Enzymatic tools for peptide ligation and cyclization

Development and applications

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CHAPTER 5

Chemo-Enzymatic Synthesis of Tricyclic Peptides

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Abstract

Constrained multicyclic peptides represent a diverse class of natural products and display an attractive class for the development of future therapeutics. However, their synthesis remains difficult and more efficient strategies for the synthesis of multi-cyclic peptide macrocycles have been a long-standing goal. Here we present a novel combination of enzyme (omniligase-1)-catalyzed peptide head-to-tail cyclization (CEPS) with another orthogonal chemical ligation strategy, namely Chemical Ligation of Peptides onto Scaffolds (CLIPS). Using a model peptide, based on the bicyclic inhibitor UK18 of the human urokinase-type plasminogen activator, we successfully demonstrated that enzymatic cyclization (CEPS) and CLIPS alkylation (using the tris-bromine scaffold TBMB) can be carried out in a one-pot procedure in less than one hour, yielding a tricyclic peptide with high efficiency. The tricyclic product fully maintained biological activity as compared to its bicyclic analogue UK18. Certainly, this new combination of CEPS and CLIPS enhances the toolbox for synthesizing and engineering new variants of tricyclic peptides.

Introduction

Constrained multicyclic peptides represent a diverse class of natural products and display a wide range of different bioactivities, which renders them an attractive format for the development of therapeutics.[1] Macrocyclic peptides have long been considered as effective agents for the inhibition of protein-protein interactions (PPI's).[2] Therefore, the modulation or inhibitions of PPI's plays an increasingly important role in drug discovery. Although multicyclic peptides represent an interesting therapeutic modality, naturally occurring macrocycles are only available for a small range of therapeutic targets. To mimic natural peptides or to create peptides with new binding modes, artificial scaffolds have been employed for the preparation of multicyclic peptides.[3] Many biologically active bicyclic peptides have been described to date and have been used for the mimicry of protein surfaces in order to address protein-protein interactions as a pharmaceutical target. For the discovery of novel bicyclic peptide leads Heinis and Winter established a workflow for the efficient generation of bicyclic peptides in a high throughput fashion using phage display.[4] Tethered to a phage, peptides containing three cysteine residues are displayed on the surface and are easily reacted with a thiol-reactive hinge to form a large variety of bicyclic peptides. Using iterative rounds of affinity screening against a particular target, several bicyclic inhibitors with nanomolar or even picomolar affinities are reported, *e.g.* the bicyclic peptide UK18, which effectively inhibits the urokinase plasminogen activator uPA.[5] Several thiol-reactive scaffolds for the use in the phase-display assisted screening of bicyclic peptides have been used such as 1,3,5-tris(bromomethyl)benzene (TBMB).[6,7] Most scaffolds are relatively small and contain a limited number of chemical groups (except reactive moieties required for tethering the scaffold to the peptide) that could perturb the overall conformation of the peptides *via* noncovalent or steric interactions. For example, in a crystal structure of UK18 with its target uPA, no noncovalent interactions between the hydrophobic TBMB mesitylene core and the peptide itself were observed.[5]

Although bicyclic peptides are already exceptionally more stable as compared to their linear counterparts, we envisioned further rigidification of CLIPS-cyclized bicyclic peptides by additional backbone cyclization, thus, resulting in the formation of tricyclic peptides with a head-to-tail cyclic backbone. In contrast, a previously described approach using a microbial transglutaminase for the preparation of tricyclic peptides generally suffered from low yields, the generation of a non-native backbone and specific sequence requirements.[8] Especially, the fact that peptiligase-mediated ligation results in the formation of a native peptide bond and does not require a specific recognition sequence encouraged us to attempt the synthesis of these even more complex peptides by combining CEPS (head-to-tail) and CLIPS (thioether) cyclization-ligation.

Results and Discussion

We used omniligase-1 for the synthesis of a constrained tricyclic peptide bound to a small molecule scaffold via three thioether bonds (Figure 1A). A 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). To investigate the possibility of a combined CEPS-CLIPS tricyclization approach we chose the potent bicyclic inhibitor UK18 as a model peptide for our studies. In order to enable efficient CEPS and CLIPS cyclization we extended the sequence of UK18 with a six amino acid spacer recognition sequence (H-SYA**C**SRYEVD**C**RGRGSA**C**GFSKL-Cam-L-OH; the *N*- and *C*- terminal linker sequences are underlined, cysteines linked to TBMB are indicated in bold). For CEPS cyclization the linear precursor peptides were dissolved in an aqueous buffer solution (500 mM potassium phosphate pH 8.5, 3.5 mM tris(2-carboxyethyl)phosphine (TCEP)). The respective reactions were started by sequential addition of omniligase-1 or the small molecule scaffold TBMB. The reaction was monitored *via* HPLC-MS. The open chain precursor UK18-ester was head-to-tail cyclized with >95% efficiency to *c*-UK18 using omniligase-1, followed by addition of TBMB to quantitatively yield the final tricyclic peptide construct *c*-UK18-TBMB (Figure 1B).

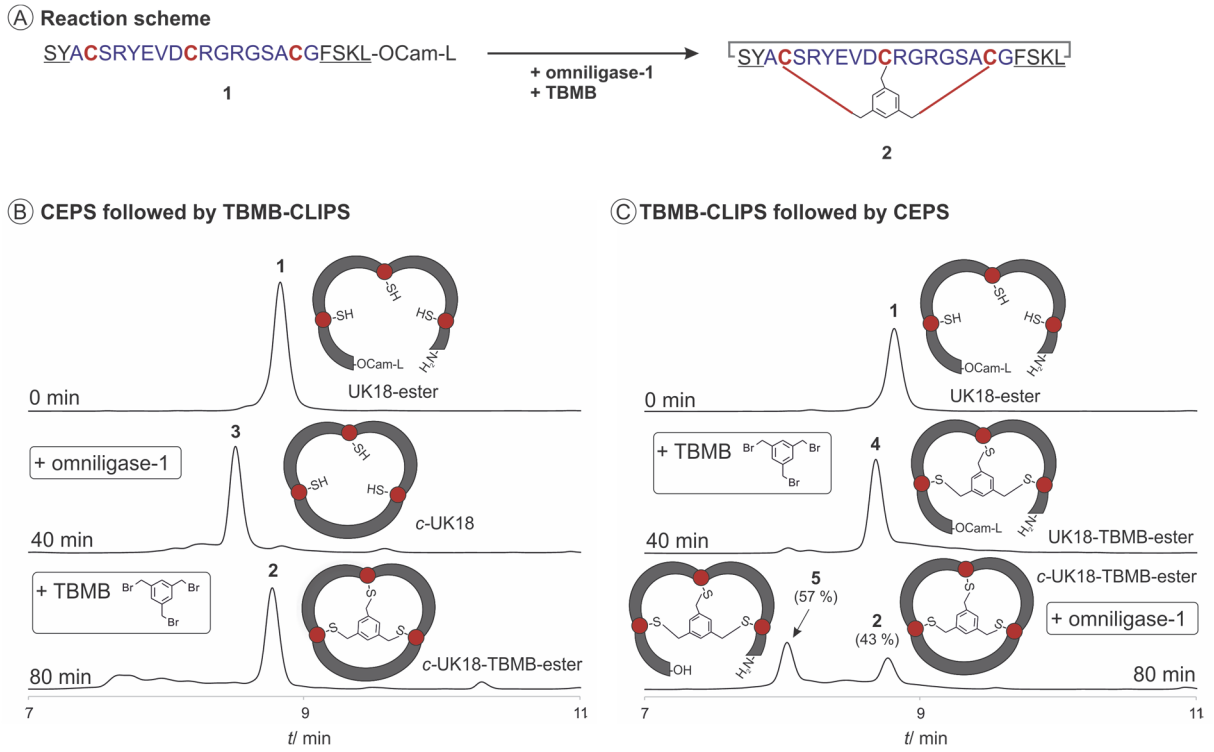

Figure 1. A) Reaction scheme for the synthesis of a tricyclic peptide based on the bicyclic inhibitor UK18 of the human urokinase-type plasminogen activator. The linker sequence is shown in black (underlined) and the sequence of UK18 in blue with the cysteines highlighted in red, respectively. The tricyclic peptide was generated *via* a combination of CEPS and CLIPS using the TBMB scaffold. B) HPLC traces of the linear precursor peptide UK18-ester. After the UK18-ester was efficiently cyclized to yield **c**-UK18, the organic small molecule scaffold (TBMB) was added directly to the reaction mixture in order to obtain the tricyclic product c-UK18-TBMB. C) HPLC traces of the linear precursor UK18-ester that was first reacted with the organic scaffold (TBMB) to yield the bicyclic precursor UK18-TBMB ester, followed by omniligase-1 mediated head-to-tail cyclization to c-UK18-TBMB (same molarities as in B).

Interestingly, the biological activity of the tricyclic variant of UK18, c-UK18-TBMB was not affected by the application of an additional constraint as compared to bicyclic UK18-TBMB (see Figure 2). Thus, we hypothesize that the bicyclic molecule is already conformationally constrained and additional backbone cyclization only poses a minor additional rigidity to the structure, therefore not resulting in a perturbation of the overall structure. However, the additional head-to-tail cyclization could potentially positively influence the overall stability of the molecule, e.g. lead to an increased protease stability. NMR analysis would allow a detailed analysis of the structure and a proper comparison with the known bicyclic UK18, but this fell beyond the scope of this proof-of-concept study.

For the reaction order CEPS followed by CLIPS neither hydrolysis of the ester moiety nor formation of multimeric species were observed. Thus, with CEPS followed by the CLIPS reaction, the final tricyclic product c-UK18-TBMB was obtained in almost quantitative HPLC peak to peak conversion. Reversely, the CLIPS reaction followed by CEPS (Figure 1C), resulted in only 43% conversion (HPLC) to the final tricyclic product c-UK18-TBMB and 57% ester hydrolysis byproduct. The CLIPS reaction proceeded efficiently to yield intermediate UK18-TBMB ester, 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,[7] such that the flexibility of the termini to be ligated by omniligase-1 is restricted, resulting (in this particular case) 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 1B) is preferred as a generally applicable strategy.

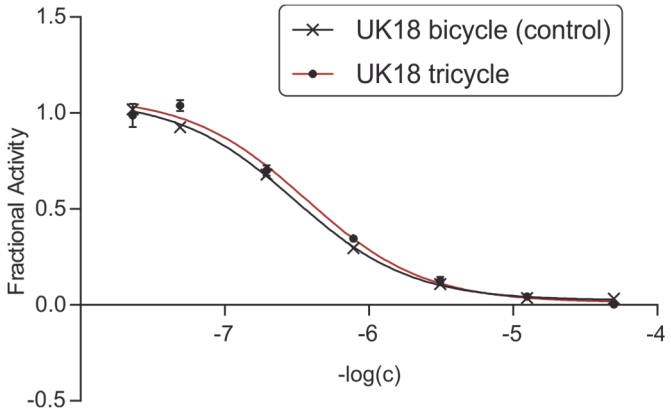

Figure 2. Inhibition curves of bicyclic and tricyclic UK18. Fractional activity is against log(c).

Conclusion

In summary, we have devised a new route for the efficient preparation of tricyclic peptides by a combination of omniligase-1-mediated backbone cyclization (CEPS) and cysteine alkylation using small molecule scaffolds (CLIPS). Using linear *C*-terminal Cam-ester peptides omniligase-1 catalyzed head-to-tail cyclization followed by CLIPS alkylation rapidly resulted in the preparation of tricyclic peptides in a one-pot fashion. Interestingly, the tricyclic construct, based on the TBMB bicyclic urokinase plasminogen activator inhibitor UK18, retained comparable inhibitory activity as compared to its bicyclic, non-head-to-tail cyclic version. This robust strategy clearly represents an expansion of the methodologies available for the preparation of tricyclic peptides containing artificial scaffolds and is well expected to be compatible with many other small molecule scaffolds. In addition, the flexibility of omniligase-1 will also enable the use of different linker sequences as well as the cyclization within the peptide sequence itself, ,as shown for UK18 (see chapter 6).

Experimental Section

Associated content

The Supporting Information of this article is available free of charge on the Adv. Synth. Catal. publications website at DOI: 10.1002/adsc.201700314.

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

Fmoc-Leu-Wang resin (0.7 g, 0.49 mmol) was washed with dichloromethane (DCM; 2 x 2 min, 10 mL) and dimethylformamide (DMF; 2 x 2 min, 10 mL) and Fmoc-deprotected using piperidine/DMF (20 %, v/v, 2 x 10 min, 10 mL). After washing with DMF (6 x 2 min flow-batch wash, 10 mL), Fmoc-Leu-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 (45 min, 10 mL). Fmoc-Leu-glycolic acid was prepared according to the previously described protocol by Nuijens *et al.*.[9] After washing with DMF (6 x 2 min flow-batch wash, 10 mL) and Fmoc deprotection, the next amino acid Fmoc-AA-OH was coupled using HBTU (4 eq.), Oxyma Pure (4 eq.) and DIPEA (10 eq.) in DMF for 45 min.. After the final Fmoc-deprotection, the resin was wished with DCM and dried under nitrogen flow. Cleavage from the resin and side-chain deprotection was performed using a 95/2.5/2.5 (v/v/v) mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIS) and water (15 mL) for 120 min, followed by filtration of the resin. The crude peptide was precipitated using a mixture of cold (-20°C) 1:1 (v/v) methyl tert-butyl ether (MTBE)/n-heptane (5 mL cleavage mixture per 45 mL MTBE/ heptane), following drying under reduced pressure. The crude peptide Cam-Leu-OH esters were purified by preparative HPLC and pure fractions were lyophilized.

Peptide Cyclization and Mass Spectrometric Analysis of Products

The purified linear precursor was dissolved at a concentration of 0.15 mM (0.5 mg /mL) in 500 mM potassium phosphate buffer pH 8.5 containing 3.5 mM TCEP. Cyclization was performed using omniligase-1 (0.95 µM, 20 µg/mL). Omniligase-1 was produced and purified as described previously.[10,11] TBMB was added as a 5 mM solution in acetonitrile to a final concentration of 1 mM. The reaction was performed at room temperature and was followed at λ= 220 nm using an HPLC-MS system (Agilent 1260 Infinity coupled with an Agilent 6130 quadrupol mass spectrometer, Agilent, Santa Clara, USA). Separation was performed using a Luna RP-C18 10 µM, 4.6 x 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. As a mobile phase a binary mixture of A (water+ 0.05% (v/v) MSA) and B (acetonitrile + 0.05% (v/v) MSA) was used. An

appropriate gradient was chosen for each sample individually. The conversion rates of the enzymatic cyclization were calculated by automatically integrating the area of the corresponding peaks in the HPLC spectrum (λ= 220 nm).

Table 1. Calculated and experimental MW (in Da) of linear precursors as well as final tricyclic products.

Code	Sequence	MW_{calc}	MW_{exp}
UK18 tricycle	c[SYA**C**SRYEVD**C**RGRGSA**C**GFSKL]-**TBMB**	2610.2	2610.9
UK18 tricycle ester precursor	H-SYACSRYEVDCRGRGSACGFSKL-Cam-L-OH	2685.2	2685.0

Residual Fluorescence Assay

Biological activities against uPA (UPA-LMW, Molecular Innovations, MW = 33000 Da) were *via* residual fluorescence polarization assays, which were carried out as previously described by Heinis et al.[5,12] uPA (5 nM, 50 µL) was incubated for 20 min with several dilutions of tricyclic c-UK18-TBMB or bicyclic UK18 control peptide (7 dilutions with final concentrations ranging from 50 µM to 12 nM, 50 µL each). Desired concentrations were achieved by dilution of the peptide (1 mM in 50% (v/v) ACN) in buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% (w/v) bovine serum albumin (BSA), 0.01% (v/v) Triton-X100). After an incubation time of 15 min, 50 µL substrate Z-Gly-Gly-Arg-AMC (final concentration of substrate= 100 µM; AMC= 4-methylcoumaryl-7-amide) was added to obtain a final volume of 150 µL. The 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. IC_{50} values were calculated (based on duplo experiments) *via* non-linear regression using GraphPad Prism. Known bicyclic inhibitor UK18[5] and FXII618 (on TATA scaffold)[12] and TBMB variant were synthesized and used for comparison of activities

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