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Ultra-Stable Peptide Scaffolds for Protein Engineering—Synthesis and Folding of the Circular Cystine Knotted Cyclotide Cycloviolacin O2

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The cyclic cystine knot motif, as defined by the cyclotide peptide family, is an attractive scaffold for protein engineering. To date, however, the utilisation of this scaffold has been limited by the inability to synthesise members of the most diverse and biologically active subfamily, the bracelet cyclotides. This study describes the synthesis and first direct oxidative folding of a bracelet cyclotide—cycloviolacin O2—and thus provides an efficient method for exploring the most potent cyclic cystine knot peptides. The linear chain of cycloviolacin O2 was assembled by solid-phase Fmoc peptide synthesis and cyclised by thioester-mediated native chemical ligation, and the inherent difficulties of folding bracelet cyclotides were successfully overcome in a single-step reaction. The folding pathway was characterised and was found to include predominating fully oxidised intermediates that slowly converted to the native peptide structure.

Introduction

Cyclotides are a family of bioactive plant proteins that contain a head-to-tail cyclised backbone and a knotted arrangement of their three disulfide bonds.[1–3] They typically comprise 28 to 31 amino acids and are the largest family of circular proteins currently known. The cystine knot in cyclotides originates as two disulfide bonds and their interconnecting backbone segments form a ring that is penetrated by the third disulfide bond.[4–6] In combination, these structural features are unique to cyclotides and define the cyclic cystine knot (CCK) motif,[4] as shown in Figure 1. This motif endows the cyclotides with exceptional stability. Their lack of N and C termini makes them insensitive both to endoproteases, including trypsin, Glu-C, pepsin and thermolysin, and to exoproteases.[7] In addition, cyclotides have high thermal and chemical stability, and it has been shown that they are stable to simulations of the acidic conditions present in the stomach.[7]

The sequences in the six backbone loops between the conserved cysteine residues are variable and provide cyclotide-containing plants with a natural combinatorial library built around the CCK motif. This variability occurs mainly in loops 2, 3, 5 and 6, with loops 1 and 4 being more conserved, reflecting their central positions in the cystine knot. The combinatorial library strategy is exemplified by that fact that at least 57 individual cyclotides have been reported in a single plant species,[8] and it was recently estimated that the total number of different cyclotides in the plant family Violaceae alone exceeds 9000.[9] To date, more than 100 cyclotides, derived either from peptides or genes, have been sequenced, and the three-dimensional structures of ten of them have been determined.[2] These structures show that the disulfide bonds occupy the interiors of the cyclotides, and that hydrophobic residues are exposed at the molecular surfaces. As a result, the native cyclotide is generally more hydrophobic than its reduced and denatured form.

Early in studies of cyclotides it was clear that they could be categorized into two main subfamilies,[10] termed the Möbius and bracelet,[4] on the basis of the presence or absence, respectively, of a conserved *cis*-Pro residue in loop 5. The two subfamilies are further distinguished by both the amino acid content and the size of certain loops. For example, signature marks for the bracelets are a cluster of positively charged amino acids in loop 5 and a small α helix structure in loop 3. At present, approximately two thirds of the known cyclotides belong to the bracelet subfamily. Notably, the bracelet cyclotides display significantly greater sequence diversity than the Möbius ones, as shown by the sequence logo representations[11] in Figure 1. In fact, the number of combinations of native loop sequences of the bracelet subfamily alone currently exceeds 10×10^6.[2] In addition to the bracelet and Möbius subfamilies there is a third minor subfamily of CCK peptides, known as the trypsin inhibitor cyclotides.[1,12] This subfamily comprises two members—MCoTI-I and –II—which are circular versions of the linear trypsin inhibitory peptides found in Cucurbitaceae plants.

Although the natural function of cyclotides is not yet fully understood, their insecticidal activity[13] suggests that they are

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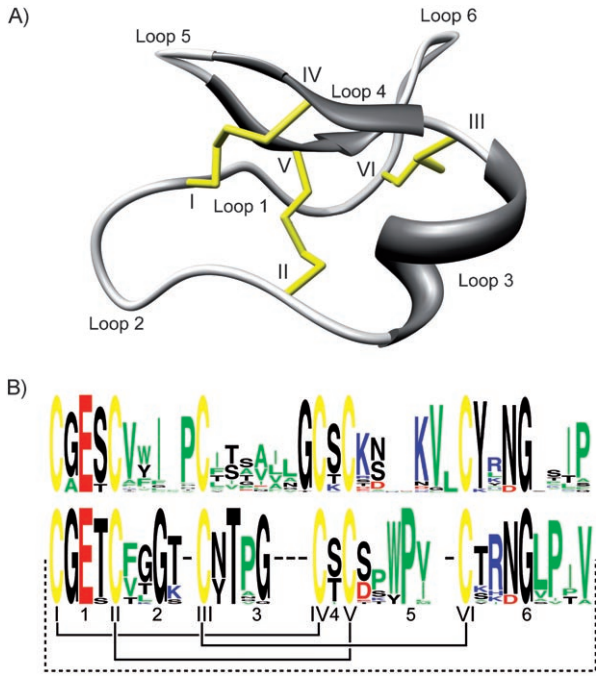
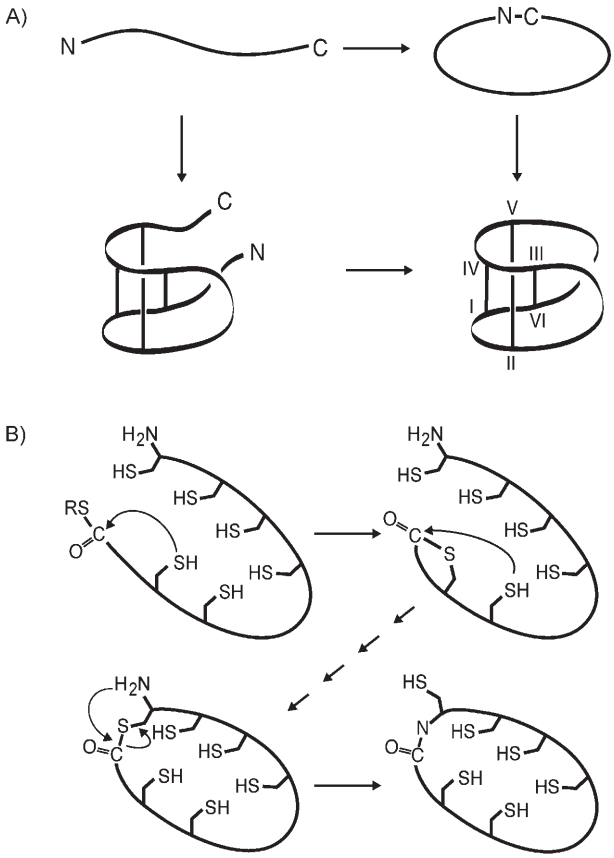


Figure 1. Cyclotide structure. A) The cyclotide scaffold and the cyclic cystine knot motif (CCK) showing the amide backbone (in grey) and the disulfide bonds I–IV, II–V and III–VI (in yellow). The β sheets and the short α helix as recognised in the bracelet subfamily are shown as ribbons. B) Sequence logos of the bracelet (top) and Möbius (below) subfamilies.

involved in host defence. Cyclotides as a group, however, have a variety of biological effects. In fact, their discovery was based on the traditional use of the plant *Oldenlandia affinis*, and its utero-contractive properties.[14] A tea from this plant was used to facilitate childbirth, and this observation led to the discovery of the prototypic (Möbius) cyclotide kalata B1. In addition, individual cyclotides have shown anti-HIV,[15] hemolytic,[16] cytotoxic[17,18] and antifouling[19] effects, and have also been reported to inhibit binding of neurotensin in a receptor-binding assay.[20] When compared in the same tests, bracelet cyclotides seem to be more potent than Möbius cyclotides, particularly in their cytotoxic activity, in which they are one order of magnitude more potent.[17,18] The reasons for these differences are just beginning to be unravelled through detailed structure activity studies, based in large part on chemical synthesis and semi-synthesis of cyclotide analogues and mutants.[21,22]

The potent and diverse biological activities of cyclotides, combined with their extraordinary stability and the sequence variability of certain loops, suggest that cyclotides could be ideal molecular scaffolds for the development of stable protein-based drugs, as well as for applications in agriculture and biotechnology.[1,2,23] However, although the trypsin inhibitory CCK peptides[24,25] and several Möbius cyclotides and mutants have been synthesised,[21,26,27] the broad application of cyclotides is hampered by the fact that bracelet cyclotides have until now been largely inaccessible to chemical synthesis. An attempt to fold this subfamily of cyclotides directly in a single step to construct the native peptide structure produced a yield of less than 5%.[28,29] The lack of a synthetic methodology has thus effectively excluded the major part of the structural variability of the CCK motif that is represented by bracelet cyclotides from being utilised as drug design templates.

There are two main strategies for the synthesis of cyclotides and the CCK motif: either the disulfide bonds are formed prior to cyclisation or vice versa, as shown in Scheme 1. So far, the



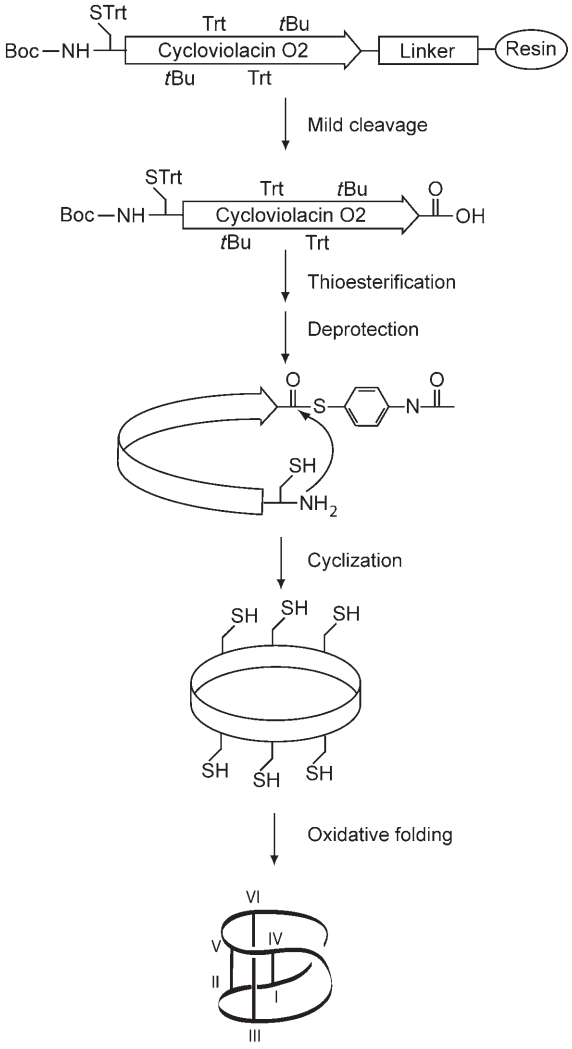
Scheme 1. A) Two routes for cyclotide synthesis: cyclisation before folding has proved to be the most successful strategy. B) NCL is assisted by the thia zip reaction, which is initiated by the Cys closest to the C terminus. The reaction proceeds through successive ring expansion by a series of thiol–thiolactone exchanges until a large thiolactone intermediate is formed with the N-terminal Cys. The amide bond linking N to C termini is then formed spontaneously through intramolecular nucleophilic attack by the Cys α-amino group.

most successful approach is the use of thioester-mediated native chemical ligation[30] (NCL) to form the circular protein prior to oxidation and folding.[26,27] The linear cyclotide protein chain is synthesised with a cysteine residue at the N terminus and a thioester linker at the C terminus. Cyclisation is subsequently performed in solution. It is postulated that this process is favoured by the so called "thia zip reaction", in which thiol–thiolactone exchange between the C-terminal thioester and the side chain thiols in the assembled peptide chain helps to connect the two ends of the linear backbone effectively.[28,31] As shown in Scheme 1, once the final thioester bond between the thiol group of the N-terminal cysteine and the C-terminal carbonyl group is formed, a spontaneous S,N acyl migration

forms an amide bond to lock the head-to-tail circular peptide backbone irreversibly.[32]

To date, solid-phase peptide synthesis (SPPS) using Boc chemistry has been the method of choice for assembling the peptide chain.[33] This chemistry has the advantage that the peptide may be connected to the resin through a thioester-based linker that subsequently forms the required C-terminal thioester after cleavage. However, such linkers are unstable under basic conditions, which rules out a similar direct route for cyclotide synthesis using Fmoc chemistry. For Möbius cyclotides, the formation of disulfides—that is, oxidative folding—is then readily achieved in solution in a high-yielding, single-step reaction using cyclised peptide. However, the Boc methodology has the disadvantage of requiring the use of toxic HF for cleavage of the peptide from the solid-phase resin.[34]

In this work we describe the first synthesis of a bracelet cyclotide, cycloviolacin O2 (CVO2), by a strategy based on Fmoc chemistry and oxidative folding of the circular protein. The Fmoc methodology relies on standard SPPS building blocks and procedures to assemble the peptide chain and cleavage from the resin. The thioester required for ligation of the N and C termini is then formed in solution after reaction of the protected peptide chain and a thiol.[35] Following deprotection, cyclisation is then achieved through NCL. Oxidative folding is then achieved by optimisation of buffer conditions, assisted by cosolvents, detergents and redox agents. Thus, in summary, we describe a strategy that is an attractive alternative to previously published Boc methods for cyclotide synthesis, and we report the first successful single-step folding conditions for bracelet cyclotides.



Scheme 2. Strategy for synthesis and folding of CVO2.

Results

The strategy to synthesise the prototypic bracelet cyclotide CVO2 is outlined in Scheme 2. In short, it first involved manual SPPS using the in situ neutralisation/HBTU activation procedure[36] and Fmoc chemistry. Fully protected peptide was then cleaved from the resin, and the thioester function required for the NCL was specifically introduced at the C terminus of the peptide in solution. In parallel, various combinations of folding conditions, including buffer systems, salt concentrations and pH, detergents, cosolvents, redox systems, temperature and time, were evaluated and optimised with reduced peptide that had been isolated from the plant. Following deprotection and cyclisation, the thus optimised conditions were then used for folding of the synthetic peptide.

CVO2 comprises 30 amino acid residues and shows all the typical features of a bracelet cyclotide, including the presence of an extended loop 3 and two Lys residues in loop 5. Although the start and endpoint of the synthesis of a circular peptide are theoretically arbitrary, the use of NCL for circularisation requires a N-terminal Cys residue in the linear precursor. Here we chose Gly16 and Cys17 as the start and endpoint, respectively, of the synthesis. Thus, besides having the N-terminal Cys residue necessary for NCL reaction, the presence of the achiral Gly residue at the C terminus efficiently avoids epimerisation during thioesterification and is also sterically favourable for the native chemical ligation reaction.[37]

The peptide chain was assembled on NovaSyn TGT resin, which was preloaded with the first residue (Gly). The first attempts were made on 2-chlorotrityl chloride resin as described in ref. [35]; however, the synthesis of this particular sequence was tedious as it required numerous recoupling reactions for each residue after a chain length of only five or six residues had been reached. This problem significantly decreased after switching to the NovaSyn TGT resin. As with the 2-chlorotrityl component, the 4-carboxytrityl linker on this resin is extremely acid-sensitive and is thus amenable for mild cleavage to release the protected peptide acid. As shown in Scheme 2, the last Cys residue to be attached—that is, the N-terminal—is Boc-protected to avoid acetylation and formation of peptide oligomers. This N-terminal protection cannot be a Fmoc group, as this cannot be removed without the thioester being affected once it is formed.

Mild cleavage, synthesis of thioester and cyclisation

Crude protected peptide acid was cleaved from the resin under mild conditions to conserve all the protecting groups, with a yield of ~96%. A sample of the protected peptide was subsequently deprotected by treatment with TFA/TIPS/water and analysed by RP-HPLC, as shown in Figure 2, and ESI-MS to confirm the integrity and identity of the peptide. In an initial attempt at thioester formation a portion of the crude protected peptide was treated with PyBOP/4-acetamidothiophenol/DIPEA (3/3/3 equiv) as reported by Eggelkraut-Gottanka et al.[35] However, for CVO2 the reported reagent equivalents did not yield any significant amount of thioester after overnight reaction. A dramatic increase in reagent equivalents—to tenfold and 50-fold, respectively, of PyBOP/4-acetamidothiophenol and 1 mL (~2200 equiv) of DIPEA (as the C-terminal Gly is not subject to racemisation, a large excess of base can be used), as reported for thioesterification of collagen-like peptide polymers[38]—resulted in an ample amount of thioester. However, under those conditions the formation of side products was considerable. Maintaining the initial equivalents of PyBOP/4-acetamidothiophenol[35] (3/3 equiv) but using DIPEA (500 μL, ~1100 equiv) resulted in a reasonable amount of thioester over 3 h. However, an appreciable amount of linear peptide without thioester was also observed. Further optimisation by increasing PyBOP to 5 equivalents and 4-acetamidothiophenol to 15 equivalents, while keeping DIPEA at 500 μL, resulted in an efficient formation of CVO2 thioester overnight. The peptide thioester was subsequently deprotected, and cyclised as described in Figure 2. The cyclisation was complete after 30 min, as judged by analytical HPLC and ESI-MS analysis of an aliquot of the reaction mixture; the reaction was then terminated by immediate purification by preparative RP-HPLC. The yield of the cyclic form was 30%.

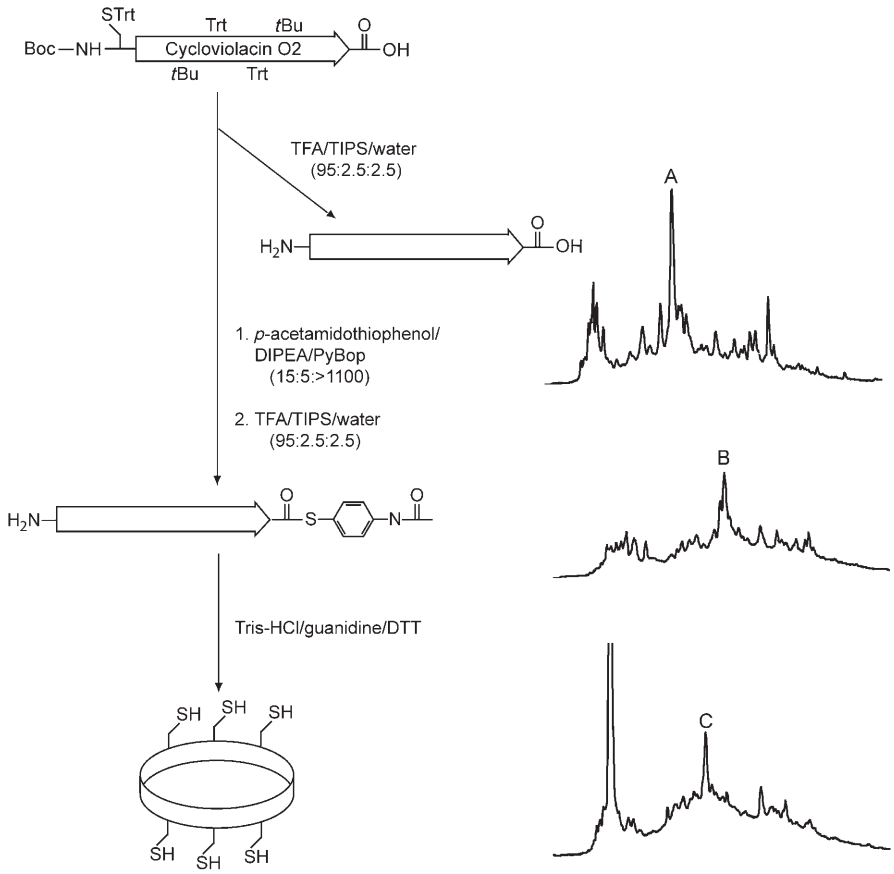


Figure 2. Synthetic scheme and RP-HPLC analyses of linear and cyclic precursors. A) Linear peptide, B) thioester, and C) cyclic reduced peptide.

Development of optimum folding conditions

In the development of folding conditions for later application to the synthesised peptide, we used CVO2 that had been isolated from the plant and then fully reduced. That peptide is equivalent to the reduced circular synthetic peptide, as obtained after cyclisation. The progress of the folding was followed by analytical RP-HPLC and ESI-MS. Initial attempts to fold this peptide were unsuccessful, due to irreversible loss of peptide by aggregation or precipitation, particularly in ammonium bicarbonate (pH 8.5, 0.1 M) with or without isopropanol (50%).[26] As a result, a series of different buffer systems were then evaluated, including NH_4Ac (pH 7, 0.5 M), $(NH_4)_2SO_4$ (pH 5, 0.5 M) and Tris-HCl (pH 8.5, 0.1 M), all containing EDTA (0.4 mM), and reduced and oxidised glutathione (GSH/GSSG; 2/1 mM), with incubation at 20 and 37 °C. The samples were assayed over 3–8 days, but no significant amounts of native peptide could be obtained.

Folding at low temperature in the presence of MeOH as a co-solvent was then explored,[39] and a very small yield of the native peptide was observed at 3 °C in Tris-HCl (0.1 M) containing EDTA (0.4 mM), MeOH (40%) and GSH/GSSG (2/1 mM) after 24 h. Increasing the concentration of EDTA from 0.4 mM to 1 mM resulted in a slight increase in yield. We also examined the effects of metal ions.[40] However, neither KCl (0.2 M) nor $CaCl_2$ (10 mM) improved the yield.

As well as co-solvents, nonionic detergents have also been reported to improve the oxidative folding of disulfide-rich hydrophobic peptides.[39] We therefore assessed the effects of different detergents in the folding system through the addition of 5% Brij 35, Tween 40 and Tween 60. All detergents favoured the formation of more hydrophobic folding products. In partic-

ular, Brij 35 quantitatively favoured more of the folded hydrophobic peptide forms, as shown by the dominance of the two late eluting peaks, marked as MI and N, in Figure 3. Notably, both of these peaks showed the same molecular mass as the native peptide (3141 Da) and both were impervious to alkylation by *n*-ethylmaleimide (Nem) and were therefore fully oxidised. However, while the second peak co-eluted with native peptide, peak MI clearly represents a misfolded peptide. As Brij 35 had quantitatively yielded the highest amount of hydrophobic folding species and because the Tween detergents became semisolid at the temperature and concentration used, we then focused on optimising the yield using Brij 35. Effects of incubation time, concentrations of different redox agents and temperature were explored as demonstrated in Figure 3. After these experiments, folding at 3 °C over 48 h with GSH/cystamine (2/2 mM) as redox agents was found to give the highest yield.

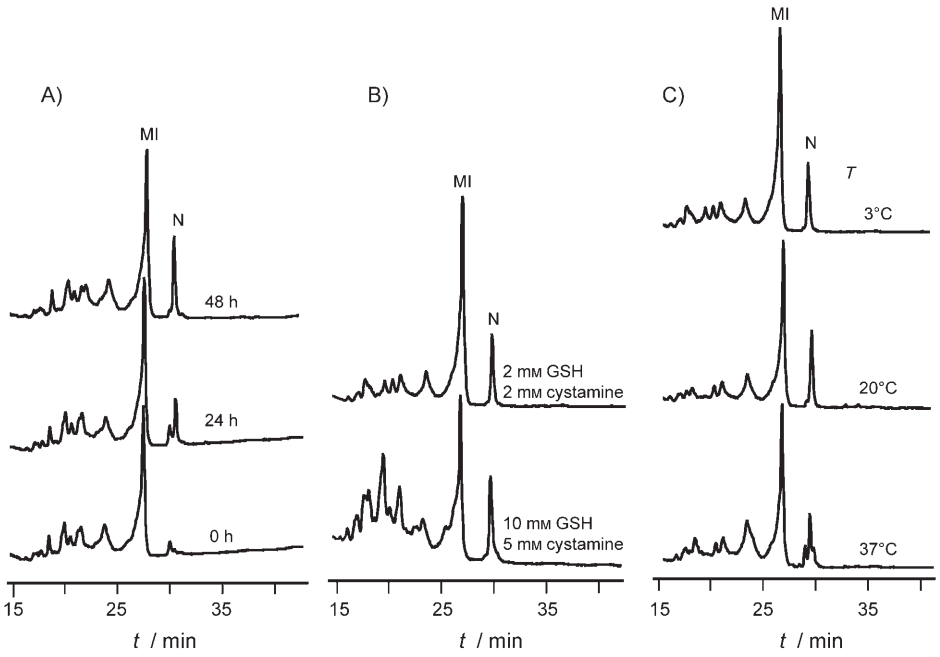


Figure 3. Effect of time, redox agents and temperature in Tris-HCl (0.1 M, pH 8.5), EDTA (1 mM), MeOH (40%) and Brij 35 (6%). A) Increasing the incubation time to 48 h improved the yield of the native peptide (N); time points beyond that did not confer any higher yield. The misfolded intermediate (MI) is dominant at all time points. B) Folding in the GSH/cystamine redox system over 24 h gave higher yields than in the GSH/GSSG system. Concentrations of 2/2 mM GSH/cystamine favoured a higher total yield of hydrophobic peptides (N, MI). C) Higher total yields were achieved at lower temperatures; hence 2/2 mM GSH/cystamine and 3 °C were used in all subsequent experiments. See the Supporting Information for RP-HPLC analytical conditions, and details of folding conditions.

However, under all experimental conditions attempted up to this point, the misfolded products, including the MI, were predominant. To increase the disulfide interchange, so as to provide more chance for the misfolded forms to shuffle to the native peptide, MeOH was replaced with DMSO. The oxidising property of DMSO has been reported to aid the folding of basic and hydrophobic peptides.[41] Accordingly, our initially attempted folding reactions in DMSO (20%) instead of MeOH (40%) resulted in an improved yield of correctly folded product. The effect of different DMSO concentrations was thus evaluated in the presence and absence of different additives and at different time intervals, as shown in Figure 4. Yields of native peptide significantly better than those obtained previously were achieved with DMSO included in the folding buffer. Incubation of the reduced peptide in DMSO (35%) with GSH/cystamine (2/2 mM) and Brij 35 (6%) over 48 h gave ~40% yields of the native peptide.

Since the reaction environment is highly oxidising, due to the presence of DMSO in a high-pH buffer (pH 8.5), most of the GSH itself might have oxidised rapidly. This would lower the rate of disulfide exchange with increased duration of incubation. Hence, addition of an equivalent amount of GSH/cystamine (2/2 mM) to the reaction mixture after 24 h could increase the native form through disulfide exchange. To check whether dilution due to the addition of extra volume caused any effect, we prepared another sample with the same volume of water. All three samples—control (undiluted), diluted (water), GSH/cystamine (2/2 mM)—were analysed by RP-HPLC, by injection of an adjusted equivalent volume, after a total of 48 h. The highest yield was obtained with addition of GSH/cystamine (2/2 mM) over 24 h. The sample diluted with water gave the same yield as the control (~40%), whereas addition of fresh GSH/cystamine (2/2 mM) after the first 24 h consistently resulted in >50% yields (52–53%), indicating a significant effect of additional GSH/cystamine.

Finally, synthetic CVO2 was folded to the native peptide structure under the optimised conditions. Large-scale folding reactions were then performed to obtain enough material to confirm the identity of the native form and to analyse the major intermediate further. The reactions were carried out under different conditions for each peptide to maximise the yields: for the native form fresh redox agents were added after 24 h according to the optimised protocol. This step was excluded for isolation of the MI (misfolded intermediate).

NMR structural studies

NMR spectral assignments for native CVO2, synthetic CVO2 and the MI were carried out by 2D techniques.[42] A comparison of the αH secondary shifts of the three peptides, shown in Figure 5, reveals that native and synthetic CVO2 have similar

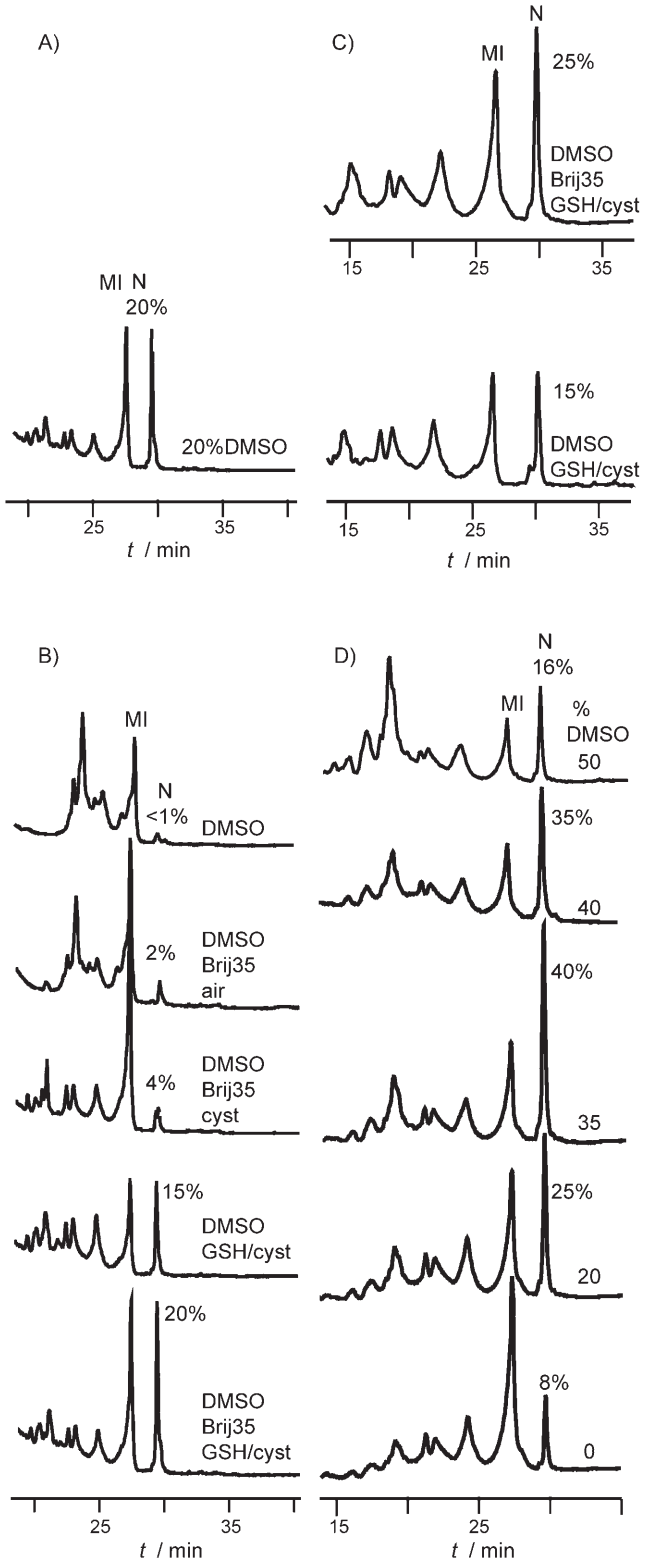


Figure 4. Folding in DMSO with Tris-HCl (0.1 M, pH 8.5) and EDTA (1 mM) at 3 °C. A) Changing co-solvent from MeOH (40%) to DMSO (20%) increased the yield of N (2/2 mM GSH/cystamine, 6% Brij 35, 24 h). Effects of DMSO with and without different reagents are shown in (B): the presence of all folding additives (DMSO/Brij 35/GSH/cystamine) gave the highest yield after 24 h. C) Increasing the incubation time to 48 h in the presence of Brij 35 gave N as the major product. D) The DMSO concentration was then optimised to 35%. See the Supporting Information for RP-HPLC analytical conditions, and details of folding conditions.

values, suggesting that they both have the same three-dimensional structure, but the misfolded form differs significantly. Both native and synthetic CVO2 show a series of positive secondary shifts for residues 17 to 18 and 23 to 26, which is indicative of the β-hairpin between loops 5 and 6 observed in proteins possessing a CCK fold. In addition, the secondary shifts also indicate a β-strand from residues 3 to 5, which is also often seen in cyclotide structures. Therefore we conclude that both native and synthetic CVO2 have the same structure and that this structure is consistent with the CCK motif.

In contrast, the secondary shifts for the misfolded peptide are not consistent with a CCK structure. Firstly, the secondary shifts are closer overall to random-coil values (the average absolute secondary shift is 0.16 ppm) than for either the native or the synthetic CVO2 (both 0.28 ppm), suggesting that the structure may be more flexible. The biggest difference in secondary shifts between native and misfolded CVO2 is for residues 3 to 5, which is the β-strand segment in native CVO2. The values for the misfolded form in this region of the molecule are negative and hence consistent with a more helical character. Interestingly, the secondary shifts from residues 24 through to residue 2 show a similar trend to native CVO2, although the magnitudes of the deviations from random coil values for each residue are not as great.

Attempts to determine the full three-dimensional structure of the misfolded form were unsuccessful, due to the lack of observable interresidue nOes under a range of different experimental conditions. This supports the hypothesis that this misfolded form has a less rigid and more poorly defined structure than the native CCK fold.

Disulfide mapping of the MI

The MI was subjected to a protocol involving partial reduction and stepwise alkylation to determine disulfide bond connectivities,[5] which gave three major partially reduced, and subsequently *N*-ethylmaleimide-modified (Nem-modified), peptides. These peptides were then fully reduced and alkylated with iodoacetamide (Iam). The series of reductions and alkylations was monitored by ESI-MS after each step. The results of these analyses were congruent: one of the partially reduced species peptides was a 2SS species—that is, two disulfides were intact after partial reduction—and the other two were 1SS species: that is, with one disulfide intact.

The peptides were cleaved with trypsin, and the positions of Nem- and Iam-alkylated Cys residues were determined by MS/MS sequencing. For the 2SS species, Nem was located on Cys1 and 5, while the remaining Cys residues were alkylated with Iam. Hence Cys1–Cys5 is one disulfide bond in the MI. The first 1SS species had Nem groups on residues 1, 5, 19 and 24, and Iam groups on residues 10 and 17; hence this species contained the disulfide bond Cys10–Cys17. These two experiments reveal two of the three disulfides, which by default define the third disulfide as Cys19–Cys24. The second 1SS species had Nem groups on Cys10, 17, 19 and 24, which reveals the same disulfide as the 1SS species (Cys1–Cys5). Hence all

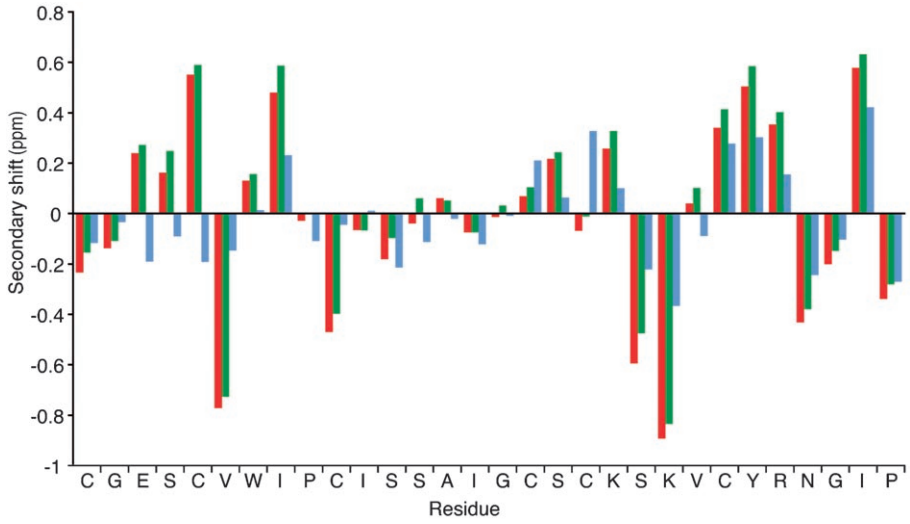


Figure 5. NMR secondary shift comparison. Isolated (in red) and synthetic (in green) CVO2 have similar values, which differ significantly from those for the MI (in blue).

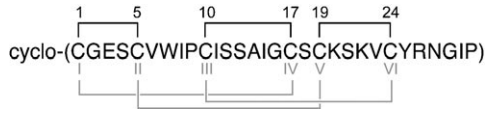


Figure 6. Disulfide mapping. The disulfide bonds of the MI are shown above the sequence, and the native bonds that define the CCK motif are shown below (in grey). Notably, the MI does not contain any native bond.

major disulfide species support the same set of disulfide bonds, as is shown in Figure 6.

Discussion

The cyclotide scaffold has become a prime target for protein engineering, due to its inherent stability and its potential for the design of drugs and of agricultural and/or biotechnological agents that can benefit from this stability. However, the exploration of the scaffold has been hampered by the fact that the major subfamily, the bracelet cyclotides, has been intractable to efficient chemical synthesis. Specifically, the oxidative folding of bracelet cyclotides with good yields had not previously been described. In this study we show that the circular peptide backbone can be synthesised by Fmoc chemistry in combination with NCL. Furthermore, we report the first successful folding of a bracelet cyclotide and identify fundamental differences in the oxidative folding pathway for that peptide in comparison with the prototypic Möbius cyclotide. In particular, a non-native three-disulfide intermediate was identified as a predominant folding product that even under optimised conditions only slowly rearranges to the native form of the protein.

Fmoc-based synthesis of peptide thioesters has attracted increased interest because of the potential to use NCL for the synthesis of glycosylated and/or phosphorylated proteins.[34,43–45] Furthermore, Fmoc chemistry does not require HF for cleavage of the peptide from the solid-phase resin, which makes the technique generally more applicable in the standard laboratory. In this work we have adapted the strategy outlined by Eggelkraut-Gottanka and co-workers[35] for the synthesis of the bracelet cyclotide CVO2. This is a simple and efficient method that uses commercially available standard Fmoc resins and reagents to produce a fully protected peptide, which then is thioesterified in solution. The method was modified to suit CVO2; in particular, the resin for SPPS and the equivalents of base (DIPEA) had to be changed to suit this peptide. Following those changes, NCL was readily done in aqueous solution.

We then turned to the folding of the cyclic reduced peptide to its native structure. The folding of cyclotides is coupled to the formation of three disulfide bonds: CysI–CysIV, CysII–CysV and CysIII–CysVI. Together with the cyclic backbone, these features define the topologically complex CCK motif, which is a challenge to fold. Two strategies to solve this problem have been used previously: namely, regioselective formation of disulfides[28,29] and direct oxidative folding.[26,27] Although the latter approach has successfully been applied for Möbius cyclotides, which fold with excellent yields, that strategy gives very poor yields for bracelet cyclotides.[28,29] In fact, not even regioselective disulfide formation produces bracelet cyclotides in high yields: a two-step oxidation procedure, in which two disulfide bonds were formed first, followed by the third bond, was reported to give only moderate yields.[28,29] Specifically, this method had the disadvantage that only one of the three two-disulfide species obtained after the first oxidation step had the native disulfide connectivity, and this was found to decrease the possible yield by two thirds.[28,29] Hence, in the current work we aimed to develop the conditions for direct oxidative folding of the prototypic bracelet cyclotide CVO2.

Initially, folding was attempted under similar conditions to those reported in the literature for Möbius cyclotides: however, in the case of CVO2 the yield of the native form was extremely low or non-existent. Instead we observed the accumulation of a series of apparently misfolded species that all had the same molecular mass as the native peptide but differed significantly in retention times. In particular, one misfolded product was predominant in the RP-HPLC analyses of the folding mixtures. This species, the MI, was formed immediately after addition of buffers and other additives to the folding buffer, and once formed it could not easily be converted to any other form.

We therefore explored a number of possible folding enhancing factors reported in the literature for cystine-rich peptides[26,39–41,46] in order to shift the equilibrium in favour of the

native conformation. Of these, the choice of co-solvent, redox agent and detergent proved to be crucial for efficient folding of CVO2.

Figure 7 summarises the folding and schematically shows the relative energy barriers to formation of the different products, together with their relative conformational stabilities. Notably, all products contained three disulfides as judged by ESI-MS. The stability, relative abundance and low level of disulfide reshuffling over time show that the MI represents a stable, low-energy conformation, which has a high energy barrier to cross to the native state. The main contribution to that high barrier is most probably associated with the fact that all cysteines are oxidised: that is, engaged in disulfide bonds. Refolding of the MI to the native form thus requires not only a major conformational change, but also breaking of disulfide bonds to form the native peptide structure. We therefore became interested in the structure and the disulfide connectivity of the MI. Firstly we attempted to characterise the MI by NMR. Contrary to the native peptide, the MI appeared more flexible in solution, as judged by the absence of nOes in NMR analysis. Because of this, NMR could not be used to determine the 3D structure nor to establish disulfide bond connectivity. We then turned to disulfide mapping by partial reduction and stepwise alkylation, which revealed that none of the MI's disulfide bonds is native; instead each cysteine residue forms a disulfide bond with a neighbouring cysteine, as shown in Figure 6.

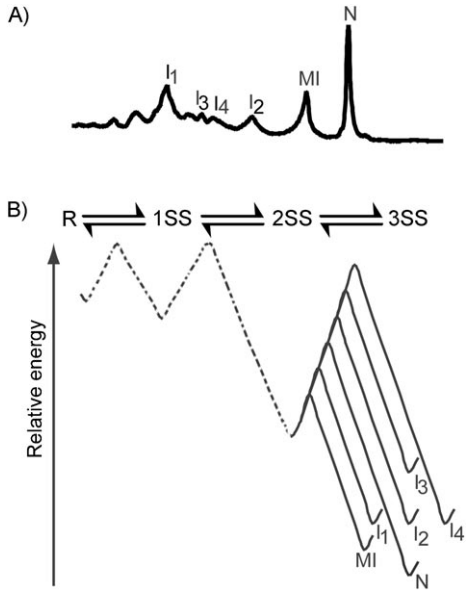


Figure 7. Schematic representation of oxidative folding of CVO2. Under optimised conditions N is the dominant product, as shown by the HPLC trace in (A); however, CVO2 folding is characterised by formation of different three-disulfide (3SS) products, including the MI and other misfolded 3SS species (labelled as I_1, I_2, I_3 and I_4, according to their relative abundances). No one-disulfide (1SS) or two-disulfide (2SS) species was observed in this study. Hence these species probably have higher relative energies than the 3SS ones, as shown by the diagram in (B). The MI, which is the dominant 3SS species under most of the tested conditions, presumably has a low relative energy and the lowest energy barrier to cross to be formed from 2SS-containing species. The conversion of misfolded 3SS species to N requires reshuffling of disulfide bonds; in the case of the MI all disulfide bonds have to be reshuffled. This conversion is favoured under optimised conditions, probably due to a decrease in the energy barrier to cross to form N. (The schematic energy diagram is based on the observed relative abundance of respective disulfide species under the different folding conditions.) See the Supporting Information for RP-HPLC analytical conditions.

Hence, all disulfide bonds in the MI have to be reshuffled to obtain the native disulfide connectivity, which most probably explains the high energy barrier between the MI and the native state. In addition, this rearrangement must be combined with major conformational changes. Here, both detergent and co-solvent assisted in the folding of CVO2. Plausibly, both of these additives play similar roles in the folding buffer: to act as stabilisers for the native conformation by interaction with hydrophobic residues. The co-solvent DMSO also has another role: namely to enhance disulfide formation, as it acts as a mild oxidising agent. Tam et al.[41] reported that oxidation by air or mixed disulfides (e.g., GSH/GSSG or GSH/cystamine) may not be satisfactory for basic and hydrophobic disulfide-rich peptides, and that use of DMSO may circumvent such problems. Our results support that observation; furthermore, our results clearly show that the oxidising property of DMSO alone is not enough for direct oxidative folding of this particular peptide. Presumably, while the presence of DMSO does facilitate formation of the native structure it is not sufficient to break the formation of the rapidly accumulating MI species.

In our experiments the relative amount of the native conformation markedly increased in the presence of redox agents. This could be due to favourable disulfide reshuffling conditions in the presence of GSH, which would facilitate breaking of non-native disulfide bonds, and finally the formation of the thermodynamically most stable product, the native form. Besides this, pH also affects disulfide reshuffling since it regulates the degree of ionisation of thiols to the reactive form—the thiolates—and reshuffling will be higher at high pH.[41,47] Hence, this may assist reshuffling of non-native disulfide bonds to native disulfide bonds. In this work, however, even with redox agents present the yield of the native form reached a plateau level: while the yield of the native product increased and that of misfolded product decreased over the first 48 h of incubation, the conversion was very slow and there was no appreciable change over the next 24 h. Plausibly, the steady state is due to oxidation of GSH, which leads to decreased disulfide reshuffling. The fact that the yield increased if redox agents were added on two occasions (at 0 and 24 h) supports this idea. Under these optimised conditions the yield of the native form was more than 50%. Thus, yields higher than 75% are easily within reach, by taking advantage of the possibility to reduce and refold non-native products again.

The pathway for the in vitro oxidative folding of CVO2 seems to be fundamentally different from that for the prototypic Möbius cyclotide, kalata B1. While the folding of kalata B1 is characterised by a mixture of one- and two-disulfide species,[48,49] the folding of CVO2 is dominated by fully oxidised peptides. Indeed, we could not observe any partly oxidised species in our experiments, in spite of the fact that they must exist at some stage during the folding (that is, from the fully reduced to fully oxidised peptide, but probably also during the

reshuffling of non-native fully oxidised peptides to the native structure). The two subfamilies also require dramatically different folding conditions in vitro. As already pointed out above, both DMSO and the detergent Brij 35 were components of the optimised buffer for folding CVO2. It should be noted that this folding buffer is optimised for that peptide in particular, and that the folding of bracelet cyclotides might be very sequence-specific. However, initial folding experiments with three other bracelet cyclotides indicate that the protocol developed for CVO2 provides a sound starting point (data not shown). In comparison, kalata B1 folds in high yields without any co-solvents present, although the folding efficiency is further enhanced in the presence of isopropanol,[26] as well as in the optimised CVO2 folding buffer (data not shown). It is interesting to speculate about the reasons for these differences, which must be connected with the structures of the corresponding subfamilies. As outlined in Figure 1, loop 5 contains one of the distinguishing factors between the subfamilies: this loop is highly conserved in Möbius cyclotides and contains the *cis*-Pro bond that defines that subfamily, while most bracelets have two Lys residues in this loop. However it is unlikely that this is the main reason for the differences in folding, as the hybrid [W19K/P20N/V21/K]kalata B1 has been successfully folded under conditions typical for Möbius cyclotides.[21] Instead it seems plausible that the differences in loop 3 may explain the need for co-solvents and detergent. This loop forms a surface-exposed hydrophobic patch in bracelet cyclotides,[4,6] and this is probably stabilised by the presence both of DMSO and of the non-ionic detergent Brij 35.

In this context it is interesting to note that the immediate precursor to the native kalata B1 structure is still unknown.[48] Although the major two-disulfide intermediate that has been characterised on the folding pathway, des[CysI–CysIV]kalata, has a native-like fold and contains two native disulfides, it is a non-productive kinetic trap.[48] In contrast, structurally closely related cystine knot peptides, such as the cyclic trypsin inhibitor MCoTI-II (which is a member of the third cyclotide subfamily), and the linear EETI-II fold via a main two-disulfide intermediate that directly collapses to the native peptide.[50,51] It has been suggested that the size of the embedded ring might explain these differences;[50] while it contains eleven residues in MCoTI-II and EETI-II it contains only eight residues in the case of kalata B1, as is also the case for CVO2. In light of this, it is possible that while the characters and oxidative states of the major products differ during the folding of CVO2 and of kalata B1, they might have similar two-disulfide direct precursors to the native fold. However, in comparison to other cystine knot peptides, the occurrence of three disulfide species during folding is not unique to CVO2. The oxidative foldings of, for example, hirudin,[52] tick coagulant protein,[52] Amaranthus α-amylase[53,54] and potato carboxypeptidase inhibitor[55] are all reported to involve highly heterogenous mixtures of one-, two- and three-disulfide intermediates, some of which contain non-native disulfide bonds.

Conclusions

In summary, we have shown that the prototypic bracelet cyclotide CVO2 is amenable to chemical synthesis. Fmoc chemistry was developed as a more user-friendly alternative to Boc chemistry, which has so far been the standard methodology for cyclotide synthesis. The synthetic strategy thus involved Fmoc-based SPPS, after which the thioester-based NCL reaction was used to produce the cyclic backbone. The peptide was then subjected to direct oxidative folding. Notably the origin of the cyclic peptide—that is, whether it comes from Fmoc or Boc chemistry—is irrelevant for the folding procedure.

The development of oxidative folding conditions for bracelet cyclotides is a potential landmark in the exploitation of the cyclotide framework, as it greatly increases the structural diversity accessible by synthesis. Indeed, a tolerance to sequence substitution and the cyclotides' inherent stability are keys for the use of the CCK motif as a scaffold for protein engineering. In view of this, it is noteworthy that the bracelet subfamily currently has twice as many members as the Möbius subfamily, and that they display a significantly different set of loop sequences, as shown in Figure 1. Loop 3, for example is a prominent feature of the bracelet cyclotides as it displays a hydrophobic α helix; this loop may now be targeted for detailed structure activity studies and for grafting of helical sequence epitopes. These results are therefore a significant step towards the overall goal of bioengineering on the cyclotide scaffold, as it is now possible to capitalise fully on the cyclotides' full diversity of sequences and their extraordinary structures for pharmaceutical and agricultural applications.

Experimental Section

Details of materials, peptide purification, MS, NMR and disulfide mapping can be found in the Supporting Information.

Synthesis of linear CVO2: The linear sequence CSCKSKVCYRNGIPCGESCVWIPCISSAIG was synthesised by manual SPPS by the in situ neutralisation/HBTU activation procedure for Fmoc chemistry on Fmoc-Gly preloaded NovaSyn TGT resin (0.22 mmol g^{-1}) on a 0.22 mmol scale. In brief, Nα-Fmoc-amino acid (1 mmol) was suspended in 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; 0.5 M, 2 mL, 1 mmol) and activated by addition of *N*,*N*-diisopropylethylamine (DIPEA; 180 μL, 1 mmol) and then immediately added to the peptide resin. The amino acid coupling cycles were 30–40 min at room temperature. Coupling reactions were monitored quantitatively as described by Sarin et al,[56] and residues were recoupled if necessary (i.e., if the yield was less than 99.6%). Residues after proline were double-coupled routinely. At each cycle, Fmoc removal was achieved with two 1 min treatments with piperidine in *N*,*N*-dimethylformamide (DMF; 50% *v*/*v*). All washings after couplings and deprotections were performed with DMF. Side-chain-protected Nα-Fmoc-amino acids were Arg(pbf)-OH, Asn(Trt)-OH, Cys(Trt)-OH, Glu(O-*t*Bu)-OH, Lys(Boc)-OH, Ser(*t*Bu)-OH, Tyr(*t*Bu)-OH and Trp(Boc)-OH. To protect the N terminus of the peptide during thioester formation and to allow simultaneous deprotection, Boc-Cys(Trt)-OH was used in the final coupling step.

Formation of protected peptide: Protected peptide (calcd. 5925 Da) was cleaved from the resin by treatment of resin-peptide (348 mg) with AcOH/2,2,2-trifluoroethanol/dichloromethane (DCM; 1/1/8, 9 mL).[35] After 3 h the resin was filtered off from the cleavage mixture and then washed twice with cleavage mixture. The combined filtrates were concentrated in vacuo. The obtained residue was repeatedly washed with *n*-hexane and dried in vacuo to remove residual AcOH. The yield of crude protected peptide was ~96% (190 mg). A sample of the protected peptide was deprotected by treatment with TFA/triisopropylsilane (TIPS)/water (95:2.5:2.5) for 2 h at room temperature. Most of the TFA was removed by blowing a stream of N_2 gas over the solution. Crude peptide was then precipitated by dropwise addition of cold diethyl ether, collected by centrifugation and analysed by RP-HPLC and ESI-MS (obs: 3163.8 Da; calcd: 3164.8 Da).

Synthesis of peptide thioester: To form the thioester of linear protected CVO2, crude protected peptide (7.5 mg, 2.5 nmol) was dissolved in DCM (20 mL), to which benzotriazol-1-yloxy tripyrrolidinophosphonium hexafluorophosphate (PyBOP; 5 equiv) was added. The solution was extensively mixed (vortex) before addition of DIPEA (500 μL). The solution was then vortexed again, followed by the addition of *p*-acetamidothiophenol (15 equiv). The reaction mixture was mixed and then incubated overnight at room temperature. After incubation, the reaction mixture was concentrated in vacuo. The concentrate/precipitate was repeatedly washed by addition of DCM and concentrated in vacuo until no more evaporation could be seen. The final sample contained a small volume of clear solution floating on the precipitate. Deprotection, precipitation and analysis were performed as described above (obs: 3313.8 Da; calcd: 3313.8 Da).

Cyclisation of linear thioester: Crude thioester, without further purification, was cyclised in Tris-HCl (0.25 M, pH 7.4), guanidine-HCl (Gdn; 6 M) and dithiothreitol (DTT; 17 mM) at room temperature for 30 min to form cyclic, but reduced, CVO2. The reaction was then terminated, and the circular protein was purified by preparative RP-HPLC and directly freeze-dried. The calculated yield for the cyclic form (obsd: 3146.4 Da; calcd: 3146.8 Da) is 30% based on UV absorbance at 280 nm ($\varepsilon = 7420$). The purity was $>95\%$ as judged by analytical RP-HPLC.

Oxidative folding: Cycloviolacin O2 (3 mg) as isolated from *Viola odorata* was reduced with DTT (13 mM) in Tris-HCl buffer (0.25 M) containing EDTA (0.4 mM), and Gdn (6 M). Fully reduced CVO2 was then isolated by RP-HPLC after 1 h incubation at 37 °C, and used to optimise the oxidative refolding. The refolding experiments were carried out in Tris-HCl (0.1 M, pH 8.5) containing EDTA (1 mM) at a peptide concentration of 30 μM. The effect of reaction temperature (3, 20 and 37 °C), co-solvents [MeOH (40%), DMSO (20, 35, 40 and 50%)], salts [$CaCl_2$ (10 mM), KCl (0.2 M)], detergents [Tween 40 (5%), 60 (5%); Brij 35 (5%)] and composition and concentration of redox system (GSH/GSSG, GSH/cystamine, 1/0.1, 10/5, 2/1 and 2/2 mM) were examined. One or more of the above combinations were used to determine optimum oxidative refolding conditions. To initiate the reactions, Tris-HCl (1 M, pH 8.5), EDTA (10 mM) and the redox system (GSH/GSSG or GSH/cystamine), detergents and/or co-solvents as appropriate for the experiments were added to the peptide solution. All samples were flushed with N_2 and incubated at appropriate temperature for the experiment. At various time intervals (24, 48, and 72 h), samples were withdrawn and quenched by addition of formic acid, to a total concentration of 2% *v/v*, diluted 4–10 times with buffer A (10% MeCN, 0.05% TFA) and analysed by RP-HPLC and ESI-MS. The accumulation of the native form relative to other folding species was calculated by integration of the HPLC peaks at 215 and 280 nm. The identity of the correctly folded species was determined by comparing HPLC retention times of the folding products with those of the native peptide isolated from the plant, as well as by co-injection. The native peptide structure of synthetic reduced CVO2 was formed from partially purified cyclic CVO2 by the optimised protocol: that is, in Tris-HCl (0.1 M, pH 8.5) containing EDTA (1 mM), DMSO (35%), GSH/cystamine (2/2 mM) and Brij 35 (6%), with addition of GSH/cystamine (2/2 mM) after 24 h) over 48 h at 3 °C.

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