Enzymatic tools for peptide ligation and cyclization

Development and applications

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CHAPTER 6

A One-Pot “Triple-C” Multicyclization Methodology for the Synthesis of Highly Constrained Isomerically Pure Tetracyclic Peptides

This work has been published as


§ authors contributed equally to this work.
Abstract

Here we report a broadly applicable one-pot methodology for the facile transformation of linear peptides into tetracyclic peptides via a CEPS/CLIPS/CuAAC ("triple-C") locking methodology. Linear peptides with varying lengths (≥ 14 amino acids), comprising two cysteines and two azidohomoalanines (Aha), were efficiently cyclized head-to-tail using the peptiligase variant omniligase-1 (CEPS). Subsequent ligation-cyclization using tetravalent (T4₁₂) scaffolds containing two bromomethyl groups and two alkyne functionalities yielded isomerically pure tetracyclic peptides. Sixteen different functional tetracycles, derived from bicyclic inhibitors against urokinase plasminogen activator (uPA) and coagulation factor XIIa (FXIIa), were successfully synthesized and their bioactivities evaluated. Two of these (FF-T4₁₂) exhibited increased inhibitory activity against FXIIa as compared to a bicyclic control peptide. The corresponding hetero-bifunctional variants (UF/FU-T4₁₂), bearing a single copy of each inhibitory sequence, exhibited micromolar activities against both uPA and FXIIa, thus illustrating the potential of the "bifunctional tetracycle peptide" inhibitor concept.
Introduction

Highly constrained peptides are abundant in nature and display a wide range of different bioactivities. Cyclotides, a class of natural peptides found in plants, provide an intriguing example of this type consisting of a head-to-tail cyclized peptide backbone that is cross-linked with multiple conserved disulfide constraints. Cyclotides, in general, and in particular cyclotides, exhibit strongly improved metabolic stabilities and target affinities as compared to their linear counterparts. This renders them highly promising as novel therapeutics, emphasized by the rapidly increasing number of cyclopeptides in (pre)clinical development over the last decade, and underscores the need for complementary synthetic strategies to manufacture similarly constrained peptide macrocycles.

Over the years, a variety of different technologies for constraining peptide structures have been described, including side-chain-to-side-chain cross-links and the use of small molecule organic scaffolds. We recently established an efficient chemo-enzymatic peptide synthesis (CEPS) strategy using omniligase-1 catalyzed head-to-tail cyclization for the preparation of various (multi)cyclic peptides, such as the cyclotide MCoTI-II. Even though the cystine-knotted core of cyclotides represents a promising scaffold for drug design, the majority of disulfide-rich peptides form isomeric mixtures upon oxidation, which further emphasizes the need for a straightforward, sequence-independent methodology that enables the manufacture of isomerically pure multicyclic constructs. We recently reported the ligation-cyclization of linear peptides on a tetravalent scaffold (T4) using orthogonal CLIPS and CuAAC reactions for the preparation of tricyclic peptides.

Results and Discussion

Here we report the rapid one-pot synthesis of tetracyclic peptides using an unprecedented combination of omniligase-1 mediated (CEPS) head-to-tail cyclization followed by CLIPS/CuAAC reactions using T4 scaffolds. This "triple-C locking" methodology provides an efficient way for the manufacturing of isomerically pure tetracyclic peptides (Figure 1). Using "triple-C locking" we successfully synthesized and identified novel bifunctional tetracycles, which display inhibitory activity against two therapeutically relevant targets, namely urokinase-type plasminogen activator (uPA) and coagulation factor XIa.
Figure 1. Schematic representation of the conversion of linear peptide 1 (four amino acids between the reactive residues) into tetracyclic construct I_T4x. Peptide head-to-tail cyclization (CEPS), yielding monocycle c1, was followed by CLIPS using the T4 scaffold (x = 1, 2). The CLIPS bicycle intermediate c1_T4x was then cyclized via double CuAAC to give the desired tetracycle I_T4x. (Azidohomoalanine is depicted as [Aha]).

We evaluated the general applicability of “triple-C” locking by synthesizing a library of peptides differing in length and sequence that contain both two cysteine and two Aha residues combined with a C-terminal elongated Cam-Leu-ester.[21] Considering the requirement for efficient omniligase-I-catalyzed cyclization to be ≥ 13 amino acids,[18] we first attempted cyclization of the linear 16-mer peptide 2_3333 (H-SYCQGA[Aha]KSE[Aha]KFGCK-Cam-L-OH). Enzymatic cyclization (CEPS) gave the monocyclic peptide c2_3333 within 30 min (Figure 2A). Reaction of purified c2_3333 with either T4_1 or T4_2 led to the formation of the corresponding CLIPS bicycles c2_3333-T4_1 and c2_3333-T4_2 within 20 min. Addition of a pre-incubated mixture of CuSO4, tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) and ascorbic acid initiated the CuAAC reaction between the peptidic azides and the alkynes in the T4 scaffold, yielding the desired tetracyclic peptides II_3333-T4_1 (Figure 2A) and II_3333-T4_2 within seconds. In both cases, the final tetracyclic peptide was formed as a single isomer (e.g. II_3333-T4_1: R_t: 1.08 min, MW_calc: 2084.4 Da, MW_exp: 2085.4 Da) owing to free rotation around either the aryl-aryl (T4_1) or aryl-amide bond (T4_2).[20] Encouraged by these results, we also tested CEPS-cyclization of the 14-mer peptide 3_2233 (H-SYCQG[Aha]KSA[Aha]KFGCK-Cam-L-OH) followed by T4_1 and T4_2 CLIPS/CuAAC-cyclizations, resulting in clean formation of the tetracyclic peptides III_2233-T4_1 and III_2233-T4_2. Similarly, “triple-C” locking of peptides with longer loop sequences (4_4444 (H-ILCQWGA[Aha]KASE[Aha]FSKVCPK-Cam-L-OH), and 5_5555 (H-ILCQKGAT[Aha]KASEK[Aha]-NHSDKVCPK-Cam-L-OH) resulted in the clean formation of tetracycles IV_4444-T4_1/2 and V_5555-T4_1/2 (T4_1: Figure 2B/C; T4_2). Only tiny amounts of unreacted (8%) and S-S oxidized (13%) 5_5555 were observed during the synthesis of V_5555-T4_1 (R_t = 0.77 and 0.78 min respectively, Figure 2C). Linear peptide 6_3454 (H-ILCQWA[Aha]KASE[Aha]DFSVKCPK-Cam-L-OH), with very different sizes of the loop sequences, was also successfully CEPS-cyclized to the monocyclic peptide V6_3454 within 30 min (isolated yield: 54%). Subsequent CLIPS/CuAAC reactions with T4_1 and T4_2 also yielded the tetracyclic peptides VI_3454-T4_1 and VI_3454-T4_2 in 64% and 99% isolated yield, respectively.
One-Pot “Triple-C” Multicyclization for the Synthesis of Tetracyclic Peptides

Figure 2. Analysis of the CEPS/CLIPS/CuAAC multicyclization reactions of peptides 2333 (A), 4444 (B), 5555 (C), and 6666 (D) with T4, carried out in either a two- (A/B/C) or one-pot (D) procedure with or without isolation of the monocyclic CEPS-intermediates (ii); UPLC-spectra of i) linear Cam-ester peptide; ii) backbone-cyclized peptide reaction samples; iii) T4-CLIPS reaction samples; iv) CuAAC reaction samples with formation of the desired tetracyclic peptides.

So far, the “triple-C” peptide locking was performed in a two-pot process, with HPLC-purification of the monocyclic intermediates. Interestingly, we observed that this multistep synthesis ran with similar efficiency when the reactions were carried out in one pot, as evident from the one-pot synthesis of VI3454-T4 in <1 hour (Figure 2D), giving tetracycles VI3454-T41 (Figure 2D) and VI3454-T42 in excellent isolated yields (47% and 50%, respectively). Many other T41 and T42 tetracycles were also synthesized successfully via the one-pot procedure, albeit the product purities (after CuAAC) were slightly lower as compared to the two-pot procedure. All natural amino acids except methionine (M) and arginine (R) were successfully incorporated (R was successfully incorporated in FF-T4x, vide infra), indicating the versatility and the wide scope of Triple-C locking.

Subsequently, we tested the methodology in a reversed order, i.e. first CLIPS/CuAAC followed by CEPS. Even though ligation-cyclization of the linear Cam-esters with T4x scaffolds via CLIPS/CuAAC was successful in all cases, the follow-up CEPS cyclization turned out to be much less efficient and mainly led to hydrolysis of the C-terminal ester moiety. We attribute this to the fact that the cyclic constraint present in the tricyclic CLIPS/CuAAC-intermediates strongly hampers efficient substrate recognition by omniligase-1, which unambiguously proves that CEPS-cyclization followed by intramolecular CLIPS/CuAAC-cyclization is clearly preferred.

In order to ensure most efficient CEPS-cyclization, our initial design of the linear Cam-ester peptides avoided the occurrence of the non-natural amino acid Aha within the binding pockets of omniligase-1. However, contrary to our expectation, we observed that Aha is well tolerated in all substrate recognition sites (S4-S1 and S1’-S2’, Figure 4), thereby significantly extending the options for connecting the peptide macrocycle.
Triggered by this, we then attempted the synthesis of potentially bioactive tetracycles via “triple-C” locking. Heinis et al. previously reported a set of bicyclic peptide inhibitors (e.g. UK18 and FXII618) against uPA and coagulation factor XIIa (αFXIIa) based on ligation-cyclizations using either 1,3,5-tris(bromomethyl)benzene (TBMB) or 1,3,5-triacyloyl-1,3,5-triazinane (TATA) as a scaffold. Inspired by this, we designed a series of homo- and hetero-bifunctional tetracycles by fusing the bioactive backbone sequences of UK18 and FXII618 into regions A or B of the T4x-tetracycle scaffold (Figure 3A). For example, the sequences of FXII618 or UK18 were duplicated in case of tetracycles FF-T4x and UU-T4x, while they were fused back-to-back in the hetero-bifunctional tetracyclic peptides UF-T4x and FU-T4x (Figure 3A). We also synthesized tetracycles containing either the UK18 or FXII618 sequence (in region A and B) together with a non-interactive (SG)n linker (UAn and FBn with n = 2 and 3, respectively). The sixteen peptide constructs designed in this manner were successfully synthesized via CEPS-cyclization using omniligase-1, followed by CLIPS/ CuAAC reactions with both T41 and T42 scaffolds. Detailed 1D- and 2D NMR analysis of FF-T4x confirmed the correct structural topology (see SI) and revealed that the construct is mainly present in one major conformation (s1a, 64% of total population, Figure 3B) in equilibrium (on a millisecond time-scale) with a minor conformer s1b (14%), wherein the linker arms of the scaffold are positioned differently with respect to each other. The presence of two different isomers is likely related to hindered rotation around the scaffold’s tertiary amide bond.

Table 1. IC50 values (µM) of the sixteen different tetracyclic constructs evaluated against uPA and αFXIIa.

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<th>FXIIa</th>
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<td></td>
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<td>&gt;10</td>
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All sixteen tetracycles were evaluated for their inhibitory activity against uPA and αFXIIa (Table 1). Thereby, the bicyclic lead inhibitors UK18 and FXII618, both cyclized on TBMB, served as controls. Cross-reactivity of FF-T4x against uPA, and similarly of UU-T4x against αFXIIa were not observed, indicating that all activities observed were target-specific and sequence-dependent. First, we evaluated the UU-T4x tetracycles against uPA. Double incorporation of the UK18 inhibitor sequence onto the T4-scaffolds resulted in an eight- and six-fold decrease in activity (Table 1; IC50: 2.4 µM (UU-T41) and 1.9 µM (IC50 UU-T42)) as compared to the bicyclic control UK18 (303 nM). The T42 scaffold proved to be slightly favorable over the more flexible T41. The monomeric reference constructs Ux-T4x, containing only a single copy of the UK18 sequence in region A (loops 1/2), combined with two (SG)3 linkers in region B (loops 3/4), maintained activities (2.4 µM (UX-T41) and 2.6 µM (UX-T42)). Interestingly, incorporation of the UK18 sequence in region B (combined with (SG)3 linkers in region A) led to tetracycles with significantly decreased activities (IC50-values >10 µM for both UX-T4 and UX-T4). We hypothesize that the elongated CuAAC linkages (region B) effectuate a sub-optimal conformation that disfavors binding to uPA. Interestingly, evaluation of the FF-T4x tetracycles against αFXIIa
revealed that the inhibitory activities of both tetracycles FF-T41 (IC50: 960 nM) and FF-T42 (IC50: 540 nM) were even slightly higher than the TBMB-cyclized bicyclic control (IC50: 2 µM), implying that the use of bivalent tetracyclic peptides over monovalent bicycles may be advantageous in this particular case. The slightly lower IC50 value observed for FF-T42 suggests that the T42 scaffold better accommodates the FXII618 loops for binding. Surprisingly, the two tetracycles Fκ-T4x and Fπ-T4x, containing only a single copy of the FXII618 sequence with two (SG)2 linkers both showed much lower activities (IC50: 8.1 µM and >10 µM for Fκ-T41/2; >10 µM and 9.5 µM for Fπ-T41/2). In contrast to the bicyclic inhibitor FXII618, both Fκ and Fπ do not contain the N- and C-terminal Arg, which have shown to increase binding affinity against its target[24] and this omission could potentially account for the loss in activity of these tetracycles. However, as the double substituted tetracyle FF replicates the N-terminal Arg (the target sequence ends with Arg), we hypothesize that both FXII618-copies in FF-T41 mutually increase binding.

Figure 3. A) Schematic structures of the most important tetracyclic constructs evaluated against uPA and FXIIa, illustrating the different regions A (loops 1&2) and B (loops 3&4); B) Energy-minimized model of the major conformer s12 of FF-T41; Bar charts of IC50's of selected tetracyclic inhibitors against αFXIIa (C) and uPA (D).

When combining the UK18 and FXII618 sequence into the hetero-bifunctional tetracyclic peptide UF and FU, we observed decent activities against both uPA and FXIIa (Figure 3C/D). UF-T41 showed almost equal activity against uPA (IC50: 1.6 µM) as compared to UU-T41, while the same construct showed much lower activity against FXIIa (IC50: 20 µM). To our surprise, the opposite trend in activity was observed for the T42-scaffold, i.e. decent activity for UF-T42 against FXIIa (IC50: 4.9 µM), while much weaker activity for UF-T42 against uPA (16 µM, Figure 3C/D). In addition, tetracycles with an inversed arrangement of sequences, i.e.
FU-T4, showed much decreased inhibitory activity against both FXIIa and uPA, independent of the choice of scaffold (see Table 1).

The observed activities for the bifunctional tetracycles were somewhat lower as compared to their bicyclic controls (UK18 and FXII618). Furthermore, the reported TATA-cyclized FXII618 bicyclic lead exhibits much higher activity than the corresponding TBMB-variant (observed IC$_{50}$ values: 140 nM and 2 µM, respectively). However, we chose the latter for comparison, since the TBMB-scaffold is structurally more similar to T4.[23] Nevertheless, the results unambiguously demonstrate the potential of “triple-C” locking for the synthesis of (b)functional tetracyclic peptides as a promising class of prospective drug leads. Furthermore, it is fair to assume that additional structural optimization of these bifunctional tetracycles will likely result in better inhibitors with further improved potencies against the aforementioned targets, potentially even containing non-natural or D-amino acids.

**Conclusion**

In conclusion, we have established a straightforward one-pot methodology for the clean and rapid generation of structurally diverse tetracyclic peptides in aqueous solution. The potential and broad applicability of the methodology is underlined by the generation of bifunctional tetracyclic peptides, displaying micromolar activities against the non-homologous enzymes FXIIa and uPA. Hence, we believe that this novel technology further enriches the toolbox for engineering multicyclic peptides and greatly boosts the development of future multicyclic peptide therapeutics.
Experimental Section

Associated content

The Supporting Information of this article is available free of charge on the ChemBioChem publications website at DOI: 10.1002/cbic.201800346.

General

Amino acids are indicated by single-letter codes; Unnatural amino acid azidohomoalanine is abbreviated as [Aha], carboxamidomethyl is abbreviated as Cam. Fmoc-azidohomoalanine-OH (Fmoc-Aha-OH) was synthesized according to a literature procedure described by Spring et al. in 2011.\textsuperscript{[25]} Progress of the CEPS cyclizations and CLIPS/CuAAC ligation-cyclizations was monitored using UPLC-ESI-MS (method: 2 min, 5-55% B or 3 min, 5-80% B) Acquity UPLC Peptide BEH C18 Column, 130Å, 1.7 µm, 2.1 x 50 mm with UV detection (λ = 215 nm) and positive ion current for MS analysis, unless stated otherwise.

Solid-Phase Peptide Synthesis of C-terminal Cam-Ester Peptides

Fmoc-L-Wang resin (0.2 mmol; 0.3 mmol/g loading) was washed with dichloromethane (DCM; 3x 2 min) and dimethylformamide (DMF; 3x 2 min) and Fmoc-deprotected using piperidine/DMF (20% (v/v), 2x 8 min). Per g of resin 10 mL of solvent was used standardly. After washing with DMF (6x 2 min), the corresponding Fmoc-AA-glycolic acid (2 eq.) was coupled to the resin using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; 4 eq.), Oxyma Pure (4 eq.) and N,N-diisopropylethylamine (DIPEA; 10 eq.) in DMF for 45 min. Fmoc-AA-glycolic acid was prepared according to Nuijens et al.\textsuperscript{[21]} After washing with DMF and Fmoc deprotection the next amino acid Fmoc-AA-OH was coupled using DIC (4 eq.) and Oxyma Pure (4 eq.) in DMF (45 min). After the final Fmoc-deprotection, the resin was washed with DCM and dried in a stream of nitrogen gas. Cleavage from the resin and side-chain deprotection was performed using a mixture of 87.5/5/2.5/2.5/2.5 (v/v/w/v/v) trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/dithiothreitol (DTT)/thioanisole/water for 120 min. The crude peptide was precipitated using methyl tert-butyl ether (MTBE)/n-heptane (1/1, v/v). The precipitated peptide was collected by centrifugation and washed three times with cold MTBE/n-heptane (1:1, v/v) followed by drying under reduced pressure. The crude peptide Cam-esters were purified by preparative HPLC (eluent-A: MilliQ-H₂O containing 0.05% (v/v) TFA; eluent-B: ACN containing 0.05% (v/v) TFA) and pure fractions were lyophilized.
Synthesis of peptide amides (P1'-P2' [Aha] test):

Peptide amides were synthesized using a Rink amide resin (0.65 mmol/g loading). After washing the resin with DMF (3x 2 min) and Fmoc deprotection using piperidine/DMF (20% (v/v), 2x 8 min), the amino acids Fmoc-AA-OH were coupled using DIC (4 eq.) and Oxyma Pure (4 eq.) in DMF (45 min). Per g of resin 10 mL of solvent was used standardly. After final Fmoc-deprotection, the resin was washed with DCM and dried in in a stream of nitrogen. Cleavage from the resin and side-chain deprotection was performed using a mixture of 87.5/5/2.5/2.5/2.5 (v/v/w/v/v) trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/dithiothreitol (DTT)/thioanisole/water for 120 min. The crude peptide was precipitated using methyl tert-butyl ether (MTBE)/n-heptane (1:1, v/v). The precipitated peptide was collected by centrifugation and washed three times with MTBE/n-heptane (1:1, v/v) followed by drying under reduced pressure. The crude peptide Cam-esters were purified by preparative HPLC and pure fractions were lyophilized.

Table 2. Codes, sequences and MWs (calculated and experimental; g/mol) of synthesized Cam esters.

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<th>Sequence</th>
<th>MW_{calc}</th>
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<tr>
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One-Pot “Triple-C” Multicyclization for the Synthesis of Tetracyclic Peptides

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**Triple-C (CEPS, CLIPS, CuAAC) reaction procedures (two-pot)**

**CEPS:**
Linear Cam-L--ester peptides were dissolved in potassium phosphate buffer solution (250 mM, pH = 8.5) to a concentration of 0.3 mM, followed by addition of omniligase-1 (0.5 µM). The reaction was followed by UPLC-MS. After completion of the reaction (usually after 30-60 min) the reaction mixture was purified via RP-HPLC. If the linear precursor peptides exhibited low solubility, guanidinium hydrochloride was added to a concentration of 1 M.

**CLIPS/CuAAC:**
Backbone cyclized peptide (1 eq.) was dissolved in DMF/H₂O (1:1) to a concentration of 0.5 mM, followed by addition of the corresponding T₄ scaffold (0.8 eq., from a 10 mM stock solution in DMF; scaffolds were synthesized as previously described[20]). The pH was adjusted to 8 with aqueous NH₄HCO₃-solution (200 mM). After complete consumption of the monocyclic peptide, a mix of CuSO₄/THPTA/sodium ascorbate was added (2 eq., ratio 1/1/5 (v/v/v), from a 100 mM stock solution in H₂O). For analysis, the reaction was quenched by adding aq. EDTA-solution (5 eq., 0.1 M)
General one-pot procedure

Linear Cam-ester (1 eq.) was dissolved in phosphate buffer/H₂O (4:1, 0.4 mM, pH = 8), followed by addition of omniligase-1 (0.5 µM final concentration). After completion of the reaction, DMF (final ratio H₂O/DMF = 2:1 (v/v)) and T4 scaffold (0.8 eq., from a 10 mM stock solution in DMF; scaffolds were synthesized as previously described[20]) were added. After complete consumption of the backbone-cyclized peptide, a mix of CuSO₄/THPTA/sodium ascorbate was added (2 eq., ratio 1/1/5 (v/v/v), from a 100 mM stock solution in H₂O). The reaction was quenched by adding aq. EDTA-solution (5 eq., 0.1 M) and directly purified on RP-HPLC.

Residual Fluorescence Assay

Biological activities against coagulation factor αFXIIa (HFXIIa, Molecular Innovations, MW = 80000 Da), and uPA (UPA-LMW, Molecular Innovations, MW = 33000 Da) were measured as IC₅₀ values via residual fluorescence polarization assays, which were carried out as previously described by Heinis et al.[22,23] Enzyme of choice (final concentrations for uPA = 5 nM, αFXIIa = 50 nM) was incubated for 20 min with several dilutions of tetracyclic or bicyclic peptide (7 dilutions with final concentrations ranging from 50 µM to 12 nM). Desired concentrations were achieved by dilutions in buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM CaCl₂, 0.1% (w/v) bovine serum albumin (BSA), 0.01% (v/v) Triton-X100). After 15 min incubation, substrate Z-Gly-Gly-Arg-AMC (final concentration of substrate = 100 µM) was added to obtain a final volume of 150 µL. Substrate was initially dissolved as a 2 mM DMSO stock solution, which was further diluted with buffer to 300 µM. Residual fluorescence measurements (excitation 355 nm, emission 460 nm) was read on a Spectramax M2 (Molecular Devices) plate reader. IC₅₀ values were calculated (based on duplo experiments) via non-linear regression using GraphPad Prism. Known bicyclic inhibitor UK18[22] and FXII618 (on TATA scaffold)[23] and TBMB variant were synthesized and used for comparison of activities.

Acceptance of [Aha] in the omniligase-1 binding pocket: model studies

Reaction procedure:

Peptides bearing an [Aha] residue in either of the positions P4-P2’ were synthesized according to the procedures described earlier. For a list of sequences including their respective molecular weights see Table 2.

For testing the acceptance of each respective substrate by omniligase-1 the acyl donor fragment (final conc.: 0.4 mM) and the acyl acceptor fragment (final concentration: 2 mM, 5 eq.) were dissolved in 0.5 mL 1 M
potassium phosphate buffer (pH 8.5) and omniligase-1 was added to a concentration of 0.14 nM. After 0 and 30 min 50 µL of reaction mixture were quenched in 450 µL of quenching solution (acetonitrile/water 3:1 (v/v) + 0.5% (v/v) methanesulfonic acid). Samples were analyzed using HPLC-MS. HPLC yields were calculated based on integration of the product peak and the remaining peak area of the acyl donor fragment. Analytical HPLC was performed on an Agilent 1260 liquid chromatography system using a reversed-phase column (Phenomenex Luna C18, 10 µm particle size, 250 × 4.6 mm) at 40°C, coupled with an Agilent 6130 quadrupole LC/MS system. UV detection was performed at 220 nm using a UV-VIS 204 linear spectrometer and peptides were identified by their mass using LC-MS. As eluents A (water+ 0.05% (v/v) MSA) and B (ACN+ 0.05% (v/v) MSA) were used.

All reactions performed are listed in Figure 4A. After a reaction time of 30 min the reactions were complete (full conversion of the acyl donor ester). In the HPLC trace the peaks of product (“synthesis”) and hydrolyzed ester (“hydrolysis”) were integrated. The results are displayed in Figure 4B.

Structure Determination of FF-T42 via NMR

NMR samples of cyclic FF-T42 were prepared as both 4.7 mM solution and 0.3 mM solution in 160 µL total volume (3 mm NMR tube) containing 25 mM NaAc-d3 buffer pH 4.7, 0.1 mM EDTA, 0.2 mM sodium azide, 6 µM DSS-d6 as chemical shift reference and 2% (v/v) D2O for deuterium lock. In addition, the 0.3 mM NMR sample was manually adjusted to pH 6.8 using small aliquots of 1N NaOH. NMR spectra (1D 1H and 13C, DIPSI 80 ms mixing time, NOESY 125ms & 350 ms mixing time, ROESY 150 ms mixing time, natural abundance 13C-1H HSQC, 13C-1H HMBC, 15N-1H HSQC, 15N-1H HMBC, DIPSI-13C-1H HSQC 70ms) of 4.7 mM FF-T42 were recorded on a Bruker Avance III HD 700 MHz spectrometer, equipped with a TCI cryoprobe. Spectra were recorded at various temperatures varying between 5°C and 51°C, in order to study
the exchange processes of FF-T4₂. Processing was done by Topspin 3.2 (Bruker, Rheinstetten) and spectral analysis as well as resonance assignment was performed using Sparky 3.115[27]. Structure generation of FF-T4₂ and NMR-restraint molecular dynamics were carried out by YASARA Structure (YASARA Biosciences, Vienna). Restraint simulated annealing during a period of 2 ns at 900 K was performed using the AMBER IPQ15 force field (NB cutoff distance 8 Angstrom) under periodic boundary conditions, after which time the system was slowly cooled down to 310 K and finally energy-minimized.
References


