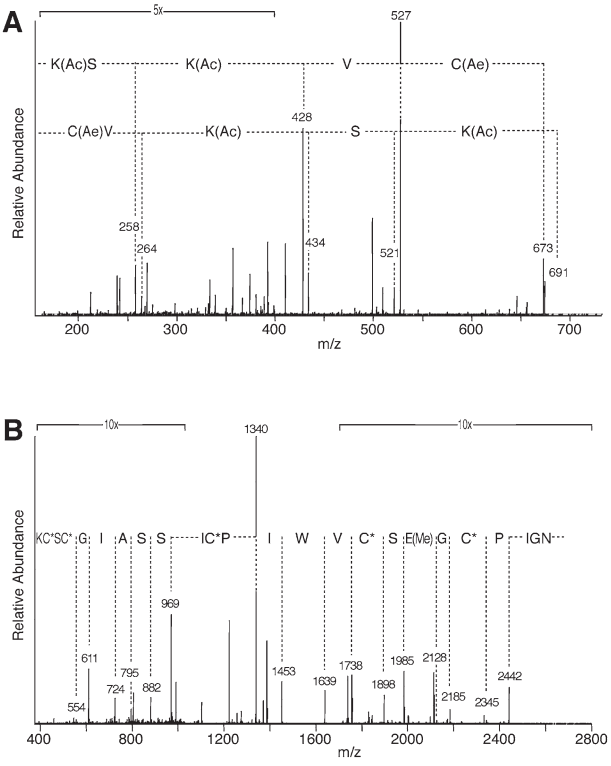

Figure 4. Examples of MS/MS sequencing of modified proteins after S-aminoethylation and S-carbamidomethylation. (*A*) Sequencing of a tryptic fragment after S-aminoethylation. In combination, the b (at the top) and y series give a complete sequence coverage and unambiguously confirm that the increase in molecular weight of $[Lys(Ac)]_2$cyO2 was due to acetylation of the two Lys residues. (*B*) Sequencing of the tryptic fragment containing the modified Glu residue. Here, the y series confirms that the Glu residue was the site of methylation. C* is Cys(CAM). See Biemann [54] for nomenclature of peptide fragment ions.

For the single Arg residue in cyO2, the guanidino group was selectively modified using CHD in a borate buffer at pH 9.0 [29], thus eliminating the positive charge, as shown in figure 2. The borate and the modified guanidino group form a stable adduct product and a bicyclic imidazole derivative is formed after addition of acid. As shown in figure 3, three major peaks were observed when purifying the crude product using RP-HPLC. All these peaks showed the same increase in molecular weight (+113 Da), which corresponds to the isomeric condensation products of CHD and the guanidino group of the Arg residue; the total yield was ~95%. The modified protein, [Arg(CHD)]cyO2, was used as an isomeric mixture in the human cancer cell line assay.

To test the stability of the modified proteins under the conditions used in the assay, all derivatives were dissolved the same way as they had been in the assay (see Materials and methods) and then kept at 37 °C for 24 h. Subsequent analyses by means of RP-HPLC and ESI-MS showed no change in either retention time or molecular weight for any of the proteins; hence the proteins were considered stable for the duration of the assay.

All proteins were sequenced by nanospray MS/MS to verify the specificity of the modifications. Before sequencing, however, the CCK motif requires that the disulfides be broken and alkylated and that the cyclic backbone be cleaved; thus the proteins were reduced with DTE and alkylated with BEA. This aminoethylation introduces six additional positively charged enzymatic cleavage sites, which lead to smaller tryptic fragments and more easily interpretable MS/MS spectra [30]. However, complete sequence coverage might sometimes be difficult to obtain, so this approach was complemented with reduction and subsequent alkylation with IAM in a separate set of experiments, in order to unambiguously determine the site of the modifications. The head-to-tail cyclic alkylated proteins were then cleaved with either endoproteinase GluC or trypsin. For sequencing, MS/MS data from both types of alkylations were used. Figure 4 shows examples of MS/MS spectra of tryptic digests in which both BEA (fig. 4A) and IAM (fig. 4B) were used as alkylation reagents. Sequencing the fragments by means of MS/MS verified that the targeted amino acid residues were selectively modified: that is, the increase in molecular weight had occurred at the fragment containing the target amino acid residue.

For the modification of both Lys and Arg residues, cyO2 was first acetylated, purified by RP-HPLC, dried, and finally subjected to the same reaction as for the modification of only the Arg residue, as described above. The change in the RP-HPLC retention time of this derivative, $([Lys(Ac)]_2[Arg(CHD)])$cyO2, followed the same pattern as for the other protein derivatives, as shown in figure 3. This derivative was used as an isomeric mixture in the human cancer cell line assay.

The non-knotted S-carbamidomethylated protein, $[Cys(CAM)]_6$cyO2, was obtained in good yields using the same protocol as used for alkylation before sequencing. The protein showed an increase in molecular weight of 348 Da, corresponding to the increase produced by the carbamidoethylation of six cysteine residues (6×58 Da). This reaction completely disrupts the overall fold of the cyclotide, as is revealed by the dramatic decrease in retention time shown in figure 3; thus, the unfolded protein is considerably more polar than the native cyO2.

Activity in the human cancer cell line assay. The cytotoxic activity of all proteins was tested using the human lymphoma cell line U-937 GTB in an FMCA [37]. This cell line is considered to be sensitive to a wide range of cytotoxic agents and has not exhibited any known drug resistance. After 24 h of treatment, the SI was calculated using the ratio of living cells in treated wells versus living cells in the control wells. The SI is a direct measurement of cell death, or of the cell toxicity of the tested substance at a particular concentration, which is then used to construct the log concentration response curves and calculate the IC_{50} values.

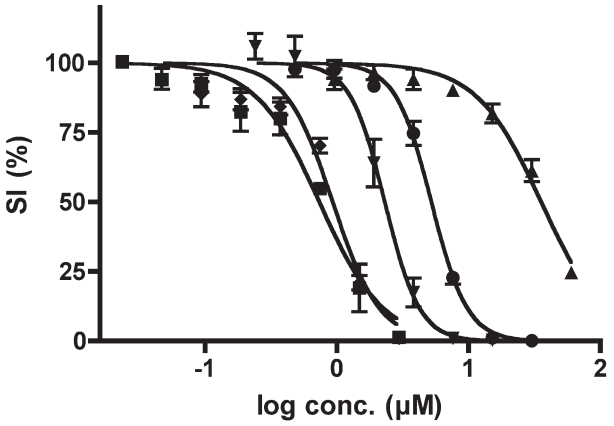

Figure 5. Effect on the human lymphoma cell line U-937 GTB. The concentration response curves of native cyO2 and its derivatives are shown as follows (from left to right): native cyO2 (squares), [Arg(CHD)]cyO2 (diamonds), $[Lys(Ac)]_2$cyO2 (downward-pointing triangles), ($[Lys(Ac)]_2$[Arg(CHD)])cyO2 (circles), and [Glu(Me)]cyO2 (upward-pointing triangles). The activity is calculated as an SI in percent as described in Materials and methods. Each point represents the mean and the error bars shows the SE.

As shown in figure 5, though all of the charge-modified proteins showed concentration-dependent cytotoxic effects, their potencies were significantly different. Native cyO2 and [Arg(CHD)]cyO2 were shown to be equipotent, while $[Lys(Ac)]_2$cyO2 and ($[Lys(Ac)]_2$[Arg(CHD)])cyO2 were three and seven times, respectively, less potent than the native protein. Thus, the protein tolerates the modification of only the guanidino group of Arg, or only the two amines of the Lys residues, without a major loss of activity. However, the cyO2 derivative without any positively charged functional groups was less potent than what would have been expected from the results obtained with [Arg(CHD)]cyO2 and $[Lys(Ac)]_2$cyO2 alone. This suggests an apparent synergistic effect of the modification reactions of the cytotoxic activity. Accordingly, the existence of at least one positively charged residue seems crucial to sustain potency in the same range as that of the native cyclotide. Indeed, a complete absence of negative charges also dramatically reduced the potency. Masking the only negative charge of cyO2 gave a considerable decrease in potency, as shown by the IC_{50} value of [Glu(Me)]cyO2, which was 48 times higher than that of the native protein. Note that all the modified proteins had concentration response curves similar in shape to that of the native protein (fig. 5), with a very steep slope suggesting an on–off effect (i.e. toxic-non-toxic).

Neither reduced and alkylated cyO2, $[Cys(CAM)]_6$cyO2, nor the six backbone loop peptides showed any cytotoxic activity at concentrations 100 times higher than that of the IC_{50} of native cyO2, and were thus considered inactive. Table 2 summarizes the results for all tested proteins and peptides.

Discussion

In the current study, we systematically and specifically masked the functional groups of the charged amino acid residues in the cyclotide cyO2. The cytotoxic effects of native and modified proteins were then tested on a human lymphoma cell line. In addition, we tested reduced and alkylated cyO2 and a set of short peptides corresponding to the amino acid sequences of the loops between the six cysteines. The results show that both the positively and negatively charged amino acid residues are important for the cytotoxic activity, and that the activity of cyO2 greatly depends on a maintained disulfide network and the overall fold of the protein.

Chemical modification of charged amino acid residues was used as a tool in specifically investigating the importance of both positively and negatively charged side chains for the interaction between the protein and its target. Hence, it was crucial that the chemical reaction should be selective for the targeted amino acid residue. To this end, we eliminated the positive charge of the Lys and Arg residues by acetylation and by reaction with CHD, respectively, and the negative charge of the single Glu residue by methylation, and all reactions were found to modify their targets specifically. As shown by the modeled structure of cyO2 in figure 1, all of the targeted amino acids are exposed on the surface of the molecule. This model shows that the positively charged residues are clustered along one edge of the molecule, while the neg-

Table 2. IC_{50} values and relative potency for native and modified proteins for the human lymphoma cell line U937 GTB.

Protein/peptide	IC_{50} (µM)	Relative potency[1]
cyO2[2]	0.75 (0.63–0.88)	1
[Glu(Me)]cyO2[2]	36 (32–40)	1/48
$[Lys(Ac)]_2$cyO2[2]	2.3 (2.0–2.5)	1/3
[Arg(CHD)]cyO2[2]	0.95 (0.82–1.1)	1
($[Lys(Ac)]_2$[Arg(CHD)])cyO2[2]	5.1 (4.8–5.5)	1/7
$[Cys(CAM)]_6$cyO2	> 80	N/A
GES[3]	>100	N/A
VWIP[3]	>100	N/A
ISSAIG[3]	>100	N/A
KSKV[3]	>100	N/A
YRNGIP[3]	>100	N/A

[1] No relative potency was calculated for proteins or peptides lacking cytotoxic activity at the concentrations used in the assay (N/A in table).
[2] Best-fit values and, in parentheses, 95% confidence intervals.
[3] The N and C termini were acetylated and amidated, respectively.

atively charged Glu residue protrudes from the otherwise mainly hydrophobic surface. None of the modifications add bulky groups, and, judged from the minor changes in RP-HPLC retention times as shown in figure 3, they do not disrupt the overall hydrophobic surface of the protein. Most likely, the derivatives also maintain the three-dimensional structure of the native peptide. Experimentally determined cyclotide structures are indeed very similar, even for cyclotides with large differences in amino acid sequence [39]. In comparison, the changes conferred by the modifications must be considered minor.

Modification of the single Arg residue did not significantly reduce the potency, whereas acetylation of the two Lys residues reduced the cytotoxic potency threefold. Although the elimination of only one or two of the three positively charged residues in cyO2 did not considerably lower the activity, the derivative lacking all three positive charges displayed a sevenfold loss of activity. The cyO2 derivative with a modified Glu residue was the protein that showed the lowest cytotoxic activity: methylation of the Glu side chain lowered the potency 48-fold, that is, more than six times as much as the derivative in which both Arg and the two Lys residues were modified.

We have previously associated a potent cytotoxic activity with a high positive net charge of the cyclotide, that is, with the bracelet cyclotides [24, 25]. For example, the bracelet cyO2 with a net charge of +2 at physiological pH showed more than 10 times the potency of the Möbius varv A with a net charge of ± 0 [24]. Hence, we were surprised to find that the derivative with only one positively charged residue (i.e. the cyO2 derivative with two masked Lys residues) maintained most of the cytotoxic activity of native cyO2, as the net charge of this derivative is ± 0. This indicates that the inherent differences explaining the higher potency of the bracelet cyclotides, as compared to the Möbius ones, are hidden in a different part of the sequence than in the positively charged loop 5 with its two Lys residues. However, the presence of at least one positively charged residue seems crucial, as masking both the Arg and Lys residues produced an apparently synergistic reduction in potency. As shown by the sequences in figure 1A, the position of the Arg residue is conserved in all cyclotides hitherto tested for cytotoxic activity, and at least one Lys or Arg residue is found in all reported cyclotides. This is a further indication of the significance of at least one cationic residue in the cyclotide; possibly, this cationic residue is conserved to sustain the natural biological function of the cyclotide. Following the same line of reasoning, the dramatically decreased effect of the Glu-modified protein was unexpected, as this protein has a net charge of +3. Instead, the results indicate that losing the only negatively charged residue removes a functional group that is vital for the proteins' interaction with its target. However, detailed nuclear magnetic resonance studies have shown that the Glu residue in cycloviolacin O1 is an acceptor of multiple hydrogen bonds from residues located at the third backbone loop [20]. Methylation of the Glu residue might possibly interfere with these interactions, meaning that the three-dimensional structure of these parts of the protein is affected.

Charge-charge interactions directly between cytotoxic proteins and their targets are one of the factors known to influence their potency, together with amphipathicity and hydrophobicity. However, our finding that Glu lowers the potency the most is rather unusual as clusters of cationic charges are strongly connected to the activity. This is, for example, the case for the antimicrobial proteins known as the defensins [40, 41]. Interesting to note is in this context that the antimicrobial activity of some synthetic cyclotides has been shown to be salt dependent, and that the cyclotides circulin A and B as well as cyclopsychotride A, all of which are of the bracelet subtype containing three basic amino acids, showed higher antimicrobial activity than did kalata B1 [22]. In that study, the single cationic residue in purified synthetic kalata B1 (an Arg residue) was blocked with p-hydroxyphenylglyoxal, which sharply reduced the potency. The single Arg residue of one of the bracelet cyclotides was also modified; the Arg residue in circulin A was protected with mesitylenesulfonyl during synthesis, which was stable in the subsequent cleavage of the protein from the solid-phase resin. This derivative showed activity in the same concentration range as the non-modified protein, and it was suggested that at least one positive charge was needed to retain the effect – which is congruent with the results of the current study. However, while we used the correctly folded native protein for our modifications, the proteins used in that study by Tam et al. [22] were all synthesized, and the folding of synthetic CCK proteins may be problematic. Another factor that should not be excluded is that charge-charge interaction may not be needed for interaction with a target *per se*, but rather for the formation of cyclotide oligomers, which in turn mediate the effect. In fact, protein-protein interactions have recently been suggested to be involved in the formation of cyclotide tetramers and octamers in solution [42]. The existence of such intermolecular interactions between the basic and the anionic parts of cyO2 could possibly provide an explanation as to why neutralization of the single negative or all positively charged residues lowered the potency the most.

As shown in table 2, no activity was observed for the protein derivative with reduced and alkylated cysteines, even at concentrations 100 times higher than that of the IC_{50} of the native protein. A similar loss of activity was indeed observed in one of the first studies of the prototypic cyclotide kalata B1: reduction and alkylation of the disulfides in that protein were then observed to lead to total loss of uterine activity [6]. More recent studies have

stressed the importance of the cystine knot for the enzymatic and chemical stability of the cyclotide kalata B1 [2]. Notably, the presence of the cyclic backbone also seems to be a prerequisite for at least the hemolytic activity of kalata B1: acyclic permutation of this cyclotide eliminates its hemolytic activity, even while the overall structure is still maintained [43–45]. These results show the functional significance of the CCK motif, but still do not exclude the possibility that the cytotoxic effects are due to only a small part of the sequence. Lacking the stabilizing effect of the cystine knot, the structure of the inactive Cys(CAM) derivative is probably more like that of a normal protein, with the hydrophobic amino acid residues buried in the core of the protein and hydrophilic amino acids exposed at the surface. Once again, this is reflected in the decrease in RP-HPLC retention time of the protein, as shown in figure 3. Hence, the distortion of the three-dimensional structure most likely causes the backbone loops to be no longer exposed at the surface of the molecule, meaning that they might not be presented to a target such as the cancer cells. Therefore, a logical step in this study was to test whether the peptides corresponding to the sequences between the cysteines in cyO2 exhibited any activity. However, as these peptides did not show any activity, even at concentrations 100 times higher than those of the IC_{50} of cyO2, it is reasonable to assume that no such small pharmacophore-like peptide exists.

The mechanism of action for the cytotoxic activity of cyO2 is still unknown, as is/are the mechanism/s underlying the biological effect/s of any other cyclotide. However, regarding the cytotoxic and hemolytic effects, it has been suggested that disruption of cell membranes is involved – by analogy to other proteins with cytotoxic activities [24, 25, 43]. Indeed, there are strong indications that this is the case. For example, a recent study has shown that cyclotides have an affinity for model lipid membranes [46], and in our previous studies [24, 25], we compared the characteristically very sharp concentration response curves exhibited by the cyclotides in the FMCA with the similarly shaped curves displayed by the defensins, which are known to form pores through both mammalian cells and bacterial membranes [47–51]. This effect is exemplified by the curves in figure 5, which show that total cell death is caused by only a small change in cyclotide concentration. In addition, the defensins share with the cyclotides features such as size (e.g. number of amino acid residues), presence of three disulfide bonds, an anti-parallel beta sheet, and an overall amphipathic structure (c.f. the cyO2 model in fig. 1). Furthermore, the pore formation displayed by defensins is preceded by interactions between cationic residues exposed on the surface of the protein and anionic lipids in the bacterial membrane [52, 53]. The results of the current study show that ionic and/or electrostatic interactions are involved in the mechanism for the cyclotides as well, another feature common to both cyclotides and defensins. Thus, even if definite proof of the mechanism of action of cyclotides is still lacking, the cyclotides may eventually be found to disrupt cell membranes through a pore-forming mechanism. However, regardless of the exact nature of the mechanism, the fact that the concentration response curves of the modified proteins examined in the current study are shifted to the right of that of the native protein (i.e. the shape of the curves is identical to that of the native protein, but the modified proteins are less potent), while the maximum effect is maintained, indicates that cyO2 and its derivatives share a common mechanism. Possibly, the chemical modifications of the charged side chains in the derivatives decrease the initial protein-membrane attraction force, which could explain their lower potency.

To conclude, in this study we have systematically and effectively probed the importance of the charged amino acid residues of the cyclotide cyO2 for its cytotoxic activity. We have shown that the Glu residue is vital for activity and that at least one positively charged amino acid is needed to maintain the potency close to that of native cyO2. We have also shown that opening the intramolecular network of disulfides eliminates the cytotoxic activity, and that the cytotoxic activity does not reside in any of the individual sequence loops existing between the cysteines.

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