Enzymatic tools for peptide ligation and cyclization

Development and applications

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CHAPTER 7

Synthesis of Constrained Tetracyclic Peptides by Consecutive CEPS, CLIPS and Oxime Ligation

This work has been published as


§ authors contributed equally to this work.
Abstract

In nature, multicyclic peptides constitute a versatile molecule class with a variety of biological functions. To exploit multicyclic peptides as pharmaceuticals, the development of chemical methodologies to enable selective consecutive macrocyclizations is required. Here we disclose a combination of enzymatic macrocyclization, CLIPS alkylation and oxime ligation for the preparation of tetracyclic peptides. Five newly designed small molecular scaffolds and differently sized model peptides featuring non-canonical amino acids were synthesized. Enzymatic macrocyclization, followed by one-pot scaffold-assisted cyclizations yielded 21 tetracyclic peptides in a facile and robust manner.
Synthesis of Constrained Tetracyclic Peptides by Consecutive CEPS, CLIPS and Oxime Ligation

Introduction

Peptides are nature’s most diverse toolkit and fulfill a plethora of functions, ranging from hormonal to antimicrobial activities.\(^{[1]}\) In the past decade, especially cyclic peptides have attracted increased attention as a highly promising class of therapeutics.\(^{[2-4]}\) Key features of macrocyclic peptides include increased metabolic stability and improved binding affinity compared to linear molecules.\(^{[5]}\) The set of macrocycles in nature ranges from small monocyclic to highly constrained multicyclic peptides such as cyclotides\(^{[6-7]}\) or the ‘last-resort’ antibiotic vancomycin, which is a prime example of a multicyclic peptide drug.\(^{[8,9]}\) While nature produces these complex, highly constrained, multicyclic compounds in a relatively straightforward manner using cascades of enzyme-catalyzed reactions, their chemical synthesis is often elaborate resulting in low overall yields.\(^{[10]}\) Clearly, there is a need to overcome these challenges by the development of novel synthetic methodologies. Thanks to their inherent properties, such as excellent regio- and stereoselectivity, the use of enzymes has recently gained increased attention as a tool for peptide head-to-tail cyclization. Enzymes such as omniligase-1\(^{[11,12]}\), butelase\(^{[13]}\) or sortase\(^{[14]}\) have been successfully employed for this purpose.\(^{[15]}\) For example, omniligase-1 efficiently catalyzes head-to-tail cyclization of linear C-terminal glycolate-ester peptides in aqueous solution.\(^{[11,12,16]}\) Additionally, peptide cyclization using small molecule organic scaffolds has been widely explored. For example, CLIPS technology (Chemical Linkage of Peptides onto Scaffolds)\(^{[17]}\) is used for peptide cyclization \textit{in vitro} and for the generation of large phage-displayed libraries of thioether bridged bicyclic peptides.\(^{[17-19]}\) However, despite its ease, the applicability is limited to the preparation of mono- and bicyclic peptides.

Recently, our group introduced a novel concept to expand the CLIPS-technology to furnish tri- and tetracyclic peptides via a one-pot procedure, by combining it with two orthogonal ligation methods; Cu(I)-catalyzed Alkyne-Azide Cycloaddition (CuAAC) and enzymatic head-to-tail cyclization using omniligase-1 (chemo-enzymatic peptide synthesis, CEPS).\(^{[11,12]}\) Here, we present a successful expansion of the set, utilizing oxime ligation. This well-established orthogonal ligation method involves the condensation reaction between an aminoxy group and a carbonyl electrophile.\(^{[20-23]}\) In contrast to CuAAC, the formed oxime bond shows E/Z isomerism, which is influenced by their substituents.\(^{[24]}\)

Results and Discussion

To explore the combination of CEPS, CLIPS and oxime ligation, a novel type of small-molecule scaffold was developed, comprising two reactive primary bromides (CLIPS) in combination with either a) an aldehyde or b) an aminoxy. Both the alkoxyamine and aldehyde were chemically protected (with Boc and diethyl-acetal groups) and are only liberated after the initial CLIPS reaction to ensure a controlled, regioselective cyclization. CEPS cyclization followed by CLIPS and subsequently oxime ligation was envisaged the most straightforward
approach based on previous experience.\cite{12} For oxime ligation, the peptide contains either a) the aminooxy or b) the ketone moiety. Therefore, we set out to investigate two different strategies (Figure 1). For strategy I, the peptide bears the aminooxy functionality, with the scaffold (T4-2(C=O)/T4-3(C=O)) bearing the aldehyde, (Figure 1A) while strategy II features a reversed orientation of the aminooxy and ketone moieties (T4-1(ONH2)/T4-2(ONH2)/T4-3(ONH2)) (Figure 1B). The set of scaffolds we studied comprises the rigid T4-1(ONH2)\cite{25} and the flexible scaffolds T4-2(ONH2)/T4-2(C=O) and T4-3(C=O)/T4-3(ONH2), all containing rotatable amide bonds in combination with a bromoacetamide.

In strategy I, the aminooxy residue is introduced in the form of aminooxy-homoserine (hS(ONH2))\cite{24}, whereas in strategy II, two different keto-amino acids, namely para-acetyl phenylalanine (F(C=O)\cite{26,27} and a tert-butyl ketone derivative of aspartic acid (D(C=O)), were incorporated into the peptide, respectively. In strategy I, (Figure 1A), scaffold aldehydes are used, presumably resulting in E-configured oximes only. In contrast, strategy II (Figure 1B) starts from the keto-containing amino acids F(C=O) and D(C=O), hence a mixture of E/Z-oximes may be expected, with an increased preference for the E-isomer (D(C=O) > F(C=O)) due to the steric hindrance of the tBu-group.

In order to thoroughly investigate the combination of CEPS, CLIPS and oxime ligation for the preparation of tetracyclic peptides, a library of eight peptides containing different number of amino acids (n) between the CLIPS and oxime junction points (n=3,4,5) was designed (Figure 1C), and the protected aminooxy/ketone amino acids, was performed using classical automated SPPS. In general, the ketone-containing peptides were easier to obtain (little to no side reactions) than peptides containing amino acid hS(ONH2), of which the latter often resulted in a low yielding synthesis and troublesome purifications. Common side-reactions were isopropylidene formation of the aminooxy functionality with traces of acetone, incomplete SPPS-coupling of Fmoc-hS(ONHBoc)-OH and elimination of the aminooxy moiety.\cite{28} Peptides containing D(C=O) exhibited low solubility in aqueous solutions, potentially requiring the addition of solubilizing agents (e.g. urea) for efficient enzymatic cyclization. Nevertheless, all linear peptide Cam-esters (n=3,4,5) were efficiently head-to-tail cyclized using omniligase-1 (see SI). Initially, we explicitly avoided to place one of the oxime-reactive non-canonical amino acids, hS(ONH2), F(C=O), D(C=O), in the enzymatic recognition sequence of the peptide (N-terminal P1’ and P2’ and C-terminal P4-P1)\cite{29} in order to ensure a high cyclization efficiency. However, it turned out that all three amino acids were well tolerated at the majority of positions (F(C=O) = all pockets; hS(ONH2)= P3, P2, P1’, P2’, D(C=O) = P3, P2; see experimental section: Figure 6) despite distinct differences in reaction rate that could in turn be compensated for by adding an increased amount of biocatalyst. In general, these results demonstrate the broad applicability and compatibility of the CEPS cyclization, even when using non-canonical amino acids.
Figure 1. Schematic representation of the CEPS/CLIPS/oxime cyclizations and the evaluated scaffold and peptides. A) Aminooxy-bearing peptides with scaffolds T4-2(C=O)/T4-3(C=O) (strategy I). B) Peptides comprising ketone-functionalized amino acids in combination with scaffolds T4-1(ONH₂)/T4-2(ONH₂)/T4-3(ONH₂) (strategy II), for the synthesis of tetracyclic peptides. C) Peptide codes and the corresponding sequences.

Peptide sequences

1333-3hS(ONH₂): H-CYKQDhS(ONH₂)SihS(ONH₂)AKGCSKL-O-Cam-L-OH
2333-F(C=O): H-CYKQDF(C=O)SIF(C=O)AKGCSKL-O-Cam-L-OH
3333-D(C=O): H-CYKQDd(C=O)SIKD(C=O)AKGCSKL-O-Cam-L-OH
4333-hS(ONH₂): H-RhS(ONH₂)FRFLPCRQLRCRFLPhS(ONH₂)RQL-O-Cam-L-OH
5444-F(C=O): H-RF(C=O)FRFLPCRQLRCRFLPhS(ONH₂)RQL-O-Cam-L-OH
66555-hS(ONH₂): H-CYKQGQhS(ONH₂)SikSAShS(ONH₂)AKVRGCKFSKL-O-Cam-L-OH
76555-F(C=O): H-CYKQDF(C=O)SikASPhS(ONH₂)AKVRGCKFSKL-O-Cam-L-OH
86555-D(C=O): H-CYKQDD(C=O)SikASD( ONH₂)AKVRGCKFSKL-O-Cam-L-OH
Next, we explored the CLIPS and oxime ligation reactions using the scaffolds given in Figure 1. Generally, CLIPS reactions with the monocyclic peptides proceeded cleanly under standard reaction conditions (aqueous NH₄HCO₃ solution (pH>8.0), within 20 min at room temperature) to give the corresponding bicyclic products. This was followed by deprotection of the scaffolds for oxime ligation. For strategy I, the acetals in T4-2(C=O)/T4-3(C=O) were hydrolyzed by the addition of a 15% (v/v) aqueous TFA solution. Under these conditions, oxime ligation occurs instantaneously. For strategy II, Boc-removal to liberate the aminooxy moiety of T4-1(ONH₂)/T4-2(ONH₂)/T4-3(ONH₂) was carried out after lyophilization of the CLIPSed bicycle, by the addition of trifluoroacetic acid in dichloromethane (2:1, v/v). The peptide was dried in vacuo before oxime ligation was instigated in aqueous DMSO (0.5 mM, 16 hours at 40°C).

Following these generalized procedures, we first investigated the reaction of hS(ONH₂)-containing monocyclic hexadecapeptides with scaffolds T4-2(C=O) and T4-3(C=O) (Strategy I, Figure 1A). The CLIPS reactions, yielding the bicyclic peptides, went smoothly. For the oxime ligation, these peptide-scaffold combinations are the least hindered as it contains the aldehyde, which, in principle should solely yield the E-isomer upon oxime ligation. Both tetracycles c13333-hS(ONH₂)⦁T4-3(C=O) gio and c13333-hS(ONH₂)⦁T4-2(C=O) gio were formed as a mixture of two different products (tᵢ = 0.82/0.83 min. and tᵢ = 0.70/0.72 min; see Figure 2) with identical molecular weights. This can likely be attributed to hindered rotation around either the aryl-C(=O) or the HN-C=O bond in the scaffolds as a result of the relatively small peptide ring-size (Figure 2A and D), matching earlier observations. Separation of the products was not attempted, and deemed impossible as the isomerism is considered conformational, rather than configurational, assuming both products are in thermodynamic equilibrium. This assumption was confirmed by the fact that for c13333-hS(ONH₂)⦁T4-3(C=O) gio, a third isomer was initially observed at tᵢ = 0.73 min., that disappeared over-night at room temperature.

Interestingly, an increased loop size (n=4) in the cyclic 20-peptide led to the formation of two broad product peaks for the tetracyclic constructs c44444-hS(ONH₂)⦁T4-2(C=O) gio and c44444-hS(ONH₂)⦁T4-3(C=O) gio (Figure 2B and E). We attribute this peak broadening to slow equilibration between several conformers on UPLC timescale, en route to coalescence, while being comparatively faster than the c13333-hS(ONH₂) tetracycles. Finally, for the largest 24-membered peptides c65555-hS(ONH₂)⦁T4-2(C=O) gio and c65555-hS(ONH₂)⦁T4-3(C=O) gio the UPLC clearly shows a single product (Figure 2C and F), confirming that conformational isomers in these macrocycles are in rapid equilibrium at the UPLC-timescale. This is in line with previous findings that the number of product isomers obtained is linearly correlated to the difference in peptide lengths and attributable to conformational/rotational rather than configurational/structural isomers.
Figure 2. UPLCs of Strategy I oxime ligations with hS(ONH₂) containing bicyclic peptides (n=3,4,5) with scaffolds T₄₋₃(C=O) (A-C) and T₄₋₂(C=O) (D-F). Peak masses: M₁ = first encountered peak (tetracycle); M₂ etc. = peaks with longer tR.

Because oxime formation within the systems studied for strategy II (Figure 1B) starts from ketones, E/Z mixtures may arise. Although intermolecular oxime formation between linear peptides containing two F(C=O) residues with methoxyamine or benzylhydroxylamine gave single products only, the prediction of the precise E/Z pattern of more constrained constructs remains challenging. Interestingly, in contrast to strategy I, not the peptide length appeared to be critical for the isomer distribution, but the type of scaffold, either T₄₋₁(ONH₂), T₄₋₂(ONH₂) or T₄₋₃(ONH₂) (Figure 3).

Using scaffold T₄₋₁(ONH₂) resulted in the formation of four products for all peptide lengths (Figure 3a-c). This was innate to the scaffold, since the quaternary ammonium center is prochiral. Upon peptide ligation in an unfavorable manner, there is no rotational relaxation of the system, yielding two isomers, which do not exchange within a time frame of one month. When using scaffold T₄₋₂(ONH₂) we did not observe thermodynamic equilibration but obtained the product with two to four different isomers, depending on the specific peptide used (Figure 3D-F). For example, in case of c₅₅₅₅₅₅F(C=O)T₄₋₂(ONH₂)c/o (Figure 3E) two distinct products were formed, that proved thermodynamically stable and did not interconvert upon separation of both peaks. In contrast, at elevated temperatures (40°C) c₂₃₃₃₃₃F(C=O)T₄₋₃(ONH₂)c/o (Figure 3G) forms one thermodynamic product from initially four isomers. For c₅₅₅₅₅₅F(C=O)T₄₋₃(ONH₂)c/o, however, very close running products were obtained and could only be separated using a slow eluting UPLC gradient (Figure 3H). Last, but not least, c₇₅₅₅₅₅F(C=O)T₄₋₃(ONH₂)c/o instantaneously yields a single product (Figure 3I), suggesting that larger cycles might also result in the formation of a single product.
Figure 3. UPLC chromatograms of strategy II oxime ligations with F(C=O) peptides and scaffolds T4-1(ONH$_2$)/ T4-2(ONH$_2$)/ T4-3(ONH$_2$) reacted for 16h at 40°C. Peak masses: M1 = first encountered peak (tetracycle); M2 etc. = peaks with longer tR.

Since the n=4 peptides were chosen based on the sequence of a previously published tetracyclic peptide\textsuperscript{19} we opted to compare the structure of c54444-AcF(C=O)$\bullet$T4-2(ONH$_2$)$^{c,b}$ with the known NMR-structure of the identical peptide fused with a T4$_2$ scaffold.[30] However, NMR studies revealed that the tetracyclic structure of c54444-AcF(C=O)$\bullet$T4-2(ONH$_2$)$^{c,b}$ is present in many conformations due to hindered rotation around the scaffold’s amide bond, as well as a slow equilibrium of the E/Z oxime bond. This prevented a detailed structure determination of the scaffold.

Similar to F(C=O), D(C=O) also contains an asymmetric ketone. However, due to the bulky tert-butyl group, it is expected that oxime ligation occurs selectively at one face of the ketone only, thus yielding single-isomeric products. Initial experiments with methoxamine revealed that the free amino acid is fairly unreactive and intermolecular reactions took weeks to complete. On the other hand, intramolecular reactions are much faster, yet slower compared to F(C=O). Since D(C=O) is very apolar and poorly reactive, only the most decisive loop lengths (c33333-D(C=O) and c85555-D(C=O)) were investigated (Figure 4). The reactivity of both peptides is quite similar and for all scaffolds (T4-1(ONH$_2$) to T4-3(ONH$_2$)) a single main product was obtained, which was especially surprising in case of scaffold T4-1(ONH$_2$) (4a-b). However, all reactions did not go to completion and mono-oximed products were still present after several weeks. It seems that only thermodynamically favorable tetracycles are formed, and unfavorable conformations of the mono-oxime do not react further. Clearly, the gain of selectivity comes to the cost of the reaction rate but is the only system with consistent single-peak results of the tetracyclic peptide for all peptide/scaffold combinations.
Conclusion

To conclude, we have devised a new route for the successful preparation of tetracyclic peptides using a unique and compatible combination of CEPS, CLIPS and oxime ligation. The majority of tetracyclic peptides were obtained as single isomers, depending on the peptide length and scaffold used. For hS(ONH$_2$)-containing peptides, their length is the critical factor determining the success of the reaction. For F(C=O)-containing peptides, the scaffold-type plays the most decisive role in the cyclization and in case of D(C=O), the amino acid itself determines the outcome. Overall, our work based on three orthogonal macrocyclization reactions gives regioselective access to tetracyclic peptides. Further studies are in progress to carry out the CEPS/CLIPS/oxime sequence in an automated fashion for applications in drug discovery.
Experimental Section

Associated content

The Supporting Information of this article will be available free of charge on the Organic Letters publications website at DOI: 10.1021/acs.orglett.9b00378.

Amino Acid Synthesis

Detailed protocols for the synthesis of the respective Fmoc-amino acid building blocks (see Figure 5) to introduce hS(ONH$_2$), F(C=O) and D(C=O) into a peptide will be available in the Supporting Information of the article as indicated above.

![Fmoc-amino acid building blocks](image)

**Figure 5.** Fmoc-amino acid building blocks synthesized to introduce hS(ONH$_2$), F(C=O) and D(C=O) into peptides.

Solid-Phase Peptide Synthesis

**General Procedure for SPPS of C-terminal Cam-ester Peptides:**

Fmoc-L-Wang resin (0.2 mmol) 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). After washing with DMF (6x 2 min), the corresponding Fmoc-AA-glycolic acid (2 eq.; AA= any amino acid) 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). Fmoc-AA-glycolic acid was prepared according to Nuijens et al.\(^{[16]}\) After washing with DMF and Fmoc deprotection the next amino acid Fmoc-AA-OH was coupled using N,N'-Diisopropylcarbodiimide (DIC; 4 eq.) and Oxyma Pure (4 eq.) in DMF (45 min). After the final Fmoc-deprotection, the resin was dried in a stream of nitrogen gas.
Synthesis of Peptide Amides:

Peptide amides were synthesized using a Rink amide resin. 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). After final Fmoc-deprotection, the resin was dried.

Cleavage of Peptides Containing Unnatural Amino Acids:

Peptides containing unnatural amino acids have been cleaved using the following protocols:

1) Aminooxy peptides containing hS(ONH₂) were cleaved using a cocktail of 90/5/2.5/2.5 (v/v/v/v) TFA/water/thioanisole/2,2’-(Ethylenedioxy)diethanethiol (DODT)/triisopropylsilane (TIS) for 2 hours at room temperature.

2) Ketone-peptides with either F(C=O) or D(C=O) were cleaved with a mixture of 80/5/5/7.5 (v/v/v/v) TFA/water/thioanisole/TIS/phenol for 2 hours at room temperature.

Precipitation of the peptide in ice-cold (-20°C) diisopropylether (5 mL cleavage mixture per 45 mL diisopropylether) followed by lyophilization of the precipitated peptide afforded the crude peptide. Purification of the crude peptide was performed by reversed-phase HPLC (mobile phase consists of gradient mixture of eluent-A (water containing 0.05% (v/v) TFA) and eluent-B (acetonitrile (ACN) containing 0.05% (v/v) TFA).

LC-MS Analysis

CEPS Reaction Samples:

Enzymatic peptide cyclizations were monitored using an HPLC-MS system. Separation was performed using a Waters XSelect® CSH C18 column (2.5 μm, 3.0 x 150 mm) and positive ion current for MS analysis was used. As mobile phase a binary mixture of solvent A (water + 0.05% (v/v) methanesulfonic acid (MSA)) and solvent B (ACN + 0.05% (v/v) MSA) was used. A linear gradient from 5-60% B in 7.5 min, followed by isocratic 95% solvent B for 3 min was used by default. Peptides were visualized at λ = 220 nm.

CLIPS/Oxime Reaction Samples:

Reaction samples were measured on a UPLC-ESMS system using a gradient of 5-55% B in 3 min. An Acquity UPLC Peptide BEH C18 Column (130 Å, 1.7 μm, 2.1x 50 mm) with UV detection (λ = 215 nm) and positive
ion current for MS analysis was used. The mobile phase consisted of eluent-A (water containing 0.05% (v/v) TFA) and eluent-B (ACN containing 0.05% (v/v) TFA).

Peptide sample preparation

Approximately 0.2 mg of peptide was dissolved in 100 µL of 1:1 (v/v) ACN/water. 10 µL of this solution was diluted with 50 µL of water and analyzed via UPLC-MS.

Reaction mixtures in ACN/water solvent mixtures

20 µL of sample was diluted with 40 µL water and filtered over a pipet-tip frit filter, before being analyzed using UPLC-MS.

Reaction mixtures in DMSO/water solvent mixtures

30 µL of sample was diluted with 30 µL water and filtered over a pipet-tip frit filter before UPLC-MS analysis.

General CLIPS/ Oxime Procedure

Chemo-Enzymatic Peptide Synthesis (CEPS):

Linear Cam-ester peptides were dissolved in potassium phosphate buffer solution (500 mM, pH 8.5) to a concentration of approx. 0.5 mg/mL (0.15- 0.25 mM), followed by the addition of omniligase-1 (0.15-0.5 µM). The reaction was followed by HPLC-MS using an individualized gradient. After completion of the reaction the reaction mixture was purified via preparative RP-HPLC. If the linear precursor peptides exhibited low solubility, guanidinium hydrochloride (Gdn.HCl) was added to a concentration of 1 M.

Example:

Linear Cam-ester 23333-F(C=O) (H-CYKQ-F(C=O)-SIK-F(C=O)-AKGCSKL-CamL-OH, 20 mg, 7.5 µmol) was dissolved in 500 mM phosphate buffer (30 mL, pH 8), followed by addition of omniligase-1 to a concentration of 0.4 µM. After a reaction time of 60 min the monocyclic peptide c23333-F(C=O) was isolated from the reaction mixture using preparative RP-HPLC.
The following linear peptide Cam-esters were cyclized into their head-to-tail cyclic counterpart:

Table 1. The sequences of linear peptide Cam-esters that were synthesized and subjected to cyclization.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>13333-hS(ONH₂)</td>
<td>H-CYKQ-hS(ONH₂)-SIK-hS(ONH₂)-AKGCSKL-O-Cam-L-OH</td>
</tr>
<tr>
<td>23333-F(C=O)</td>
<td>H-CYKQ-F(C=O)-SIK-F(C=O)-AKGCSKL-O-Cam-L-OH</td>
</tr>
<tr>
<td>33333-D(C=O)</td>
<td>H-CYKQ-D(C=O)-SIK-D(C=O)-AKGCSKL-O-Cam-L-OH</td>
</tr>
<tr>
<td>44444-hS(ONH₂)</td>
<td>H-R-hS(ONH₂)-FRLPCRQLRCFLP-hS(ONH₂)-RQL-O-Cam-L-OH</td>
</tr>
<tr>
<td>54444-F(C=O)</td>
<td>H-R-F(C=O)-FRLPCRQLRCFLP-F(C=O)-RQL-O-Cam-L-OH</td>
</tr>
<tr>
<td>65555-hS(ONH₂)</td>
<td>H-CYKGKQ-hS(ONH₂)-SIKAS-hS(ONH₂)-AKVRGCKFSKL-O-Cam-L-OH</td>
</tr>
<tr>
<td>75555-F(C=O)</td>
<td>H-CYKGKQ-F(C=O)-SIKAS-F(C=O)-AKVRGCKFSKL-O-Cam-L-OH</td>
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<tr>
<td>85555-D(C=O)</td>
<td>H-CYKGKQ-D(C=O)-SIKAS-D(C=O)-AKVRGCKFSKL-O-Cam-L-OH</td>
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Table 2: The synthesized cyclic peptides with the corresponding m/z values.

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<th>Name</th>
<th>Sequence</th>
<th>m/z found</th>
<th>m/z calc</th>
<th>Species</th>
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<tr>
<td>c13333-hS(ONH₂)</td>
<td>cycCYKQ-hS(ONH₂)-SIK-hS(ONH₂)-AKGCSKL</td>
<td>1771.91</td>
<td>1771.46</td>
<td>[M+H]+</td>
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<tr>
<td>c23333-F(C=O)</td>
<td>cycCYKQ-F(C=O)-SIK-F(C=O)-AKGCSKL</td>
<td>1918.32</td>
<td>1918.64</td>
<td>[M+H]+</td>
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<tr>
<td>c33333-D(C=O)</td>
<td>cycCYKQ-D(C=O)-SIK-D(C=O)-AKGCSKL</td>
<td>983.02</td>
<td>983.85</td>
<td>[M+2H]2+</td>
</tr>
<tr>
<td>c44444-hS(ONH₂)</td>
<td>cycR-hS(ONH₂)-FRLPCRQLRCFLP-hS(ONH₂)-RQL</td>
<td>859.04</td>
<td>859.18</td>
<td>[M+3H]3+</td>
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<tr>
<td>c54444-F(C=O)</td>
<td>cycR-F(C=O)-FRLPCRQLRCFLP-F(C=O)-RQL</td>
<td>907.80</td>
<td>907.90</td>
<td>[M+3H]3+</td>
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<td>c65555-hS(ONH₂)</td>
<td>cycCYKGKQ-hS(ONH₂)-SIKAS-hS(ONH₂)-AKVRGCKFSKL</td>
<td>1325.27</td>
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<tr>
<td>c75555-F(C=O)</td>
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<td>c85555-D(C=O)</td>
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<td>1420.30</td>
<td>1420.43</td>
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</table>
Strategy I

In a glass vial the corresponding monocyclic peptide (0.2 mg) was dissolved in a 1:1 (v/v) mixture of DMSO/water at a concentration of 0.5 mM. The scaffold (10 to 20 mg/mL) was added with a molar equivalent relative to the peptide whereby the peptide weight was taken uncorrected for any TFA-salts present in the dry material (T4-2(C=O) at 0.95 eq., T4-3(C=O) at 1.05 eq.). A solution of 1M NH₄HCO₃ (30 µL) was added to reach a pH > 8. The reaction mixture was analyzed after 20 min. Upon completion, the reaction mixture was acidified with a 15% (v/v) TFA solution to remove the acetal protecting groups of the aldehydes. Oxime ligation occurred simultaneously, and the reaction mixture was analyzed at certain time intervals until completion of the reaction.

Strategy II

CLIPS

In a glass vial the respective peptide (0.2 mg) was dissolved in a 1:1 (v/v) mixture of ACN/ water at a concentration of 0.5 mM. The scaffold (10 to 20 mg/mL) was added with a molar equivalent relative to the peptide, whereby the peptide weight was taken uncorrected for any TFA-salts present in the dry material (T4-1(ONH₂)) at 0.95 eq., T4-2(ONH₂) at 1.05 eq. and T4-3(ONH₂) at 0.85 eq.). A solution of 1M NH₄HCO₃ (30 µL) was added to reach pH > 8. The reaction mixture was analyzed via HPLC-MS after 20 min and upon completion the reaction mixture was lyophilized. The reaction can also be performed in DMSO/ water mixtures, but, although the presence of DMSO does not affect follow-up reactions, it is less suitable for lyophilizing.

Scaffold Deprotection

To remove the Boc-groups on the scaffold aminooxy, the lyophilized product was treated with an excess of 2:1 (v/v) TFA/ dichloromethane (DCM) (300 µL in total). The solution was left for 2 hours at room temperature, after which the volatiles were evaporated under a flow of N₂. For larger scale reactions, the material was repeatedly dissolved in DCM and evaporated to dryness to remove all remnants of TFA.

Oxime Ligation

The free-aminooxy peptide was dissolved in 1:1 (v/v) mixture of DMSO/ water to reach a peptide concentration of 0.5 mM (same as for the CLIPS reaction). If necessary, the pH was adjusted to 4.5 1M acetate buffer of pH 4.5. The reaction can be carried out at room temperature, but some systems showed the best results at 40°C. In the latter the glass reaction vessel was placed on a heating plate with a temperature set to 40°C.
Tolerance of Unnatural amino Acids hS(ONH₂), F(C=O) and D(C=O) by Omniligase-1

Reaction Procedure

Peptides bearing a \( \text{hS}(\text{ONH}_2) \), \( \text{F}(\text{C}=\text{O}) \) or \( \text{D}(\text{C}=\text{O}) \) residue in either of the positions P4-P2’ were synthesized according to the procedures described above. For a list of sequences including their respective molecular weights see Table 3.

Table 3. Codes, sequences and exact masses (calculated and experimental) of synthesized Cam esters.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MW(_{\text{calc}})</th>
<th>MW(_{\text{exp}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>hS(ONH₂)-S4</td>
<td>Ac-D-hS(ONH₂)-SKL-Cam-L-OH</td>
<td>790.4</td>
<td>790.3</td>
</tr>
<tr>
<td>hS(ONH₂)-S3</td>
<td>Ac-DF-hS(ONH₂)-KL-Cam-L-OH</td>
<td>850.4</td>
<td>850.3</td>
</tr>
<tr>
<td>hS(ONH₂)-S2</td>
<td>Ac-DFS-hS(ONH₂)-L-Cam-L-OH</td>
<td>809.4</td>
<td>809.3</td>
</tr>
<tr>
<td>hS(ONH₂)-S1</td>
<td>Ac-DFSK-hS(ONH₂)-Cam-L-OH</td>
<td>843.4</td>
<td>*</td>
</tr>
<tr>
<td>hS(ONH₂)-S1’</td>
<td>H-hS(ONH₂)-LKKF-NH₂</td>
<td>649.4</td>
<td>649.3</td>
</tr>
<tr>
<td>hS(ONH₂)-S2’</td>
<td>H-A-hS(ONH₂)-KKF-NH₂</td>
<td>607.4</td>
<td>607.3</td>
</tr>
<tr>
<td>F(C=O)-S4</td>
<td>Ac-D-F(C=O)-SKL-Cam-L-OH</td>
<td>863.4</td>
<td>863.3</td>
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<td>F(C=O)-S3</td>
<td>Ac-DF-F(C=O)-KL-Cam-L-OH</td>
<td>923.5</td>
<td>923.4</td>
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<tr>
<td>F(C=O)-S2</td>
<td>Ac-DFS-F(C=O)-L-Cam-L-OH</td>
<td>882.4</td>
<td>882.3</td>
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<tr>
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<td>722.3</td>
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<td>680.3</td>
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<tr>
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<td>Ac-D-D(C=O)-SKL-Cam-L-OH</td>
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<td>886.3</td>
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<tr>
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<td>*</td>
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<tr>
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<td>745.3</td>
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<tr>
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<td>703.3</td>
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<td>Control ester</td>
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<td>821.3</td>
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<tr>
<td>Control amine</td>
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<td>604.4</td>
<td>604.3</td>
</tr>
</tbody>
</table>

*synthesis failed.

To test the acceptance of each respective substrate by omniligase-1, stock solutions of acyl donor (ester) fragment (10 mM) and acyl acceptor fragment (amine) (15 mM) were prepared in ultrapure water. 25 µL of both stock solutions were combined and the mixture was diluted with 100 µL 1 M potassium phosphate...
buffer pH 8.5. The final concentration of the ester fragment was 1.66 mM and of the amine fragment 2.5 mM (5 eq.), respectively. To initiate the reaction 2 µg of Omniligase-1 (final concentration 0.5 µM) was added. After 0 and 30 min 25 µL of reaction mixture were quenched in 475 µL of quenching solution (0.5% (v/v) MSA in water). Samples were analyzed using HPLC-MS. All reactions performed are listed in Figure 6A. HPLC yields were calculated based on the peak area of the respective peaks: product ("synthesis"), hydrolyzed ester ("hydrolysis") as well as potentially remaining starting material ("ester") were integrated. The results are displayed in Figure 6B, C and D. The reaction was deliberately performed under sub-optimal reaction conditions and stopped after 30 min in order to highlight differences between the substrates. After 30 min the control "benchmark" reaction (Ac-DFSKL-Cam-L-OH + H-ALKKF-NH₂) was usually (almost) complete with exclusive formation of the desired ligation product.

![Figure 6](image_url)

**Figure 6.** A) Model reactions for testing the acceptance of the unnatural amino acids hS(ONH₂), F(C=O) or D(C=O) in each respective peptide binding pocket of omniligase-1. The reaction Ac-DSFKL-Cam-L-OH + H-ALKKF-NH₂ served as a control ligation. Reaction yields after 30 min (blue bars), corresponding hydrolysis of the Cam-ester (red bars) and remaining Cam-ester peptide (grey bars) is given. B) F(C=O) containing peptides. C) hS(ONH₂)-containing peptides and C) D(C=O)-containing model peptides.
Synthesis of Constrained Tetracyclic Peptides by Consecutive CEPS, CLIPS and Oxime Ligation

References


