Efficient Enzymatic Cyclization of Disulfide-Rich Peptides by Using Peptide Ligases


Disulfide-rich macrocyclic peptides—cyclotides, for example—represent a promising class of molecules with potential therapeutic use. Despite their potential their efficient synthesis at large scale still represents a major challenge. Here we report new chemoenzymatic strategies using peptide ligase variants—intral alia, omniligase-1—for the efficient and scalable one-pot cyclization and folding of the native cyclotides MCoTI-II, kalata B1 and variants thereof, as well as of the θ-defensin RTD-1. The synthesis of the kB1 variant T20K was successfully demonstrated at multi-gram scale. The existence of several ligation sites for each macrocycle make this approach highly flexible and facilitates both the larger-scale manufacture and the engineering of bioactive, grafted cyclotide variants, therefore clearly offering a valuable and powerful extension of the existing toolbox of enzymes for peptide head-to-tail cyclization.

In the last decade macrocyclic peptides have gained increased traction as a promising class of therapeutics. High metabolic stability than their linear analogues, often accompanied by higher potency, make them excellent leads in drug design. Several mono- and multicyclic peptides are currently under clinical evaluation.[1] Disulfide-rich macrocycles such as cyclotides or θ-defensins are particularly promising classes of compounds for therapeutic application, and cyclotides also offer potential as crop-protection agents in the agricultural industry.[2] Cyclotides are characterized by a combination of a head-to-tail cyclic backbone and three conserved disulfide bonds forming a distinctive, knotted pattern, which makes them exceptionally stable (metabolically, thermally, and chemically).[3]

In contrast, θ-defensins such as the rhesus θ-defensin (RTD-1)[4] are cyclic peptides of 18 residues in length with two anti-parallel β-strands stabilized by a laddered arrangement of three disulfide bonds (I–VI, II–V, III–IV). θ-Defensins are of mammalian origin and possess a range of antimicrobial activities.[4]

Both cyclotides and θ-defensins are being used as pharmaceutical tools, particularly as scaffolds onto which bioactive epitopes can be fused.[5, 6] Thanks to their highly conserved structure they can accommodate the introduction of bioactive epitopes (“grafting”) between the cysteine residues while retaining their native structural and biophysical characteristics.[6]

Despite their pharmaceutical potential, the preparation of disulfide-rich macrocyclic peptides, especially at large scale, still poses a significant challenge, and new tools for facilitating their design and (scalable) manufacture are highly desired. Although chemical approaches, such as native chemical ligation (NCL)[7] or carbodiimide-mediated couplings using entirely protected peptides,[8] have been applied for peptide head-to-tail cyclization in many studies, the requirement for careful optimization of reaction conditions, the lack of scalability, and often the low yields of the syntheses limit their application on larger scale.[8] Thus, there is a clear need for new, more efficient methods for peptide head-to-tail cyclization and for the preparation of macrocyclic disulfide-rich peptides.

In recent years, various research groups have investigated enzymatic strategies to fill this gap.[10–12] Several examples have been described, including the use of sortase A,[13–17] split in- tenses,[18–22] trypsin,[23, 24] asparaginyl endopeptidases such as butelase-1,[25–27] or OaAEP1b,[28] or engineered subtilisin[29] such as peptiligase variants.[10, 30, 31] In addition, recombinant expression of disulfide-rich peptides in bacteria has been reported.[32–36]

We have recently employed the peptide ligase (peptiligase) variant omniligase-1 to efficiently prepare several mono-, tri-, and tetracyclic peptides, as well as the naturally occurring cyclotide MCoTI-II.[10, 17] Omniligase-1 is a broad-specificity subtili- sin variant, which efficiently and tracelessly catalyzes inter- or intramolecular peptide bond formation of unprotected peptides in aqueous solution. On the basis of earlier studies performed with MCoTI-II we sought to expand the scope of peptide cyclization mediated by omniligase-1 to other disulfide-rich macrocycles, including kalata B1 (kB1) and RTD-1. Additionally, the presence of multiple cyclization sites in every molecule, as well as the possibility of cyclizing pre-oxidized/folded...
cyclotides, would certainly expand the scope of this methodology for the benefit of the chemical biology community.

Various studies in which bioactive epitopes were grafted into flexible loops of the squash trypsin inhibitor cyclotide MCoTI-II, particularly into loops 1 and 6 (see Figure 1A), have been reported.[5,6,38,39] Because the grafted epitopes typically vary in their sequence, a cyclization site distinct from the established coupling site in loop 1 (ligation site L1: …CRGN//G Y…) and, moreover, outside loop 6, such as in loop 5, for example, would be desired. In this way, the generation of chemically synthesized libraries would clearly be facilitated. Encouraged by this idea, we attempted to identify suitable ligation sites in loop 5, using a sequence-based evaluation. By this approach we identified a second ligation point (site L5: …CRGN//G Y…) that should enable efficient cyclization catalyzed by omniligase-1.

After the preparation of the open-chain (oc) elongated carboxamidomethyl (Cam) ester precursor MCoTI-II-L5-Cam-Leu-OH by using classical solid-phase peptide synthesis (SPPS),[30,40] our first cyclization attempts using omniligase-1 successfully led to the formation of cyclic c-MCoTI-II-L5. However, the linear C-terminal ester precursor oc-MCoTI-II-L5-Cam-L-OH was not entirely stable during the course of the reaction, with a base-induced dehydration-type side reaction of the C-terminal P1-asparagine residue being observed (Figure S11 in the Supporting Information).[41] In view of this finding, we decided to use a less activated, more stable C-terminal aryl-type ester [a hydroxymethylbenzoic acid (HMBA) ester, Figure 2A] that had been demonstrated in previous studies to be well suited for chemoenzymatic peptide synthesis (CEPS) catalyzed by omniligase-1 on larger scale (unpublished results) and can be easily introduced by using classical SPPS (Supporting Information). In addition, peptiligase variant #1015, optimized for the recognition of small, polar amino acid residues in position P1, was expected to deliver a better performance than omniligase-1 in this particular ligation. Indeed, the HMBA ester was found to be much less susceptible to Asn dehydration, and efficient conversion of oc-MCoTI-II-L5 HMBA-L-OH into its cyclic counterpart in the presence of peptiligase #1015 was observed within 30 min. Overnight air oxidation in combination with the addition of 5 mM reduced glutathione (GSH)[42] yielded native cyclic folded cf-MCoTI-II (Figure 1B) in a straightforward manner through a one-pot cyclization/folding procedure.

The correct disulfide connectivity was confirmed by the HPLC retention time (tR), which was identical to that of chemically synthesized MCoTI-II (see Figure S9). The trypsin inhibition activity of cf-MCoTI-II-L5 was subsequently determined and compared with that of chemically synthesized MCoTI-II (Figure S13). The K values of 0.0041 and 0.0050 nm, respectively, were comparable with the previously reported K value of native MCoTI-II (0.0023 nm).[43] In general, the identification of this second ligation site significantly expands the scope of using peptiligases for the rapid and simple preparation of MCoTI-II variants for grafting studies: that is, the C-terminal part of MCoTI-II (loops 1–5) can be synthesized at larger scale and upon splitting of the resin, derivatization with diverse sequences in loop 6 can be performed conveniently.

Many disulfide-rich macrocycles adopt their native conformation only when formation of the correct disulfide pattern precedes cyclization. Hence, using MCoTI-II as a model system, we also investigated whether the reaction order of enzymatic cyclization followed by oxidative folding could be reversed. This would significantly expand the scope of using peptiligases for the rapid and simple preparation of MCoTI-II variants for grafting studies: that is, the C-terminal part of MCoTI-II (loops 1–5) can be synthesized at larger scale and upon splitting of the resin, derivatization with diverse sequences in loop 6 can be performed conveniently. Many disulfide-rich macrocycles adopt their native conformation only when formation of the correct disulfide pattern precedes cyclization. Hence, using MCoTI-II as a model system, we also investigated whether the reaction order of enzymatic cyclization followed by oxidative folding could be reversed. This would significantly expand the scope of using peptiligases for the rapid and simple preparation of MCoTI-II variants for grafting studies: that is, the C-terminal part of MCoTI-II (loops 1–5) can be synthesized at larger scale and upon splitting of the resin, derivatization with diverse sequences in loop 6 can be performed conveniently.
In contrast to our expectations, cyclization of oc-f-MCoTI-II-L1 ester proceeded with high efficiency and selectivity (Figures S4 and S5), again demonstrating the high flexibility of ligations mediated by omniligase-1. The cyclization of an aprefolded MCoTI-II precursor clearly represents an advancement from the previously described approach starting from reduced, linear MCoTI-II.

We anticipated that the Cys8–Cys25 disulfide bridge, which has to accommodate Cys8 in pocket S3, would render the peptide rigid and sterically hindered to adopt a conformation for binding that would be favorable for efficient cyclization. To examine catalytically competent binding modes we performed biased molecular dynamics (MD) simulations. The fully cyclized cf-MCoTI-II had very restricted conformational freedom and despite the bias, only omniligase-1 was partially distorted and not the cyclic product. Upon binding, the enzyme was distorted at β-strand Val95–Gln103, which flanks the active-site groove. This strand forms an extended β-sheet with linear peptide substrates, so this does not appear to be an optimal binding mode. Moreover, superimposition of omniligase-1 from this binding mode with 83 structures of subtilisins available from the Protein Data Bank[44] shows that no experimentally determined structure exhibits such extensive distortion (Figure S8). Superimposition of cf-MCoTI-II from this binding mode with 83 structures of subtilisins available from the Protein Data Bank[44] shows that no experimentally determined structure exhibits such extensive distortion (Figure S8). Superimposition of omniligase-1 from this binding mode with 83 structures of subtilisins available from the Protein Data Bank[44] shows that no experimentally determined structure exhibits such extensive distortion (Figure S8).

Encouraged by the successful chemoenzymatic synthesis of MCoTI-II by using two different connection points, we focused next on another prototypic example of the Möbius family of cyclotides: kalata B1. On the basis of a sequence-guided evaluation we identified two potential ligation sites: L2 (…VGTT//CN…) and L6 (…CTRN//GL…) (Figure 2A and B). Our initial studies focused on ligation site L2 and use of omniligase-1. After extensive screening of various sets of reaction conditions c-kB1-L2 was obtained in a reasonable yield of 60% (HPLC), albeit accompanied by 40% hydrolysis of the glycolate ester moiety. We identified Asn in the P2-position to be suboptimal, because in this position hydrophobic amino acids are

Figure 2. Chemoenzymatic synthesis of kB1. A) Cartoon representation of folded kB1 (PDB ID: 1NB1) and sequence with potential ligation sites. Disulfide bonds are shown in yellow. B) Linear C-terminal esters for cyclization of kB1 catalyzed by omniligase-1. C) HPLC traces of one-pot cyclization/folding of [T20K]kB1 on multigram scale: crude oc-[T20K]kB1-L6-HMBA-L-OH (0 min) and crude reaction mixture after oxidative folding to [T20K]kB1 are depicted. D) Comparison of secondary Hα chemical shifts of enzymatically (red) and chemically (gray) cyclized [T20K]kB1.
generally preferred by omniligase-1. Indeed, introduction of an N14L mutation into k81 increased the ligation yield to over 85% (Figure S15). On the basis of this result we switched our focus to cyclization site L6, identical to the natural cyclization site in the biosynthesis of k81.[48] Again, by using a C-terminal HMBA ester P1-Asn dehydration was minimized and head-to-tail cyclization was clearly dominant, with efficient conversion of oc-k81-L6 HMBA ester to c-k81-L6 (Figure S16).

In view of these promising results, we set out to establish a one-pot cyclization/folding procedure for the scalable manufacture of k81-L6. For this we used the [T20k81 variant of k81, which is under preclinical evaluation as an immunosuppressive agent for the treatment of multiple sclerosis.[49] After chemical synthesis of linear [T20k81-L6-HMBA ester on a larger scale (5 mmol) we achieved efficient cyclization in a one-pot reaction with omniligase-1 and crude, non-lyophilized [T20k81-L6-HMBA ester. To minimize the occurrence of P1-Asn dehydration the pH of the ligation mixture was lowered to 8.0 and the amount of enzyme was increased to 0.006 molar equivalents with respect to the linear precursor peptide. After cyclization, upon addition of propan-2-ol to a final concentration of 50%, complemented with 2 mM GSH/GSSG,[47] and incubation for 15 h, native [T20k81 was obtained (Figure 2C). Pure chemoenzymatically synthesized [T20k81 was shown by NMR spectroscopy and LC-MS analysis to be identical to fully chemically synthesized [T20k81 (Figure 2D, Figures S19 and S20). Besides structural verification by NMR, the chemoenzymatically synthesized [T20k81 proved to have cytotoxicity (IC50 = 2.21 µM) towards HeLa cells similar to that of its chemically synthesized counterpart (IC50 = 2.42 µM).

In order to aid easier processing of large quantities and to reduce the amount of ligase further, we investigated the possibility of dosing crude linear [T20k81-L6 ester over time in a batchwise manner to increase the concentration of final product drastically and thus facilitate the downstream processing significantly. Although omniligase-1 is, in contrast with naturally occurring cyclases such as butelase 1,[40] easily available in large quantities through recombinant expression, a further decrease in the required quantity of catalyst will result in an additional cost/price reduction of the synthesis at scale. Whereas most cyclization reactions catalyzed by omniligase-1 are performed in the 0.5–1 g L⁻¹ (low-µM) range, we were able, when starting from 0.5 g of crude linear [T20k81-L6 ester at a concentration of 1 g L⁻¹, to increase the concentration of linear ester by repeated dosing (5 × 0.5 g each) to 6 g L⁻¹. The amount of omniligase-1 required could be reduced to ≤0.002 molar equivalents. After oxidative folding and direct HPLC purification, c-[T20k81 was obtained with a cyclization/folding purification isolated yield of 35%. Clearly, this chemoenzymatic strategy is a viable approach for the production of larger quantities of [T20k81 or variants thereof.

In addition to cyclotides, the non-mechanically interlocked θ-defensins, which display a range of antimicrobial activities,[44] are also being pursued as pharmaceutically exciting scaffolds, onto which bioactive epitopes can be grafted. Rhesus θ-defensin-1 (RTD-1) was the first θ-defensin discovered, in leukocyte extracts from rhesus macaques,[49] and can be considered a prototypical example of this class of ultrastable compounds. To date, several approaches for the chemical synthesis of RTD-1 have been described, including chemical ring closure by using standard coupling reagents,[49,50] bacterial expression,[52,53] and the use of native chemical ligation.[51] Although the last of these represents a powerful approach for the generation of large libraries of RTD-1 variants, it is not suitable for their production on industrial scale. To overcome this limitation, we further explored the possibility of using peptiligase variants for the chemoenzymatic one-pot synthesis of RTD-1. We identified two potential ligation sites: A (…LCCR//GV…) and B, located in the respective β-turn loops of the molecule (…ICTR//GF…, Figure 3A). After the synthesis of both linear precursors as elongated C-terminal Cam esters—namely RTD-1 A-Cam-L-OH and RTD-1 B-Cam-L-OH—we evaluated the possibility of cyclizing them by using omniligase-1.

As expected, both RTD-1 A ester and RTD-1 B ester were rapidly and efficiently cyclized in less than 30 min (Figure 3B and C). Because ligations can also be carried out by starting from crude, non-lyophilized linear peptide, as demonstrated for [T20k81 (see above) and MCoTI-II,[30] the initial HPLC purification can be avoided if suitable crude qualities of the linear precursor are obtained. To ensure efficient cyclization in initial studies, reducing conditions were applied by addition of 3.5 mM tris(2-carboxyethyl)phosphine (TCEP). Interestingly, we observed that RTD-1 has a very high propensity rapidly to adopt its native, oxidized fold. This led to the surprising discovery that after completion of the cyclization the excess of TCEP initially present is entirely consumed and hence cannot maintain a reducing environment. Thus, as confirmed by LC-MS, native cf-RTD-1 is obtained after a reaction time of only 2–6 h.

In contrast with these observations, rapid oxidative folding of oc-RTD-1 ester was observed under nonreducing cyclization conditions. However, no enzymatic ligation occurred, but only fast hydrolysis of the C-terminal Cam ester moiety. Identical observations were made when cyclization of oxidized, linear RTD-1 was attempted. We assume that the excess of TCEP required for efficient cyclization can be lowered and delicately adjusted to the specific batch of linear precursor used in order to obtain native RTD-1 in an even shorter time frame. The native folding of RTD-1 B was confirmed by one-dimensional 1H NMR. The Hα chemical shifts of RTD-1 B were essentially identical to those of chemically synthesized RTD-1 reported by Craik et al. (Figure 3D),[51] thus confirming that c-RTD-1 B had adopted a native θ-defensin fold. c-RTD-1 A was unequivocally identified by LC-MS, and c-RTD-1 A and c-RTD-1 B showed identical retention times and mass spectra (Figure 3B, C). In conclusion, here we have presented a new approach for the efficient synthesis of the θ-defensin RTD-1, characterized by its simplicity and high flexibility. The linear ester precursors can easily be obtained by using Fmoc SPPS, and the two ligation sites offer high flexibility for the generation of grafted RTD-1 variants, similarly to the flexible system for MCoTI-II grafting.

The work described clearly demonstrates the applicability of CEPS in the presence of peptiligase variants—omniligase-1, for example—for the cyclization of diverse classes of native disulfide-rich peptides without the need to add a recognition se-
The identification of multiple ligation sites together with the possibility of cyclizing prefolded disulfide-rich precursors by use of a more stable HMBA ester significantly increases the synthetic flexibility of this enzymatic approach. We have achieved the efficient chemoenzymatic synthesis of the l-defensin RTD-1, as well as those of the cyclotides MCoTI-II, kB1, and [T20K]kB1 in combination with concomitant folding in a one-pot procedure involving two different ligation sites per molecule. This chemoenzymatic strategy with peptiligase variants can be scaled to the multi-gram range and therefore represents a promising methodology for larger-scale manufacture of this class of compounds. Head-to-tail cyclization mediated by peptiligase variants represents a valuable alternative to other previously reported methodologies, such as the use of sortase A, and offers a powerful extension to the currently existing toolbox of enzymes for cyclization reactions. Our findings provide proof of concept for the potential broad applicability of enzymatic macrocyclization for the efficient preparation of disulfide-rich peptides.

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Conflict of Interest

The authors declare no conflict of interest.

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