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DOI
10.1002/cbic.201900033

Publication date
2019

Document Version
Final published version

Published in
ChemBioChem

ChemPubSoc Europe

DOI: 10.1002/cbic.201900033

CHEMBIOCHEM Communications

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

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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—inter 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 makes 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.[3,5] 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.[9] 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 inteins,[18–22] trypsin,[23,24] asparaginyl endopeptidases such as butelase-1[25–27] or OaAEP1b,[28] or engineered subtilisins[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.[30,37] Omniligase-1 is a broad-specificity subtilisin 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

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Supporting information and the ORCID identification numbers for the authors of this article can be found under https://doi.org/10.1002/cbic.201900033.

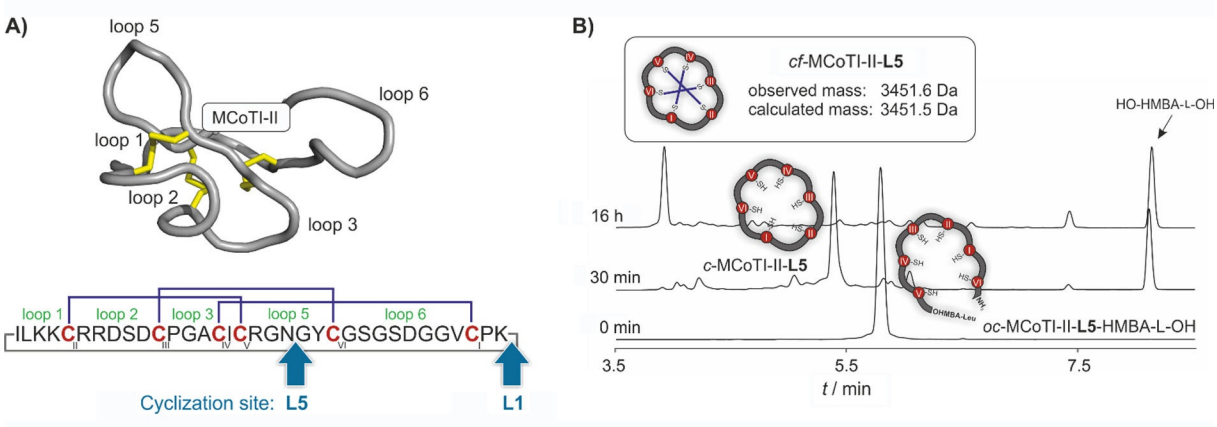

Figure 1. A) Cartoon representation of MCoTI-II (PDB ID: 1IB9) and sequence with potential cyclization sites. Disulfide bonds are shown in yellow. B) HPLC trace of the MCoTI-II-L5 one-pot cyclization/folding: oc-MCoTI-II-L5 HMBA-L-OH is readily converted into cyclic c-MCoTI-II-L5, followed by folding (2 mM GSH, pH 8.3) to adopt its native, oxidized conformation.

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 1 A), 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**: …VCPK//IL…)[30] 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//GY…) 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 2 A] 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)[30,42] yielded native cyclic folded *cf*-MCoTI-II (Figure 1 B) in a straightforward manner through a one-pot cyclization/folding procedure.

The correct disulfide connectivity was confirmed by the HPLC retention time (t_R), 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_i values of 0.0041 and 0.0050 nM, respectively, were comparable with the previously reported K_i value of native MCoTI-II (0.0023 nM).[43] In general, the identification of this second ligation site in loop 5 of MCoTI-II (**L5**: …CRGN//GY…) represents a viable alternative to the one described in loop 1 (**L1**: …VCPK//IL…)[30] and opens up the possibility of a modular synthesis of MCoTI-II. In addition, 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 the CEPS macrocyclization platform and minimize the amounts of misfolded isomers, thus resulting in higher overall yields. Again, a C-terminal HMBA-L-OH ester was chosen, having proven to be stable under the basic and oxidative conditions required for oxidative folding. Oxidized open-chain *oc-f*-MCoTI-II-**L1** HMBA ester was obtained after application of oxidative folding conditions (pH 7.8, 5 mM GSH)[30] for several hours. 1D ^{1}H NMR revealed that MCoTI-II-**L1** HMBA ester adopted a native-like fold (I–IV, II–V, III–VI, Figure S3).

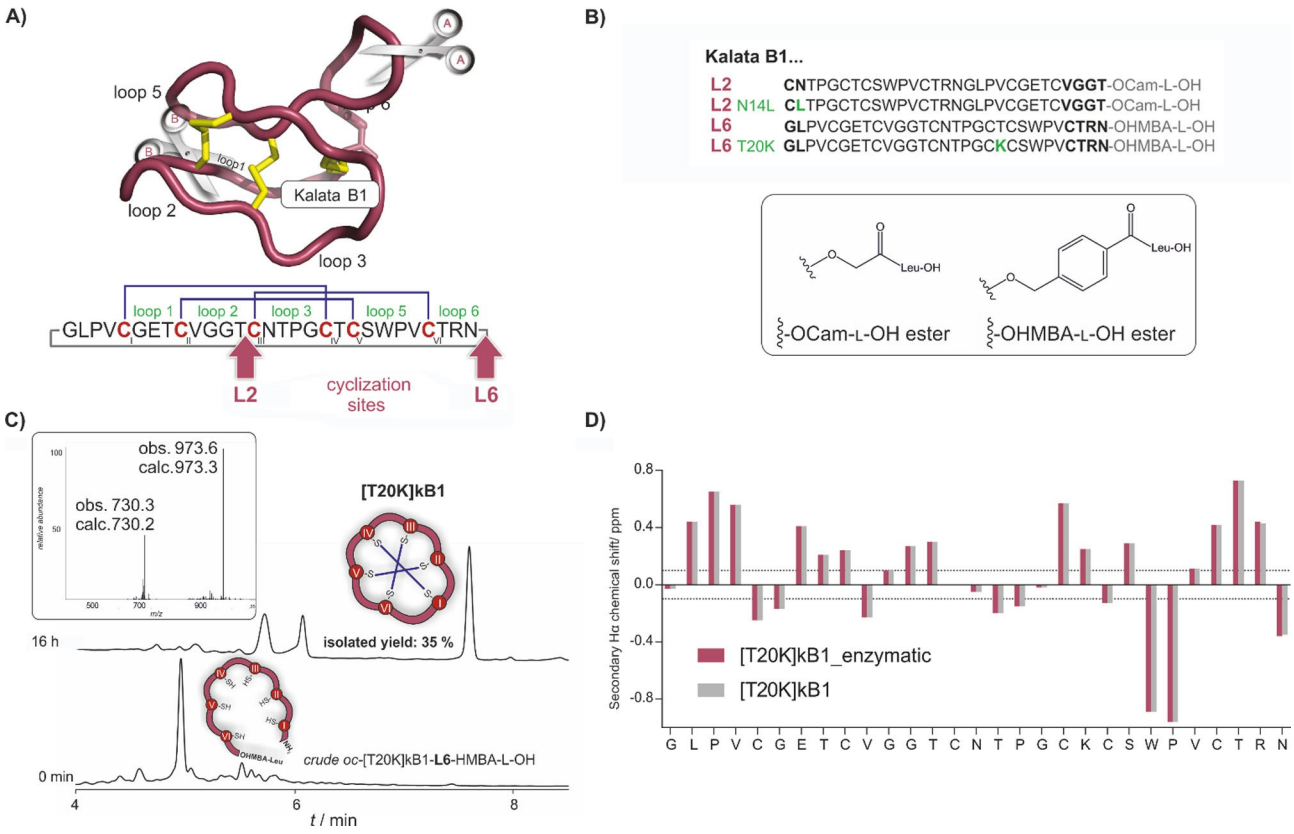

Figure 2. Chemoenzymatic synthesis of kB1. A) Cartoon representation of folded kB1 (PDB ID: 1NB1) and sequence with potential ligation sites. Disulfide bonds are shown in yellow. B) Linear C-terminal esters for cyclization of kB1 catalyzed by omniligase-1. C) HPLC traces of one-pot cyclization/folding 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.

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 a prefolded MCoTI-II precursor clearly represents an advancement from the previously described approach starting from reduced, linear MCoTI-II.[30] 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 30 NMR structures (PDB ID: 1HA9) reveals similar conformations, underlining the structural rigidity of *cf*-MCoTI-II. Indeed, molecular dynamics simulation revealed that although native MCoTI-II might bind omniligase-1, it does not appear to be the most favorable binding mode. The MD simulations suggest that breaking the Cys8–Cys25 disulfide bond, resulting in a partially reduced MCoTI-II, would be required for favorable binding and more efficient cyclization (Supporting Information). Interestingly, when the reaction was performed without any GSH present, it still resulted in the exclusive formation of *cf*-MCoTI-II, although at a significantly reduced rate in relation to the reaction with GSH present (reaction time of 18 h vs. 2 h). This finding indicates that cyclization most likely proceeds via a partially unfolded version of *oc-f*-MCoTI-II, potentially promoted by GSH as a disulfide shuffling agent.

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** (…VGGT//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

generally preferred by omniligase-1. Indeed, introduction of an N14L mutation into kB1 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 kB1.[45] 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*-kB1-**L6** HMBA ester to *c*-kB1-**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 kB1-**L6**. For this we used the [T20K]kB1 variant of kB1, which is under preclinical evaluation as an immunosuppressive agent for the treatment of multiple sclerosis.[46] After chemical synthesis of linear [T20K]kB1-**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 [T20k]kB1-**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 [T20K]kB1 was obtained (Figure 2C). Pure chemoenzymatically synthesized [T20K]kB1 was shown by NMR spectroscopy and LC-MS analysis to be identical to fully chemically synthesized [T20K]kB1 (Figure 2D, Figures S19 and S20). Besides structural verification by NMR, the chemoenzymatically synthesized [T20K]kB1 proved to have cytotoxicity ($IC_{50}=2.21$ μM) towards HeLa cells similar to that of its chemically synthesized counterpart ($IC_{50}=2.42$ 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 [T20K]kB1-**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,[48] 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 [T20K]kB1-**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 $\leq$0.002 molar equivalents. After oxidative folding and direct HPLC purification, *cf*-[T20K]kB1 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 [T20K]kB1 or variants thereof.

In addition to cyclotides, the non-mechanically interlocked θ-defensins, which display a range of antimicrobial activities,[4] 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,[22,35] 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** (…LCRR//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 [T20K]kB1 (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 ^{1}H 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),[50] thus confirming that *cf*-RTD-1 **B** had adopted a native θ-defensin fold. *cf*-RTD-1 **A** was unequivocally identified by LC-MS, and *cf*-RTD-1 **A** and *cf*-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-

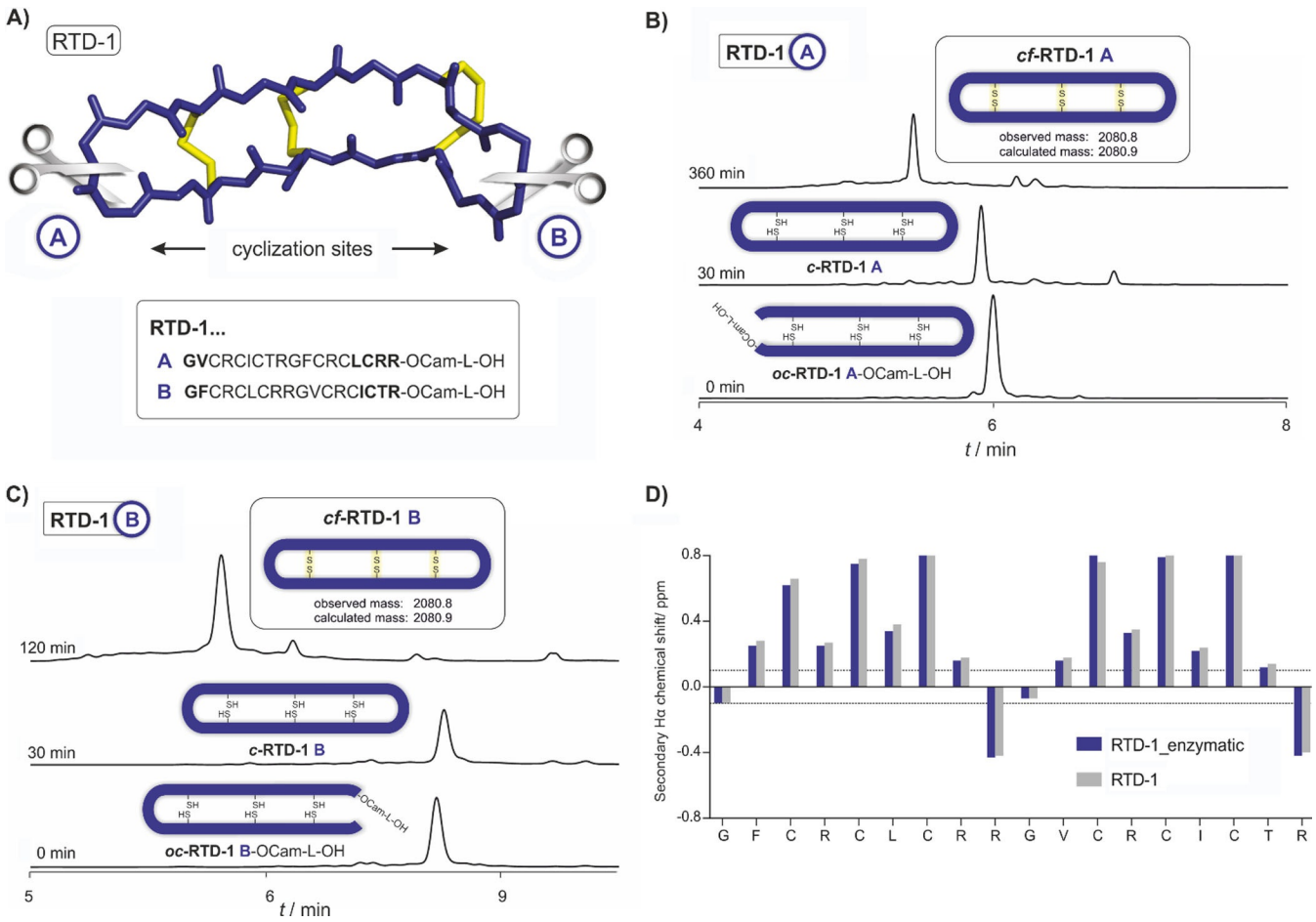

Figure 3. Chemoenzymatic synthesis of θ-defensin RTD-1. A) Stick representation of RTD-1 (PDB ID: 1HVZ). Disulfide bonds are shown in yellow. Ligation sites (A/B) and the individual sequence of each linear ester precursor peptide are given. B), C) HPLC traces for cyclization of *oc*-RTD-1 ester A and B, catalyzed by omniligase-1, followed by folding into their corresponding native forms *cf*-RTD-1. D) Comparison of secondary chemical shifts of enzymatically (blue) and chemically (gray) cyclized RTD-1 B.

quence. 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 θ-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.

Acknowledgements

We would like to thank Dr. Rodney Lax for fruitful discussions and for providing corrections to this manuscript. D.J.C. is supported by an Australian Research Council Laureate Fellowship (FL150100146).

Conflict of Interest

The authors declare no conflict of interest.

Keywords: chemoenzymatic peptide synthesis (CEPS) · cyclotides · enzyme catalysis · macrocycles · omniligase-1

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Manuscript received: January 17, 2019
Accepted manuscript online: February 8, 2019
Version of record online: April 25, 2019

