



UvA-DARE (Digital Academic Repository)

Omniligase-1: A Powerful Tool for Peptide Head-to-Tail Cyclization

Schmidt, M.; Toplak, A.; Quaedflieg, P.J.L.M.; Ippel, H.; Richelle, G.J.J.; Hackeng, T.M.; van Maarseveen, J.H.; Nuijens, T.

DOI

[10.1002/adsc.201700314](https://doi.org/10.1002/adsc.201700314)

Publication date

2017

Document Version

Final published version

Published in

Advanced Synthesis & Catalysis

License

Article 25fa Dutch Copyright Act

[Link to publication](#)

Citation for published version (APA):

Schmidt, M., Toplak, A., Quaedflieg, P. J. L. M., Ippel, H., Richelle, G. J. J., Hackeng, T. M., van Maarseveen, J. H., & Nuijens, T. (2017). Omniligase-1: A Powerful Tool for Peptide Head-to-Tail Cyclization. *Advanced Synthesis & Catalysis*, 359(12), 2050-2055. <https://doi.org/10.1002/adsc.201700314>

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (<https://dare.uva.nl>)

VIP Very Important Publication

Omniligase-1: A Powerful Tool for Peptide Head-to-Tail Cyclization

Marcel Schmidt,^{a,b} Ana Toplak,^a Peter J. L. M. Quaedflieg,^a Hans Ippel,^c Gaston J. J. Richelle,^b Tilman M. Hackeng,^c Jan H. van Maarseveen,^b and Timo Nuijens^{a,*}

^a EnzyPep B.V., Brightlands Campus, Urmonderbaan 22, 6167 RD Geleen, The Netherlands
E-mail: timo@enzypep.com

^b Van 't Hoff Institute of Molecular Sciences, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands

^c Department of Biochemistry, CARIM, University of Maastricht, Universiteitsingel 50, 6229 ER Maastricht, The Netherlands

Received: March 14, 2017; Revised: April 20, 2017; Published online: May 8, 2017



Supporting information for this article can be found under <https://doi.org/10.1002/adsc.201700314>.

Abstract: Strategies for the efficient synthesis of peptide macrocycles have been a long-standing goal. In this paper, we demonstrate the use of the peptide ligase termed omniligase-1 as a versatile and broadly applicable enzymatic tool for peptide cyclization. Several head-to-tail (multi)cyclic peptides have been synthesized, including the cyclotide MCoTI-II. Cyclization and oxidative folding of the cyclotide MCoTI-II were efficiently performed in a one-pot reaction on a 1-gram scale. The native cyclotide was isolated and the correct disulfide bonding pattern was confirmed by NMR structure determination. Furthermore, compatibility of chemo-enzymatic peptide synthesis (CEPS) using omniligase-1 with methods such as chemical ligation of peptides onto scaffolds (CLIPS) was successfully demonstrated by synthesizing a kinase-inhibitor derived tricyclic peptide. Our studies indicate that the minimal ring size for omniligase-1 mediated cyclization is 11 amino acids, whereas the cyclization of peptides longer than 12 amino acids proceeds with remarkable efficiency. In addition, several macrocycles containing non-peptidic backbones (e.g., polyethylene glycol), isopeptide bonds (amino acid side-chain attachment) as well as D-amino acids could be efficiently cyclized.

Keywords: chemo-enzymatic peptide synthesis (CEPS); cyclic peptides; cyclization; cyclotide synthesis; cyclotides; enzyme catalysis; head-to-tail cyclization; ligases; macrocycles; omniligase-1; peptides

Peptide macrocycles represent an extremely diverse class of molecules that attract increased attention as drug leads and prospective pharmaceuticals, with currently over 30 cyclic peptides registered or in clinical trials.^[1–3] Together with linear peptides, they fill the gap between small-molecule drugs (less than 500 Da) and biologics (over 5000 Da) and have the potential to address previously “undruggable” targets. Many cyclic peptides are characterized by their unique structural and enhanced biopharmaceutical properties, such as an improved metabolic stability due to a reduced sensitivity to proteolytic cleavage. The increasing number of cyclic peptides used as therapeutics is accompanied by the need for efficient and cost-effective routes that enable their synthesis on a large scale. Moreover, efficient synthesis of large libraries of cyclic peptides (e.g., for screening purposes) or of cyclic peptides containing non-canonical amino acids (e.g., D- or unnatural amino acids to increase stability and diversity) is of importance. However, efficient cyclization of peptides using traditional synthetic methods is still a challenge.^[4] Classical coupling reagents are often used to cyclize side-chain protected peptides in anhydrous organic solvents. However, heavy dilution to prevent polymerization, risk of epimerization and a poor solubility of side-chain protected peptides limit this approach, especially for peptides longer than 20 amino acids.^[5] Native chemical ligation (NCL)^[6] is often used to cyclize unprotected peptides in aqueous solution. However, not all peptides contain the cysteine needed for this ligation.^[7] Thus, due to their favourable properties such as excellent chemo-selectivity, the use of enzymes for the head-to-tail cyclization of peptides has been extensively examined and provides an elegant link between chemistry

and biology.^[8] The currently existing set of enzymes used for peptide cyclization is comprised of enzymes such as sortases,^[9,10] trypsin,^[11] asparaginyl endoproteases (AEP) like butelase-1^[12,13] or OaAEP1b^[14] and subtilisin variants like peptiligase.^[15,16] Most of the enzymatic approaches investigated suffer from incomplete ligation reactions and low catalytic efficiency, and in addition leave a ligase “footprint”, an unavoidable enzyme recognition sequence at the coupling site. In contrast, peptiligase based enzyme variants have recently emerged as a very powerful tool for traceless (footprint free) enzyme-mediated peptide bond formation.^[8] Peptiligase variants have been reported to efficiently catalyze the head-to-tail cyclization of a linear peptide bearing a C-terminal (glycolate) ester introduced during routine solid-phase peptide synthesis (SPPS).^[17] An improved variant of peptiligase, termed omniligase-1, which recently became commercially available, has a very broad substrate scope that provides an excellent basis for efficient head-to-tail peptide cyclization.

Herein, we describe the chemo-enzymatic peptide synthesis (CEPS) of several (multi)cyclic peptides using omniligase-1. To investigate the scope and limitations of omniligase-mediated peptide cyclization, a library of linear model peptides with different lengths was synthesized, namely SY-(SG)_n-FSKL-OCam-L, with n being 2–6 for peptides of 10, 12, 14 and 16 amino acids, respectively, and cyclized using omniligase-1 (Figure 1). The N- (SY) and C-termini (FSKL) of the model peptides were known to be

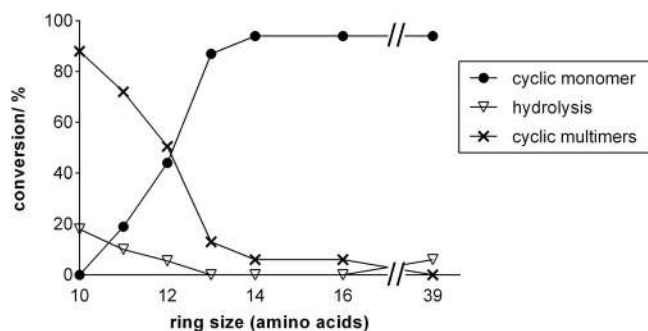


Figure 1. Conversion after full consumption of ester starting material vs. peptide length. The library of model peptides [SY-(SG)_n-FSKL-OCam-L with n being 2–6 for peptides of 10, 12, 14 and 16 amino acids in length] was cyclized using omniligase-1. For a ring size of 11 and 13 amino acids the peptides SY-G(SG)_n-FSKL-OCam-L were used with n being 1–2. The reaction was carried out in 1 M potassium phosphate buffer pH 8.5 containing 3.5 mM *tris*(2-carboxyethyl)-phosphine (TCEP) and 10 μg mL⁻¹ of omniligase-1. The linear precursor Cam-ester peptides were cyclized at a concentration of 0.5 mg mL⁻¹ and the cyclizations were followed by HPLC (λ = 220 nm). Conversion to cyclic multimers is given as the sum of cyclic dimers and cyclic trimers. Reactions were completed within 30 minutes, except for the formation of the 11-mer cycle, which took approx. 90 min.

good substrates for omniligase-1 and are deliberately used as an enzyme recognition sequence throughout this study. Enzymatic cyclizations were performed in aqueous solution [1 M potassium phosphate, 3.5 mM *tris*(2-carboxyethyl)phosphine (TCEP)] at slightly basic pH (8.5) with a substrate concentration of 0.5 mg mL⁻¹. The reaction progress was monitored *via* RP-HPLC-MS. It was found that cyclic peptides with a ring size of over 12 amino acids could be generated with very high efficiency (i.e. >90% HPLC conversion to peptide cycles of 14 and 16 amino acids, Figure 1) without the formation of significant amounts of dimeric or trimeric cyclic by-products. Peptide esters resulting in ring sizes of 12 amino acids or less clearly led to an increased formation of multimeric species, predominantly cyclic dimers and trimers. In addition, a decreased cyclization efficiency corresponded to an increased rate of hydrolysis of the C-terminal ester moiety. To investigate the minimal chain length for (efficient) peptide cyclization, two peptides were synthesized of 11 and 13 amino acids length, respectively [SY-G(SG)_n-FSKL-OCam-L, with n being 1 and 2]. It was shown that the minimal number of amino acids needed for cyclization is 11 (25% HPLC conversion to monocyclic product, Figure 1), but for very efficient cyclization (>85% HPLC conversion to monocyclic product) the cyclic product needs to be at least 13 amino acids long (Figure 1).

In order to enhance the cyclization efficiency for peptides with 12 or less amino acids, several attempts were undertaken. For example, the introduction of a turn-inducing D-Pro/L-Pro (pP) β-hairpin motif at the 4–5 position was investigated [SY-(SG)₂-p⁶P⁵F⁴S³K²L¹-OCam-L, 12 amino acid ring size]. However, using this substrate, the cyclization efficiency could not be improved and with approximately 40% HPLC conversion to monocyclic product the results are similar to the cyclization efficiency without this turn-inducing template (see the Supporting Information). Even poorer monocyclization results were obtained when cyclizing an 12-mer peptide with the pP motif at positions 5–6 directly after the enzymatic recognition sequence [SY-(SG)₂-p⁵P⁴S³K²L¹-OCam-L]. In fact, the reaction was slower and a significant amount of cyclic multimers (cyclic dimers, trimers) was formed. We reasoned that the minimal ring size is pre-determined by the 6 amino acid recognition pockets of omniligase-1 (P₄-P₁*P₁'-P₂', with * being the coupling position), leading to a minimal ring size of 12 to 13 amino acids because (besides the 6 amino acids bound to the enzyme pockets) an additional 6 amino acids are needed to loop back for efficient peptide cyclization. With increasing peptide length a more efficient cyclization is observed, as shown for the 39-mer incretin mimetic drug exenatide (N-terminus: FI, C-terminus: AVRL-OCamL), which was cy-

cyclized in an efficiency of 94% (Figure 1). This clearly shows that virtually any amino acid sequence can be cyclized efficiently and that the deliberately used model recognition sequence (SY...FSKL-OCamL) is not a prerequisite. Many other sequences can be used as previously reported for peptiligase.^[16,18]

The scope and versatility of omniligase-1 mediated head-to-tail peptide cyclization was further elaborated by the successful cyclization of a number of peptides containing, for example, non-peptidic moieties [i.e., polyethylene glycol, SY-NH-CH₂-CH₂(-O-CH₂-CH₂)_n-CH₂-CH₂-CO-FSKL-OCam-L, with *n* = 11, Figure 2],

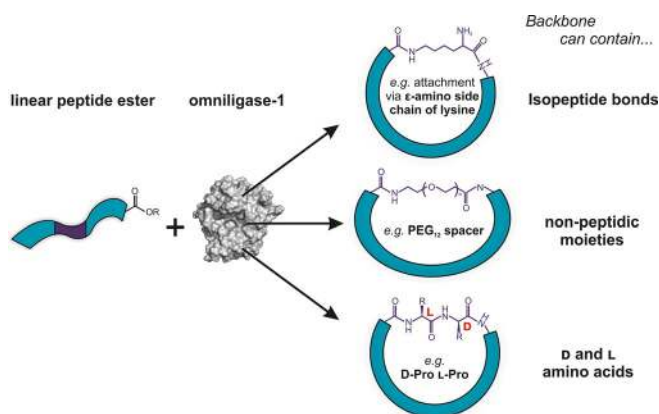


Figure 2. Cyclization of linear peptide esters using omniligase-1. The backbone (outside the enzyme recognition sites) can contain isopeptide bonds, non-peptidic moieties as well as D-amino acids.

D-amino acids (see D-Pro peptides above) or isopeptide bonds (e.g., attachment of the following amino acids sequences *via* the ε-amino side chain of K*, H-SYG-K*-SGSGFSKL-OCam-Leu-OH, Figure 2) outside the enzymatic 6 amino acid recognition sequence. The recognition motif needs to consist exclusively of L-amino acids. The results support the finding that the backbone between the N- and C-terminal recognition sequences is not crucial for efficient peptide cyclization. In summary, omniligase-1 can serve as a tool for almost quantitative head-to-tail cyclization of peptides over 12 amino acids in size, without the formation of multimers. This underlines the fact that intramolecular ligation is much faster than the corresponding intermolecular ligations.

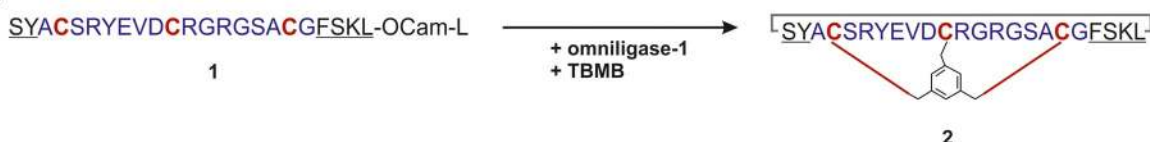
The above results encouraged us to attempt the synthesis of even more complex peptides. For instance, omniligase-1 was used for the synthesis of a constrained tricyclic peptide bound to a small molecule scaffold *via* three thioether bonds (Figure 3A). The three cysteine residues containing peptide was synthesized and cyclized using CEPS, followed by a CLIPS^[19] reaction of the free thiol functionalities with tris(bromomethyl)benzene (TBMB). The in-

creased conformational rigidity of such constrained cyclic peptides offers the opportunity to amplify the affinity to a pharmaceutical target by reducing entropy and to increase target specificity. Such peptide derivatives can be used for the mimicry of protein surfaces in order to address protein-protein interactions as a pharmaceutical target. Based on the potent bicyclic inhibitor UK18 of the human urokinase-type plasminogen activator (uPA)^[20] the sequence was extended by a 6 amino acid spacer sequence to enable formation of the tricyclic product (SYACS-RYEVD_CCRGRGSAC_CGFSKL-OCam-L; the N- and C-terminal linker sequences are shown in *italics* and the underlined cysteines are linked to TBMB). The linear precursor peptides were dissolved in aqueous solution (500 mM potassium phosphate pH 8.5, 3.5 mM TCEP). The respective reactions were started by sequential addition of omniligase-1 or the small molecule scaffold TBMB. The reaction was followed *via* RP-HPLC-MS. The open chain precursor **1** was head-to-tail cyclized with >95% efficiency to **3** using omniligase-1, followed by addition of the small molecule ligand TBMB to quantitatively yield the final tricyclic peptide construct **2** (Figure 3B). Neither hydrolysis of the ester moiety nor formation of multimeric species were observed. Thus, with CEPS followed by the CLIPS reaction, the final product **2** was obtained with almost quantitative HPLC peak to peak conversion.

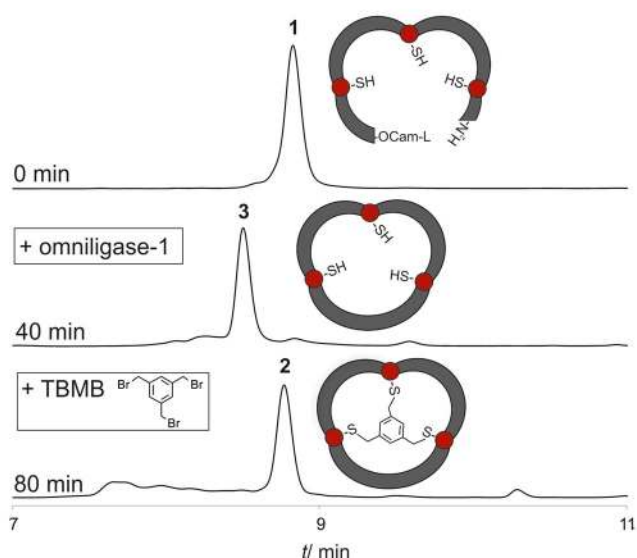
Reversely, CLIPS reaction followed by CEPS (Figure 3C), resulted in an only 43% conversion (HPLC) to the final tricyclic product **2** and 57% hydrolysis by-product **5**. The CLIPS reaction proceeded efficiently to yield intermediate **4**, but the head-to-tail macrocyclization of the CLIPS-constrained peptide using omniligase-1 was hampered. The addition of the TBMB scaffold to the open chain precursor imposes conformational constraints to the linear backbone,^[21] such that the flexibility of the termini to be ligated by omniligase-1 is restricted, thus resulting in a reduced cyclization efficiency. It must be noted that conformational restriction might depend on the amino acid sequence, position of the cysteines, peptide length and the organic scaffold, and that CEPS followed up by CLIPS reaction (Figure 3B) is preferred as a generally applicable strategy. In comparison, a previously described approach using a microbial transglutaminase for such reactions suffers from low yields, the generation of a non-native backbone and specific sequence requirements.^[22]

Next, we embarked on the synthesis of a member of the complex family of multicyclic peptides, termed cyclotides. Cyclotides contain a cyclic cystine knot (CCK) scaffold, a configuration of three conserved disulfide bonds in a knotted structure, which enhances the topological complexity as well as the chemical, enzymatic and thermal stability of the cyclic back-

A) Reaction scheme



B) CEPS followed by TBMB-CLIPS



C) TBMB-CLIPS followed by CEPS

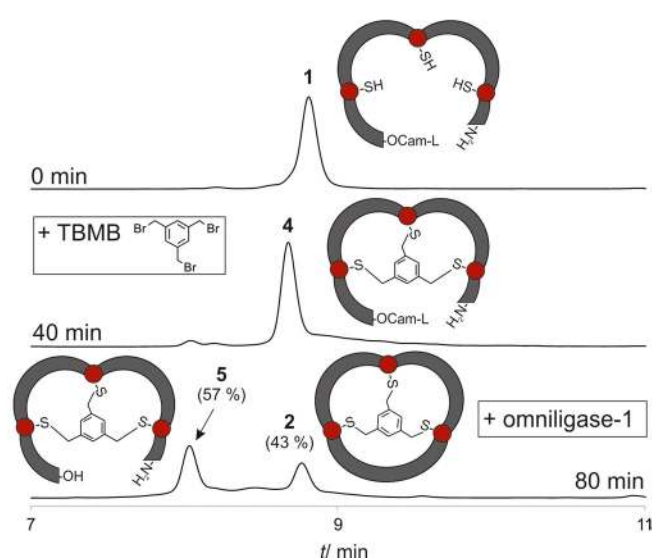


Figure 3. A) Reaction scheme for the synthesis of the tricyclic peptide based on the bicyclic inhibitor UK18 of the human urokinase-type plasminogen activator. The linker sequence is shown in black (underlined) and the sequence of UK18 in blue with the cysteines highlighted in red. The tricyclic peptide was generated using a combination of CEPS (SPPS + omniligase-1 mediated cyclization) and CLIPS (TBMB scaffold). The purified linear precursor was dissolved at a concentration of 0.15 mM (0.5 mg mL^{-1}) in 500 mM potassium phosphate buffer pH 8.5 containing 3.5 mM TCEP. Cyclization was performed using omniligase-1 ($0.95 \mu\text{M}$, $20 \mu\text{g mL}^{-1}$). TBMB was added as a 5 mM solution in acetonitrile to a final concentration of 1 mM. B) HPLC chromatogram ($\lambda = 220 \text{ nm}$) of the linear precursor peptide **1**. After **1** was efficiently cyclized to yield **3**, the organic small molecule scaffold (TBMB) was added directly to the reaction mixture in order to obtain the tricyclic product **2**. C) HPLC chromatogram ($\lambda = 220 \text{ nm}$) of the linear precursor **1** that was first reacted with the organic scaffold (TBMB) to yield the bicyclic precursor **4**, followed by omniligase-1 mediated head-to-tail cyclization (same molarities as in B). The enzymatic cyclization reaction was started by adding the enzyme to the CLIPS reaction mixture, resulting in the formation of the tricyclic product **2** (43% HPLC conversion) and the hydrolysis by-product **5** (57% HPLC conversion, bicyclic open chain product).

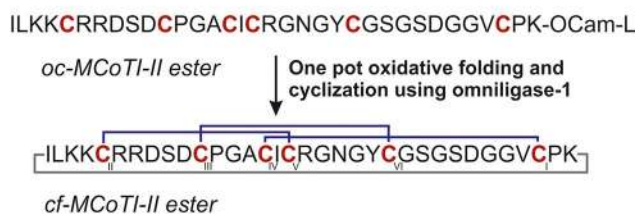
bone.^[23,24] Due to their excellent properties (e.g., oral bioavailability^[25]), cyclotides have gained increasing attention for their application as molecular scaffolds in drug design.^[26,27] A well described example is MCoTI-II, a 34-mer head-to-tail cyclic trypsin inhibitor isolated from *Momordica cochinchinensis*.^[28]

Although several synthetic protocols for cyclotides are well established,^[29–32] efficient head-to-tail cyclization still remains a challenge, especially at a larger scale. However, a CEPS approach for the synthesis of cyclotides bears great potential. The linear open chain (*oc*-) MCoTI-II precursor peptide Cam-ester was assembled using standard Fmoc-SPPS (Figure 4A). The coupling position was chosen with a hydrophobic amino acid in the P_4 position, which is important for an efficient ligation, and in addition, proline was avoided in positions P_1' and P_2' . Subsequently, at

a concentration of 1 mg mL^{-1} the purified precursor peptide ester could be efficiently cyclized ($>90\%$ HPLC conversion, Figure 4B) to reduced cyclic (*c*-) MCoTI-II within 30 minutes using omniligase-1. Stirring the reaction mixture for another 14 hours in 1 M potassium phosphate solution (pH 8.5) containing 5 mM reduced glutathione resulted in the formation of the intact, native (*cf*-) MCoTI-II. The final product was isolated using preparative HPLC.

Although MCoTI-II is known to efficiently fold into its correct, naturally occurring structure,^[31] the correct disulfide connectivity as well as the correct overall structure was confirmed *via* NMR (see the Supporting Information). Both chemical shifts and NOESY spectra indicate the same native structure of MCoTI-II as determined before by means of X-ray crystallography^[33] and NMR spectroscopy.^[28,33,34] One

A) Reaction Scheme



B) one pot cyclization and oxidative folding of MCoTI-II:

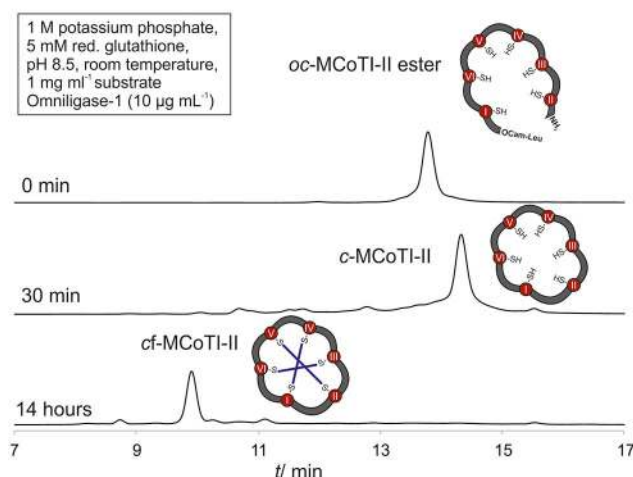


Figure 4. A) Reaction scheme for the cyclization and oxidative folding of open chain (*oc*)-MCoTI-II in a one-pot reaction to yield cyclic folded (*cf*)-MCoTI-II. B) HPLC chromatograms ($\lambda=220$ nm) after 0 min (*oc*-MCoTI-II), 30 min [cyclic (*c*)-MCoTI-II] and after 14 hours (*cf*-MCoTI-II). The correct folding of *cf*-MCoTI-II was confirmed *via* NMR structure determination.

single set of amide and amino resonances was observed, and most resonances appeared sharp in the various spectra. Exceptions are NH Lys10 and NH Lys13, positioned in the inhibitor region of MCoTI-II, that exhibit increased dynamic line broadening at low temperature.

After the successful formation of native *cf*-MCoTI-II in a one-pot reaction, the process was further simplified. For example, *crude*, non-HPLC purified, *oc*-MCoTI-II ester was cyclized and oxidatively folded in a one-pot reaction (Figure 4A). The scalability of the one-pot CEPS approach was demonstrated by the preparation of the native cyclotide *cf*-MCoTI-II at a 1 gram scale. At a concentration of 4 mg mL⁻¹ of the crude substrate the reduced intermediate *c*-MCoTI-II was formed efficiently (see the Supporting Information). Upon subsequent oxidative folding for 20 hours in 250 mM potassium phosphate buffer containing 5 mM reduced glutathione the final, native *cf*-MCoTI-II was obtained. Thus, the number of steps required could be significantly reduced, that is, only one final preparative HPLC run was required instead of

two separate purification steps for the precursor and product. The crude reaction mixture was purified *via* a single RP-HPLC purification and *cf*-MCoTI-II was obtained in an overall yield of 8.0% based on the loading of the first amino acid on the resin with over 95% HPLC purity. For a peptide of this length (34 amino acids) and complexity this yield is good and may be improved even further by optimizing the SPPS, cyclization/folding and purification protocol.

In conclusion, the efficiency of omniligase-1 combined with its broad substrate scope, that allows a traceless ligation, makes CEPS technology a flexible and generally applicable tool, which provides a viable and economically attractive route for the synthesis of cyclotides and other head-to-tail cyclic peptides. Moreover, since other processes using CEPS technology have been successfully performed on the multi-gram scale^[16] we believe that omniligase-1-mediated cyclization could serve as a general methodology for the head-to-tail cyclization of peptides at a large scale.

Experimental Section

Peptide Cyclization and Mass Spectrometric Analysis of Products

Peptides were dissolved in phosphate buffer (1M, pH 8.5, 3.5 mM TCEP) to the desired concentration (0.5 mg mL⁻¹ to 4 mg mL⁻¹) and omniligase-1 (10 µg omniligase/mg peptide) was added. The ligation was carried out at room temperature (20 °C). The reaction was followed at $\lambda=220$ nm using an HPLC-MS system (Agilent 1260 Infinity coupled with an Agilent 6130 quadrupole mass spectrometer, Agilent, Santa Clara, USA). Separation was performed using a Luna RP-C18 10 µM, 4.6 × 250 mM column (Phenomenex, Torrance, USA), eluting with 0.05% methanesulfonic acid (MSA) in a water-acetonitrile gradient at a flow rate of 1 mL min⁻¹. As a mobile phase a binary mixture of A (water + 0.05% MSA) and B (acetonitrile + 0.05% MSA) was used. An appropriate gradient was chosen for each sample individually (see the Supporting Information). The purity of peptides and the conversion rates of the enzymatic cyclization were calculated by automatically integrating the area of the corresponding peaks in the HPLC spectrum ($\lambda=220$ nm).

Omniligase-1 is commercially available *via* Iris Biotech (Art. No. EZK2020.0000, Marktredwitz, Germany).

Solid-Phase Peptide Synthesis

Peptides were assembled on Fmoc-Leu-Wang resin using standard Fmoc-SPPS. Cam-esters were introduced as Fmoc-AAx-glycolic acid according to Nuijens et. al.^[35] For experimental details see the Supporting Information.

NMR Spectroscopy on *cf*-MCoTI-II

The NMR sample of MCoTI-II was prepared as a 2.3 mM solution in 160 µL of 25 mM NaAc-d₃ buffer (3 mm NMR

tube, pH 3.5), containing 0.1 mM EDTA, 0.2 mM sodium azide, 3 μM DSS- d_6 as chemical shift reference and 2% (v/v) D_2O for deuterium lock. NMR spectra (1D ^1H and ^{13}C , DIPS1 80 ms, NOESY 200 ms, natural abundance ^{13}C - ^1H HSQC, ^{13}C - ^1H HMBC and ^{15}N - ^1H HSQC)^[36] were recorded on a Bruker Avance III HD 700 MHz spectrometer, equipped with a TCI cryoprobe. Spectra were recorded at both 12°C and 23°C to allow comparison to known resonance assignments of MCoTI-II.^[28,34] Processing was done by Topspin 3.2, spectral analysis and resonance assignment was carried out by Sparky 3.115.^[37] Our final complete ^1H , ^{13}C , and ^{15}N assignments of MCoTI-II are listed in the Supporting Information, Table SH1.

Acknowledgements

The authors wish to thank Dr. Rodney Lax for useful discussions and providing corrections to this manuscript.

References

- [1] A. M. White, D. J. Craik, *Expert Opin. Drug Discov.* **2016**, *11*, 1151–1163.
- [2] F. Giordanetto, J. Kihlberg, *J. Med. Chem.* **2014**, *57*, 278–295.
- [3] A. Zorzi, K. Deyle, C. Heinis, *Curr. Opin. Chem. Biol.* **2017**, *38*, 24–29.
- [4] C. J. White, A. K. Yudin, *Nat. Chem.* **2011**, *3*, 509–524.
- [5] O. Cheneval, C. I. Schroeder, T. Durek, P. Walsh, Y. Huang, S. Liras, D. A. Price, D. J. Craik, *J. Org. Chem.* **2014**, *79*, 5538–5544.
- [6] R. H. Kimura, A.-T. Tran, J. A. Camarero, *Angew. Chem.* **2006**, *118*, 987–990; *Angew. Chem. Int. Ed.* **2006**, *45*, 973–976.
- [7] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776–779.
- [8] M. Schmidt, A. Toplak, P. J. L. M. Quaedflieg, T. Nuijens, *Curr. Opin. Chem. Biol.* **2017**, *38*, 1–7.
- [9] X. Jia, S. Kwon, C.-I. Anderson Wang, Y.-H. Huang, L. Y. Chan, C. C. Tan, J. K. Rosengren, J. P. Mulvenna, C. I. Schroeder, D. J. Craik, *J. Biol. Chem.* **2014**, *289*, 6627–6638.
- [10] K. Stanger, T. Maurer, H. Kaluarachchi, M. Coons, Y. Franke, R. N. Hannoush, M. De La Rosa, *FEBS Lett.* **2014**, *588*, 4487–4496.
- [11] P. Thongyoo, N. Roqué-Rosell, R. J. Leatherbarrow, E. W. Tate, *Org. Biomol. Chem.* **2008**, *6*, 1462–1470.
- [12] G. K. T. Nguyen, S. Wang, Y. Qiu, X. Hemu, Y. Lian, J. P. Tam, *Nat. Chem. Biol.* **2014**, *10*, 732–738.
- [13] R. Yang, Y. H. Wong, G. K. T. Nguyen, J. P. Tam, J. Lescar, B. Wu, *J. Am. Chem. Soc.* **2017**, *139*, 5351–5358.
- [14] K. S. Harris, T. Durek, Q. Kaas, A. G. Poth, E. K. Gilding, B. F. Conlan, I. Saska, N. L. Daly, N. L. van der Weerden, D. J. Craik, et al., *Nat. Commun.* **2015**, *6*, 1–10.
- [15] T. Nuijens, A. Toplak, M. B. A. C. van de Meulenreeck, M. Schmidt, M. Goldbach, P. J. L. M. Quaedflieg, D. B. Janssen, *Chem. Today* **2016**, *34*, 16–19.
- [16] T. Nuijens, A. Toplak, P. J. L. M. Quaedflieg, J. Drenth, B. Wu, D. B. Janssen, *Adv. Synth. Catal.* **2016**, *358*, 4041–4048.
- [17] T. Nuijens, A. Toplak, M. B. A. C. van de Meulenreeck, M. Schmidt, M. Goldbach, P. J. L. M. Quaedflieg, *Tetrahedron Lett.* **2016**, *57*, 3635–3638.
- [18] A. Toplak, T. Nuijens, P. J. L. M. Quaedflieg, B. Wu, D. B. Janssen, *Adv. Synth. Catal.* **2016**, *358*, 2140–2147.
- [19] P. Timmerman, J. Beld, W. C. Puijk, R. H. Meloen, *ChemBioChem* **2005**, *6*, 821–824.
- [20] A. Angelini, L. Cendron, S. Chen, J. Touati, G. Winter, G. Zanotti, C. Heinis, *ACS Chem. Biol.* **2012**, *1*, 817–821.
- [21] S. Chen, J. Morales-Sanfrutos, A. Angelini, B. Cutting, C. Heinis, *ChemBioChem* **2012**, *13*, 1032–1038.
- [22] J. Touati, A. Angelini, M. J. Hinner, C. Heinis, *ChemBioChem* **2011**, *12*, 38–42.
- [23] N. L. Daly, D. J. Craik, *Curr. Opin. Chem. Biol.* **2011**, *15*, 362–368.
- [24] M. L. Colgrave, D. J. Craik, *Biochemistry* **2004**, *43*, 5965–5975.
- [25] K. P. Greenwood, N. L. Daly, D. L. Brown, J. L. Stow, D. J. Craik, *Int. J. Biochem. Cell Biol.* **2007**, *39*, 2252–2264.
- [26] S. E. Northfield, C. K. Wang, C. I. Schroeder, T. Durek, M. W. Kan, J. E. Swedberg, D. J. Craik, *Eur. J. Med. Chem.* **2014**, *77*, 248–257.
- [27] D. J. Craik, J. Du, *Curr. Opin. Chem. Biol.* **2017**, *38*, 8–16.
- [28] M. E. Felizmenio-Quimio, N. L. Daly, D. J. Craik, *J. Biol. Chem.* **2001**, *276*, 22875–22882.
- [29] O. Cheneval, C. I. Schroeder, T. Durek, P. Walsh, Y. Huang, S. Liras, D. A. Price, D. J. Craik, *J. Org. Chem.* **2014**, *79*, 5538–5544.
- [30] N. L. Daly, R. J. Clark, D. J. Craik, *J. Biol. Chem.* **2003**, *278*, 6314–6322.
- [31] M. Čemažar, N. L. Daly, S. Häggblad, P. L. Kai, E. Yulyaningsih, D. J. Craik, *J. Biol. Chem.* **2006**, *281*, 8224–8232.
- [32] T. L. Aboye, R. J. Clark, R. Burman, M. B. Roig, D. J. Craik, U. Göransson, *Antioxid. Redox Signal.* **2011**, *14*, 77–86.
- [33] N. L. Daly, L. Thorstholm, K. P. Greenwood, G. J. King, K. J. Rosengren, B. Herast, J. L. Martin, D. J. Craik, *J. Biol. Chem.* **2013**, *288*, 36141–36148.
- [34] A. Heitz, J.-F. Hernandez, J. Gagnon, T. T. Hong, T. C. Pham, T. M. Nguyen, D. Le-Nguyen, L. Chiche, *Biochemistry* **2001**, *40*, 7973–7983.
- [35] T. Nuijens, A. Toplak, M. B. A. C. van de Meulenreeck, M. Schmidt, M. Goldbach, P. J. L. M. Quaedflieg, *Tetrahedron Lett.* **2016**, *57*, 3635–3638.
- [36] E. E. Kwan, S. G. Huang, *Eur. J. Org. Chem.* **2008**, *2008*, 2671–2688.
- [37] T. D. Goddard, D. G. Kneller, SPARKY 3, University of California, San Francisco (USA).