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Head-to-Tail Cyclized Cystine-Knot Peptides by a Combined Recombinant and Chemical Route of Synthesis

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Cyclic peptides form an important class of naturally occurring or synthetic compounds with a large variety of biological activities as, for example, hormones, ion carriers, cancerostatics, antibiotics, antimycotics, or toxins.[1] Biological studies with cyclopeptides have often indicate increased metabolic stability, improved receptor selectivity, and improved activity profiles in comparison with their linear counterparts.[2] Among the group of natural circular peptides and proteins isolated in the last few years from microorganisms, plants, and even from humans,[3] cyclotides provide an especially interesting topology.[4] This family of circular plant proteins displays a head-to-tail cyclized peptide backbone together with a cystine knot (CK) motif based on disulfide bonds formed by six conserved Cys residues (Figure 1). Two disulfide bonds and their connecting backbone segments form a ring that is penetrated by the third disulfide bond to give a pseudo-knot structure that is invariably associated with the nearby β sheet structure.[5] The cystine knot in combination with the cyclic backbone appears to be a highly efficient motif for structure stabilization, resulting in exceptional conformational rigidity, together with stability against denaturing conditions, as well as against proteolytic degradation. CK-containing peptides are found in almost 20 different protein families with activities such as ion channel blocking (conotoxins and spider toxins), protease inhibition (squash inhibitors), and antiinsecticidal activity (plant cyclotides). Head-to-tail macrocyclic cystine knot peptides have been isolated from plants in the *Rubiaceae*, *Violaceae*, and *Cucurbitaceae* families. Several members of these family have been introduced as versatile scaffolds in drug design and biomolecular engineering.[6]

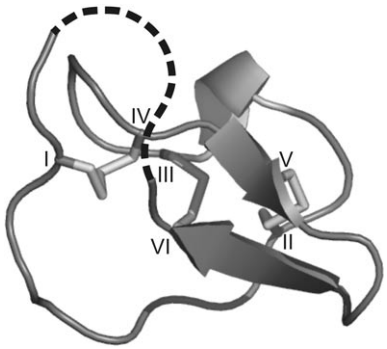

Figure 1. Structure of the cystine knot peptide McoEeTI.[7] β Strand secondary structure elements are indicated as arrows. The disulfide bonds forming the cystine knot architecture are indicated as sticks; cysteine residues are numbered from I to VI beginning from the N terminus. The macrocycle-forming loop was tentatively added and is shown as a dashed line.

Because of their sizes, in the range of 30–40 amino acids, cyclotides are amenable both to recombinant production through bacterial expression and to chemical synthesis.[8] In both routes, two steps of post-synthetic processing—oxidation of six cysteines to form three disulfide bonds and head-to-tail cyclization—are required to obtain the final cyclic product. Although the processes by which cyclotide backbone cyclization occurs naturally are largely unknown,[9] two major strategies have been applied to generate synthetic macrocyclic CK peptides. The first approach relies on recombinant synthesis and makes use of modified protein splicing elements known as inteins to form a C-terminal thioester that reacts with the N terminus to result in macrocyclization.[10,11] The second strategy is based on a solid-phase synthesis of the target peptide, followed by oxidation and cyclization. Fully deprotected peptides have successfully been "zipped" into macrocycles, followed by oxidation and cystine knot formation.[12]

Here we present a strategy for the backbone cyclization of already folded miniproteins based on the formation of a stable hydrazone.[13] This method takes advantage of the combination of cheap and high-yielding recombinant production of linear peptide precursors that are already folded and oxidized. Chemical synthesis efficiently provides the artificial linkage of the termini, not interfering with the fold of the knotted motif stabilized by disulfide bonds. In a comparison of linear and cyclized derivatives, an increased efficiency in tryptase inhibition is reported for a representative iminocyclotide; this also indicates

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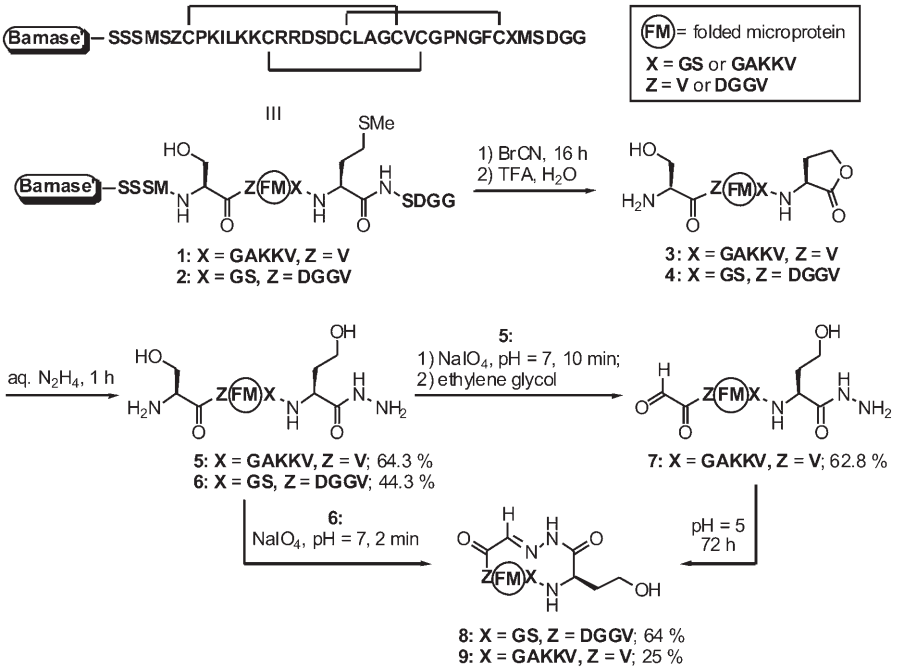
that the structurally artificial macrocyclization is functionally tolerated.

The new hydrazone peptide head-to-tail macrocyclization was first developed for cyclotide McoEeTI[7] (Figure 1), which consists of the N-terminal part of trypsin inhibitor MCoTI-II[14] and the C-terminal part of squash inhibitor EETI-II.[15] This hybrid miniprotein was chosen as we had serendipitously found that it is an inhibitor of human β-II tryptase, a tetrameric serine protease located almost exclusively in mast cells and which presumably plays an important role in allergic asthma.[16] The McoEeTI-based variants McoEeTI^{SDG} and McoEeTI^{KKV}, differing in the amino acid sequences of the loops that connect the terminal ends, bridging the distance of eight amino acids between knot-forming cysteines VI and I (Figure 2), have been designed. In McoEeTI^{SDG} we kept the whole linking sequence of natural



Scheme 1. Route of synthesis of iminocyclotides cyclo-McoEeTI^{SDG} and cyclo-McoEeTI^{KKV}.

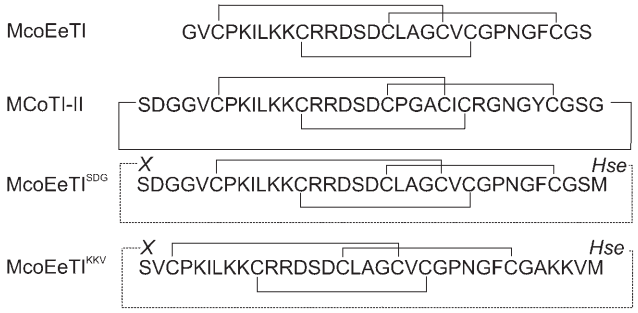


Figure 2. Amino acid sequences and disulfide connectivity of the hybrid miniprotein McoEeTI[7] consisting of the N-terminal part of MCoTI-II[14] and the C-terminal part of EETI-II,[15] and of the recombinantly produced variants McoEeTI^{SDG} and McoEeTI^{KKV}. The disulfide connectivity is shown as lines, as well as the additional amide bond of MCoTI-II connecting N and C termini. Hydrazone linkages of McoEeTI^{SDG} and McoEeTI^{KKV} are indicated as dashed lines. The N-terminal glyoxylyl residue (X) and the C-terminal homoserine (Hse) that are generated by chemical conversion are represented above the corresponding amino acid residue from which they are derived (Scheme 1).

MCoTI-II, while in McoEeTI^{KKV} the tripeptide sequence KKV was introduced in place of SDG, as we reasoned that positively charged residues preceding the inhibitor loop spanning Cys I and Cys II may contribute to tryptase binding and inhibition.[17]

The general outline of the cyclization strategy for McoEeTI is shown in Scheme 1. Recombinant bacterial synthesis is used to generate a fusion protein, which can be released by cyanogen bromide (CNBr) cleavage at engineered flanking methionine residues. CNBr cleavage at the C-terminal methionine is well known to result in formation of a homoserine lactone[18] along with its hydrated open-chain form, the C-terminal homoserine. Macrocyclization can be achieved by C-terminal hydrazinolysis,[19] followed by hydrazone formation with the N-terminal ketoaldehyde formed upon mild oxidation of the N-terminal serine residue.

McoEeTI variants were produced recombinantly in *Escherichia coli* by use of a recently described expression system that relies on peptide fusion to an enzymatically inactivated variant of the RNase barnase from *Bacillus amyloliquefaciens*.[20] The fused barnase directs the fusion to the *E. coli* periplasm, where oxidative conditions support disulfide bond formation. Detailed data on cystine knot miniprotein production through barnase fusion can obtained from ref. [20].

In the construct used here, the McoEeTI sequence is preceded by SSS**M** and followed by **M**SDGG. For both McoEeTI^{KKV} and McoEeTI^{SDG} homoserine lactones **3** and **4**, a folded miniprotein was released upon chemical cleavage of the fusion protein with cyanogen bromide at the flanking methionine residues and was then purified by reversed-phase HPLC with TFA-acidified aqueous acetonitrile (Scheme 1).[21] When the CNBr cleavage of the barnase-McoEeTI fusion protein was performed under strongly acidic conditions the lactone/homoserine equilibrium was shifted completely towards γ-lactone formation.

As it is known that hydrazides are also able to retain their nucleophilic reactivity in slightly acidic aqueous media,[22] we envisaged that the C-terminal hydrazide might selectively attack the N-terminal ketoaldehyde. This should be the case even in the presence of primary amino groups, which are protonated and not nucleophilic, therefore tolerating the three lysine residues in the McoEeTI scaffold. Miniproteins **3** and **4**, each bearing a γ-lactone moiety, were smoothly converted

into the corresponding hydrazides **5** and **6** in the presence of excess aqueous hydrazine within 1 h at room temperature (see the Supporting Information). As the pH of aqueous hydrazine is basic, the formation of a miniprotein with C-terminal homoserine was detected during the hydrazinolysis of **4** (see the Supporting Information). To avoid this by-product in the case of homoserine lactone **3** the reaction was carried out in a slightly acidic medium; this significantly increased the yield of hydrazide **5** in relation to **6** (Scheme 1).

Sodium periodate oxidation–cyclization

In the two open-chain precursors McoEeTI^{SDG} and McoEeTI^{KKV}, the 2-amino alcohol of the N-terminal Ser residue functions as a masked aldehyde. After oxidative periodate cleavage (Scheme 1), the N-terminal ketoaldehyde reacted with the C-terminal hydrazide, thereby closing the macrocycle through the formation of a hydrazone. In order to avoid side reactions during oxidation an excess of only 10 equivalents of periodate was used. The desired oxidation was rapid and highly specific for the N-terminal serine.[23,24]

The hydrazone cyclization to cyclo-McoEeTI^{SDG} **8** initiated by periodate oxidation proceeded within 5 min, directly yielding the cyclic hydrazone **8** in 64% yield,[25] without the intermediate N-terminal ketoaldehyde being detected. The fast macrocyclization is probably due to conformational preorganization of the folded open-chain precursor of cyclo-McoEeTI^{SDG}, orienting the N and the C termini in close proximity, and thereby establishing a high effective concentration.[26] Macrocyclization of McoEeTI^{SDG} hydrazide **6** was followed by HPLC (Figure 3A). As soon as 5 min after initiation of oxidation a signal at 17.22 min indicated the formation of hydrazone **8**. Product formation was easily detectable, since the hydrazide **6** shows no absorption at 280 nm whereas product **8** provides a UV absorption that can be attributed to hydrazone formation.[27] In general, hydrazone formation is reversible, but it is known to be stable at physiological pH.[28] Since the hydrazone of knotted miniprotein **8** also turned out to be stable, its reduction with $NaCNBH_3$ to ensure the integrity of a formed conjugate[28,29] was not required. The structural integrities of the hydrazone linked miniproteins were corroborated by high-resolution ESI mass spectrometry (Figure 4). No efforts to determine the ratio of *syn*-/*anti*-hydrazone isomer formation have yet been undertaken.

The open-chain precursor of cyclo-McoEeTI^{KKV} was obtained as the hydrazide **5** in 64% yield. It was oxidized with sodium periodate to the N-terminal ketoaldehyde **7**, which reacted with the C-terminal hydrazide at slightly acidic pH within 72 h to provide the iminocyclotide **9**. The cyclization of cyclo-McoEeTI^{KKV} **9** was significantly slower that that of iminocyclotide **8**, combined with a much lower yield of only 25% (Figure 3B). The cyclization efficiency is reported to depend on the amino acid sequence of the termini forming the connecting loop.[30]

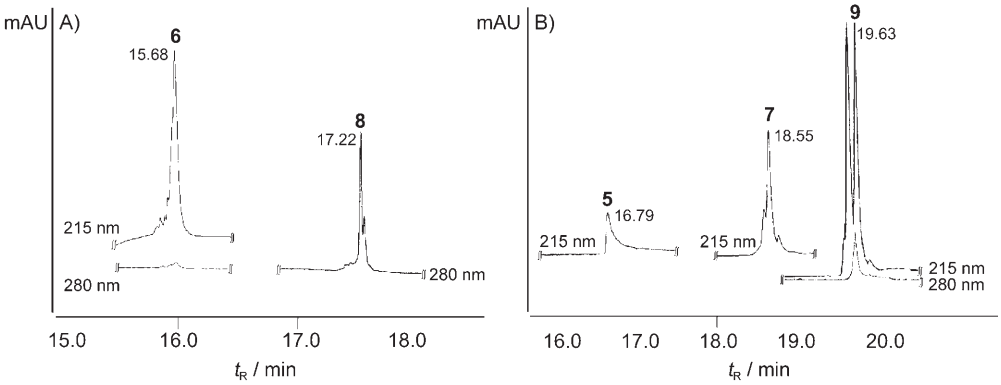


Figure 3. A) HPLC profile of the oxidation and macrocyclization of McoEeTI^{SDG} linear precursor in its hydrazide form **6**. Left: Elution profile of hyrazide **6** after purification. No absorption is detected at 280 nm. Right: Reaction mixture after 2 min incubation with sodium periodate monitored at 280 nm. B) HPLC monitoring at 215 and 280 nm of oxidation and cyclization of McoEeTI^{KKV} hydrazide **5** (left), N-terminal aldehyde **7** formed after 4 min of oxidation (middle), cyclization progress after 2 days of reaction at pH 5 (right). Only the macrocyclic product **9** would be expected to show absorbance at 280 nm. Detailed analytical data can be found in the Supporting Information.

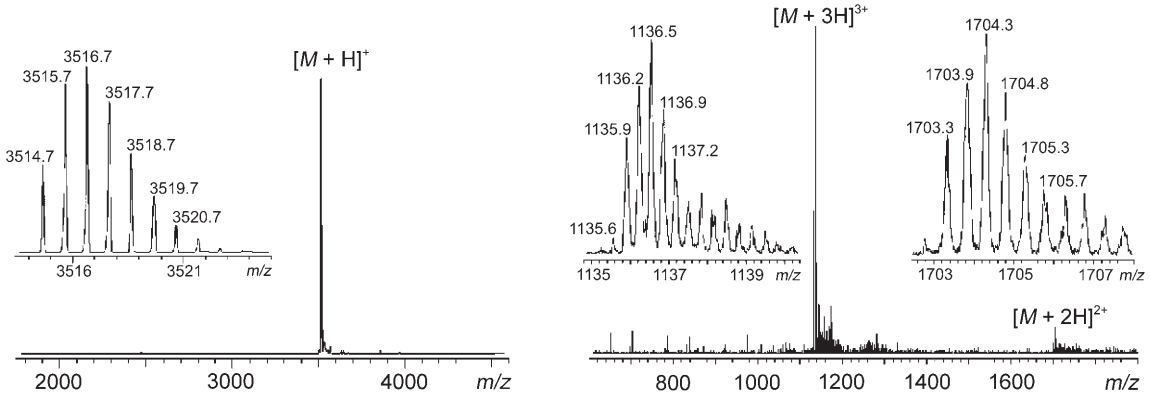


Figure 4. ESI-MS of cyclo-McoEeTI^{KKV} **9** (left) and cyclo-McoEeTI^{SDG} **8** (right).[31] Analytical data can be found in the Supporting Information.

Preorganization of the terminal ends in close proximity within the linear but folded precursors also seems to be decisive for the different cyclization efficiencies of the synthesized iminocyclotides **8** and **9**.

Further verification of correct folding and successful linkage of the termini to form cyclo-McoEeTI^{SDG} **8** and cyclo-McoEeTI^{KKV} **9** was obtained from the finding that the cyclized products and, in the case of the McoEeTI^{KKV} variant, also the barnase-fusion protein and the homoserine lactone **3** act as inhibitors of human β-II tryptase (Table 1). In comparison with linear McoEeTI, which shows a K_i constant of about 100 nM, we observed a 40-fold decrease in inhibitory activity for the cyclo-McoEeTI^{SDG}. In contrast, cyclization of the McoEeTI^{KKV} derivatives resulted in a 20-fold increase in inhibitory activity, which is reflected in a K_i of 20 nM for the linear variant compared to 1 nM for the cyclic miniprotein. Upon cyclization, the KKV tripeptide sequence becomes positioned in close proximity to the inhibitory loop (spanning Cys I and Cys II), which may provide favorable electrostatic interactions with tryptase, while an aspartate residue in cyclo-McoEeTI^{SDG} results in a lower tryptase affinity.

Table 1. Inhibition of human β-II tryptase by McoEeTI^{SDG} and McoEeTI^{KKV} as barnase-fusion proteins, cleaved miniproteins, and cyclized final products. K_i values were determined as described[31] and are given in nM.

Variant	Barnase fusion	CNBr cleaved	Cyclized
McoEeTI	100	100	–
McoEeTI^{SDG}	n.d.	n.d.	4000
McoEeTI^{KKV}	600	20	1

n.d.: Not determined.

In conclusion, a convenient method for the backbone cyclization of recombinantly produced cystine knot peptides that results in correctly folded macrocyclic disulfide-bridged peptides is reported. It does not require protecting groups, takes place in aqueous solution, and is devoid of racemization and solubility problems. One of the major advantages of the proposed method is the potential to cyclize fully deprotected peptides that can be produced conveniently by recombinant synthesis, with oxidative folding occurring under physiological conditions, leading to correctly disulfide-bonded linear precursors. As the hydrazide moiety retains its activity over a wide pH-range, at neutral or slightly acidic pH the termini could be coupled into the cyclic hydrazone without interference with lysine side chains. Limitations of general applicability lie in the facts that this cyclization strategy relies on CNBr cleavage at flanking methionine residues, which excludes the presence of internal methionines, and that periodate oxidation may give rise to oxidative side products with, for example, cysteine or tryptophan residues, which requires careful control of oxidation reaction conditions. Protein expression, purification, and cyclization procedures are amenable to further optimization; nevertheless, 0.5 mg of cyclized cystine knot miniprotein McoEeTI^{KKV} and 1.0 mg of McoEeTI^{SDG} were obtained from 5 L initial bacterial liquid culture. At each step, substantial amounts of synthesis product were used for analysis and biological tests. Scaling up to production of multi-milligram amounts of iminocyclotides seems feasible. Although a non-natural hydrazone linkage is formed by macrocyclization, the resulting iminocyclotides are biologically active proteinase inhibitors. Imino-cyclo-McoEeTI^{KKV} was identified as the most potent proteinaceous inhibitor of human mast cell tryptase known to date, capable of binding to all four active sites of the tryptase tetramer simultaneously, thereby blocking the access of natural substrates (unpublished data). Despite the limitations, as previously discussed, this method may be applicable to the synthesis of large cyclic peptides in general, as well as to that of the cystine knot motif.

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Keywords: cyclic peptides · cystine knot miniproteins · hydrazones · inhibitors · macrocyclization · protein modifications

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