

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

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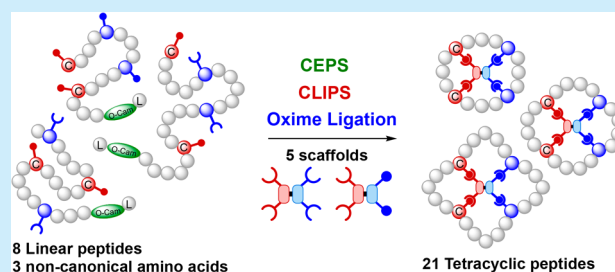
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Supporting Information

ABSTRACT: In Nature, multicyclic peptides constitute a versatile molecule class with various biological functions. For their pharmaceutical exploitation, chemical methodologies that enable selective consecutive macrocyclizations are required. We disclose a combination of enzymatic macrocyclization, CLIPS alkylation, and oxime ligation to prepare tetracyclic peptides. Five new small molecular scaffolds and differently sized model peptides featuring noncanonical amino acids were synthesized. Enzymatic macrocyclization, followed by one-pot scaffold-assisted cyclizations, yielded 21 tetracyclic peptides in a facile and robust manner.



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 regioselectivity 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, butelase, or sortase^{11–13} have been successfully employed for this purpose.¹⁴ For example, omniligase-1 efficiently catalyzes head-to-tail cyclization of linear C-terminal glycolate-ester peptides in aqueous solution.^{11,15} In addition, peptide cyclization using small molecule organic scaffolds has been widely explored. For example, CLIPS technology (Chemical Linkage of Peptides onto Scaffolds)¹⁶ is used for peptide cyclization in vitro and for the generation of large phage-displayed libraries of thioether-bridged bicyclic peptides.^{16–20}

However, despite its ease, the applicability is limited to the preparation of monocyclic and bicyclic peptides.

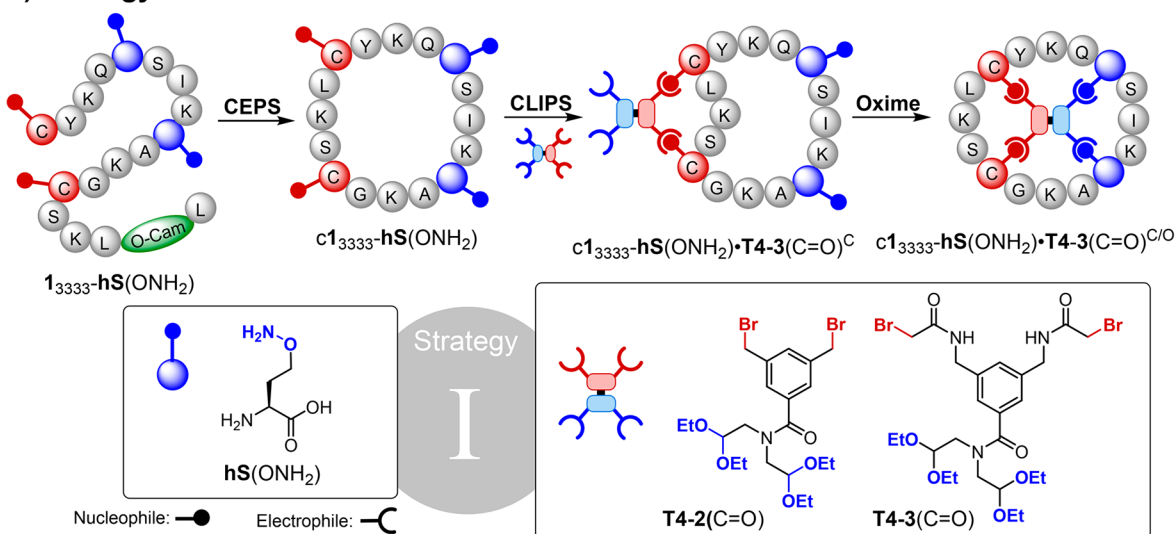
Recently, our group introduced a novel concept to expand the CLIPS technology to furnish tricyclic and tetracyclic peptides via a one-pot procedure, by combining it with two orthogonal ligation methods: Cu(I)-catalyzed alkyne–azide cycloaddition (CuAAC) and enzymatic head-to-tail cyclization using omniligase-1 (chemo-enzymatic peptide synthesis, CEPS).^{11,21} 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.^{22–25} In contrast to CuAAC, the formed oxime bond shows *E/Z* isomerism, which is influenced by their substituents.²⁶

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 aminoxy 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.²¹ For oxime ligation, the peptide contains either (a) the aminoxy or (b) the ketone moiety. Therefore, we set out to

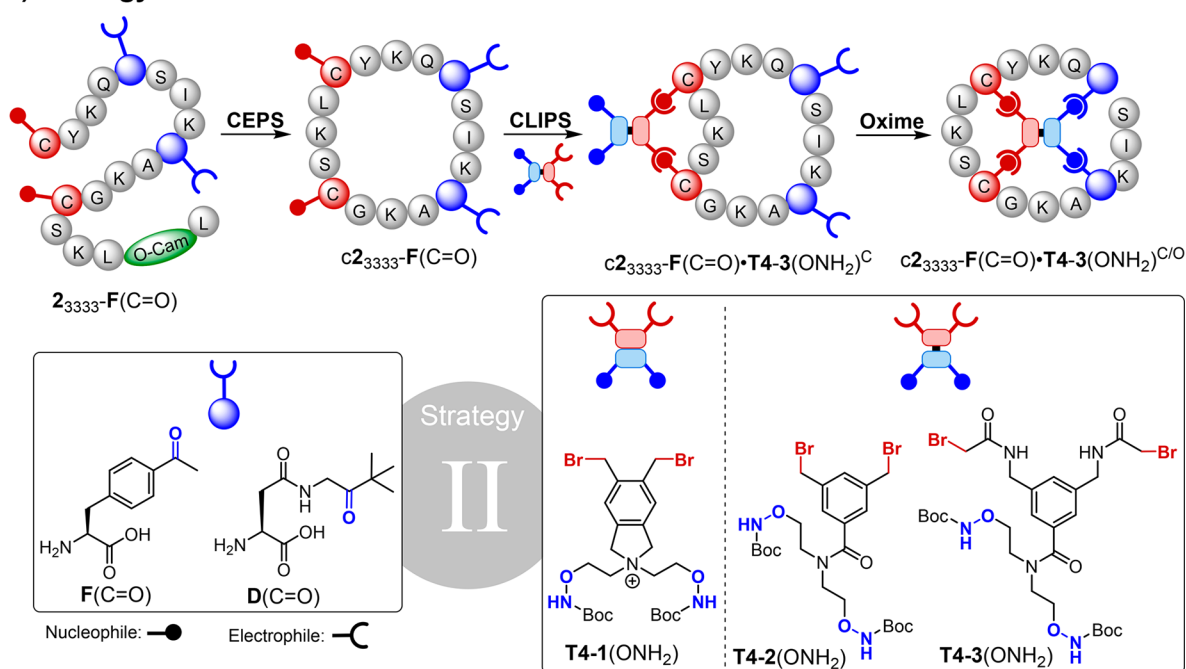
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a) Strategy I



b) Strategy II



c) Peptide sequences

- 1₃₃₃₃-hS(OH₂): H-CYKQhS(OH₂)SIK**hS(OH₂)**AKG**C**SKL-O-Cam-L-OH
- 2₃₃₃₃-F(C=O): H-CYKQ**F(C=O)**SIK**F(C=O)**AKG**C**SKL-O-Cam-L-OH
- 3₃₃₃₃-D(C=O): H-CYKQ**D(C=O)**SIK**D(C=O)**AKG**C**SKL-O-Cam-L-OH
- 4₄₄₄₄-hS(OH₂): H-R**hS(OH₂)**FRLP**CR**QLR**CF**RLP**hS(OH₂)**RQL-O-Cam-L-OH
- 5₄₄₄₄-F(C=O): H-R**F(C=O)**FRLP**CR**QLR**CF**RLP**F(C=O)**RQL-O-Cam-L-OH
- 6₅₅₅₅-hS(OH₂): H-CYK**GKQhS(OH₂)**SIK**AShS(OH₂)**AKVRG**C**KFSKL-O-Cam-L-OH
- 7₅₅₅₅-F(C=O): H-CYK**GKQF(C=O)**SIK**ASF(C=O)**AKVRG**C**KFSKL-O-Cam-L-OH
- 8₅₅₅₅-D(C=O): H-CYK**GKQD(C=O)**SIK**ASD(C=O)**AKVRG**C**KFSKL-O-Cam-L-OH

Figure 1. Schematic representation of the CEPS/CLIPS/oxime cyclizations and the evaluated scaffold and peptides: (a) aminoxy-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(OH₂)/T4-2(OH₂)/T4-3(OH₂) (Strategy II), for the synthesis of tetracyclic peptides; and (c) peptide codes and the corresponding sequences.

investigate two different strategies (Figure 1). For Strategy I, the peptide bears the aminoxy 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

aminoxy and ketone moieties (T4-1(OH₂)/T4-2(OH₂)/T4-3(OH₂)) (Figure 1b). The set of scaffolds we studied comprises the rigid T4-1(OH₂)²⁷ and the flexible scaffolds T4-2(OH₂)/T4-2(C=O) and T4-3(C=O)/T4-3(OH₂),

all containing rotatable amide bonds in combination with a bromoacetamide.²⁸ [For the synthesis of the scaffolds, see the Supporting Information (SI).]

In Strategy I, the aminoxy residue is introduced in the form of aminoxy-homoserine ($\text{hS}(\text{ONH}_2)$),²⁶ whereas in Strategy II, two different keto-amino acids, namely *para*-acetyl phenylalanine ($\text{F}(\text{C}=\text{O})$), being well-studied^{29–33} and a *tert*-butyl ketone derivative of aspartic acid ($\text{D}(\text{C}=\text{O})$), 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 $\text{F}(\text{C}=\text{O})$ and $\text{D}(\text{C}=\text{O})$; hence, a mixture of *E/Z*-oximes may be expected, with an increased preference for the *E*-isomer ($\text{D}(\text{C}=\text{O}) > \text{F}(\text{C}=\text{O})$), because of the steric hindrance of the *t*Bu 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 numbers of amino acids (n) between the CLIPS and oxime junction points ($n = 3, 4, 5$) was designed (Figure 1c). The synthesis of peptides containing both the C-terminal glycolate-type ester, as required for the CEPS macrolactamization,¹⁵ and the protected aminoxy/ketone amino acids, was performed using classical automated SPPS (see the SI). Generally, the ketone-containing peptides were easier to obtain (little to no side reactions) than peptides containing amino acid $\text{hS}(\text{ONH}_2)$, of which the latter often resulted in a low-yielding synthesis and troublesome purifications. Common side-reactions included isopropylidene formation of the aminoxy functionality with traces of acetone, incomplete SPPS-coupling of $\text{Fmoc-hS}(\text{ONHBoc})-\text{OH}$, and elimination of the aminoxy moiety.³⁴ Peptides containing $\text{D}(\text{C}=\text{O})$ exhibited low solubility in aqueous solutions, potentially requiring the addition of solubilizing agents (e.g., urea) for efficient enzymatic cyclization. Nevertheless, all linear peptide Camesters ($n = 3, 4, 5$) were efficiently head-to-tail cyclized using omniligase-1 (>85% average efficiency; see the SI). Initially, we explicitly avoided placing one of the oxime-reactive non-canonical amino acids, $\text{hS}(\text{ONH}_2)$, $\text{F}(\text{C}=\text{O})$, $\text{D}(\text{C}=\text{O})$, in the enzymatic recognition sequence of the peptide (N-terminal P1' and P2' and C-terminal P4–P1)³⁵ 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 ($\text{F}(\text{C}=\text{O}) =$ all pockets; $\text{hS}(\text{ONH}_2) =$ P3, P2, P1', P2', $\text{D}(\text{C}=\text{O}) =$ P3, P2; see the SI), despite distinct differences in reaction rate that could, in turn, be compensated for by adding an increased amount of biocatalyst. Generally, these results demonstrate the broad applicability and compatibility of the CEPS cyclization, even when using noncanonical amino acids.

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 (0.5 mM peptide solution, ~0.9 equiv of scaffold), aqueous NH_4HCO_3 solution (pH >8.0), within 20 min at room temperature (rt) to give the corresponding bicyclic products (see the SI). This was followed by deprotection of the scaffolds for oxime ligation. For Strategy I, the acetals in $\text{T4-2}(\text{C}=\text{O})/\text{T4-3}(\text{C}=\text{O})$ 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 aminoxy moiety of $\text{T4-1}(\text{ONH}_2)/\text{T4-2}(\text{ONH}_2)/\text{T4-3}(\text{ONH}_2)$ was performed 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 dimethylsulfoxide (DMSO) (0.5 mM, 16 h at 40 °C). Reactions were monitored via UPLC analysis (column temperature = 50 °C).

Following these generalized procedures, we first investigated the reaction of $\text{hS}(\text{ONH}_2)$ -containing monocyclic hexadecapeptides with scaffolds $\text{T4-2}(\text{C}=\text{O})$ and $\text{T4-3}(\text{C}=\text{O})$ (Strategy I, Figure 1a). The CLIPS reactions, yielding the bicyclic peptides, went smoothly (see the SI). 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 $\text{c1}_{3333}\text{-hS}(\text{ONH}_2)\cdot\text{T4-2}(\text{C}=\text{O})^{\text{c/o}}$ and $\text{c1}_{3333}\text{-hS}(\text{ONH}_2)\cdot\text{T4-3}(\text{C}=\text{O})^{\text{c/o}}$ were formed as a mixture of two different products ($t_{\text{R}} = 0.82/0.83$ min and $t_{\text{R}} = 0.70/0.72$ min) 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 (Figures 2a and 2d), matching earlier

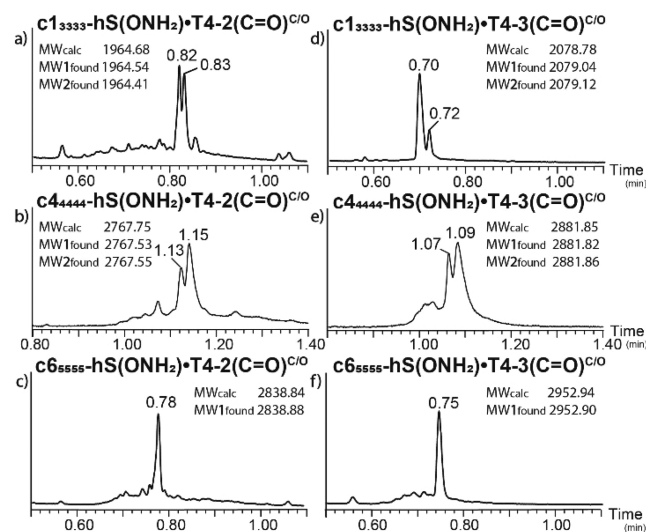


Figure 2. UPLC traces of Strategy I oxime ligations with $\text{hS}(\text{ONH}_2)$ -containing bicyclic peptides ($n = 3, 4, 5$) with scaffolds (a–c) $\text{T4-3}(\text{C}=\text{O})$ and (d–f) $\text{T4-2}(\text{C}=\text{O})$. Peak masses: M1 = first encountered peak (tetracycle); M2, etc. = peaks with longer t_{R} .

observations.²¹ Separation of the products was not attempted and was deemed impossible, because the isomerism is considered conformational, rather than configurational, assuming both products are in thermodynamic equilibrium. This assumption was confirmed by the fact that, for $\text{c1}_{3333}\text{-hS}(\text{ONH}_2)\cdot\text{T4-3}(\text{C}=\text{O})^{\text{c/o}}$, a third isomer was initially observed at $t_{\text{R}} = 0.73$ min, that disappeared overnight at rt.

Interestingly, an increased loopsize ($n = 4$) in the cyclic 20-peptide led to the formation of two broad product peaks for the tetracyclic constructs $\text{c4}_{4444}\text{-hS}(\text{ONH}_2)\cdot\text{T4-2}(\text{C}=\text{O})^{\text{c/o}}$ and $\text{c4}_{4444}\text{-hS}(\text{ONH}_2)\cdot\text{T4-3}(\text{C}=\text{O})^{\text{c/o}}$ (see Figures 2b and 2e). We attribute this peak broadening to slow equilibration between several conformers on UPLC time scale, en route to coalescence, while being comparatively faster than the $\text{c1}_{3333}\text{-hS}(\text{ONH}_2)$ tetracycles. Finally, for the largest 24-membered peptides $\text{c6}_{5555}\text{-hS}(\text{ONH}_2)\cdot\text{T4-2}(\text{C}=\text{O})^{\text{c/o}}$ and $\text{c6}_{5555}\text{-hS}(\text{ONH}_2)\cdot\text{T4-3}(\text{C}=\text{O})^{\text{c/o}}$, the UPLC clearly shows a single product (see Figures 2c and 2f), confirming that

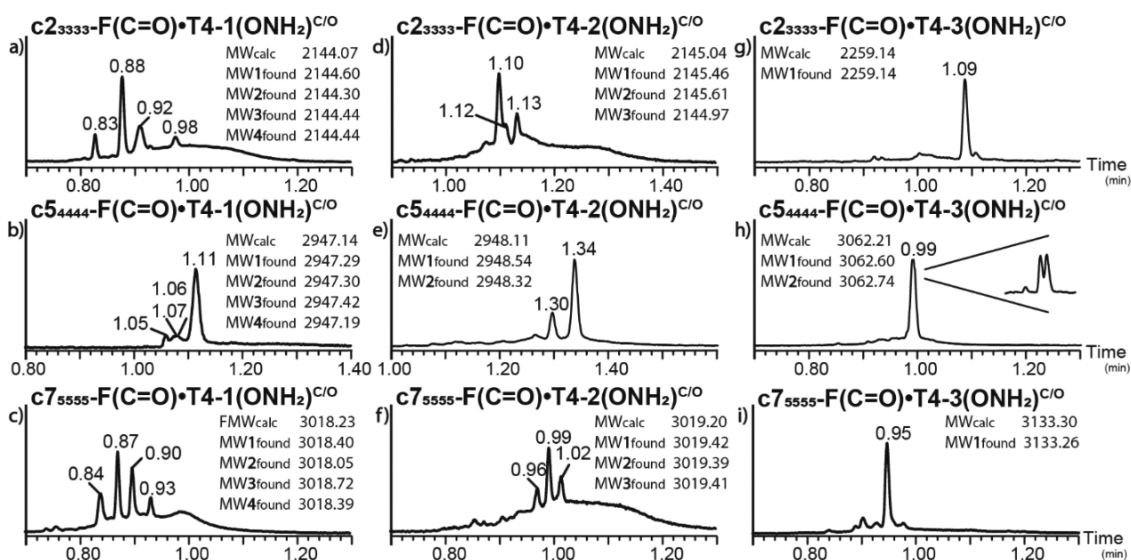


Figure 3. UPLC chromatograms of Strategy II oxime ligations with F(C=O) peptides and scaffolds T4-1(OH₂)/T4-2(OH₂)/T4-3(OH₂) reacted for 16 h at 40 °C. Peak masses: M1= first encountered peak (tetracycle); M2, etc.= peaks with longer t_R .

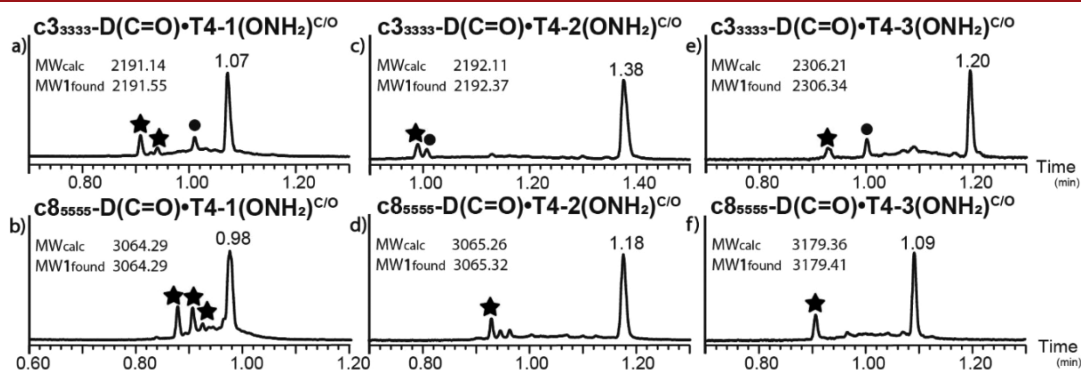


Figure 4. UPLC chromatograms of Strategy II oxime ligations with D(C=O) peptides and scaffolds T4-1(OH₂)/T4-2(OH₂)/T4-3(OH₂) reacted for 16 h at 40 °C. Peak masses: M1= first encountered peak (tetracycle); M2, etc. = peaks with longer t_R . [Legend: (★) mono-oxime still present; (●) disulfide starting material not reacted during CLIPS.]

conformational isomers in these macrocycles are in rapid equilibrium at the UPLC-time scale. This is consistent 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.²¹

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 only single products, 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(OH₂), T4-2(OH₂), or T4-3(OH₂), did seem to have an effect (Figure 3).

Using scaffold T4-1(OH₂) resulted in the formation of four products for all peptide lengths (Figures 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(OH₂), we did not observe

thermodynamic equilibration, but obtained the product with 2–4 different isomers, depending on the specific peptide used (see Figures 3d–f). For example, in the case of c5₄₄₄₄-F(C=O)•T4-2(OH₂)^{c/o} (Figure 3e), two distinct products were formed, that proved thermodynamic stability and did not interconvert upon separation of both peaks. In contrast, at elevated temperatures (40 °C), c2₃₃₃₃-F(C=O)•T4-3(OH₂)^{c/o} (Figure 3g) forms one thermodynamic product from initially four isomers. For c5₄₄₄₄-F(C=O)•T4-3(OH₂)^{c/o}, however, very close running products were obtained and could only be separated using a slow eluting UPLC gradient (Figure 3h). Lastly, c7₅₅₅₅-F(C=O)•T4-3(OH₂)^{c/o} instantaneously yields a single product (Figure 3i), suggesting that larger cycles might also result in the formation of a single product.

Since the *n* = 4 peptides were chosen based on the sequence of a previously published tetracyclic peptide,²¹ we opted to compare the structure of c5₄₄₄₄-F(C=O)•T4-2(OH₂)^{c/o} with the NMR structure of the identical peptide fused with a T4₂ scaffold.^{21,36} However, NMR studies revealed that the tetracyclic structure of c5₄₄₄₄-F(C=O)•T4-2(OH₂)^{c/o} is present in many different conformations, because of 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 (see the SI).

Similar to F(C=O), D(C=O) also contains an asymmetric ketone. However, because of 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 methoxyamine 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 divisive 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(OH₂) to T4-3(OH₂)), a single main product was obtained, which was especially surprising in the case of scaffold T4-1(OH₂) (see Figures 4a and 4b). 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 at 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.

To conclude, we have devised a regioselective preparation of tetracyclic peptides using a combination of CEPS, CLIPS, and oxime ligation. Most tetracyclic peptides were obtained as single isomers, depending on the peptide length and scaffold used. For hS(OH₂)-containing peptides, two conformational isomers were obtained for short sequences emerging from hindered rotation. For F(C=O)-containing peptides, also thermodynamically stable isomers were found arising from oxime *E/Z* configurational mixtures. Although oxime *E/Z* mixture formation was not observed with the sterically demanding ketone in amino acid D(C=O), its low reactivity hampers the applicability. Overall, our work that is based on three orthogonal macrocyclization reactions gives regioselective access to tetracyclic peptides. Further studies are in progress to perform the CEPS/CLIPS/oxime sequence in an automated fashion for applications in drug discovery.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b00378.

Detailed procedures for the synthesis of amino acids, peptides, and the scaffolds are given, accompanied by the characterization data (including NMR spectra); procedures and results of all peptide cyclization reactions, including CEPS, CLIPS, and oxime ligation (PDF).

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Notes

The authors declare the following competing financial interest(s): PT is employee of Pepscan Therapeutics at which part of the work has been carried out. The methodology described may be commercialized in the future.

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■ REFERENCES

- (1) Bhardwaj, G.; Mulligan, V. K.; Bahl, C. D.; Gilmore, J. M.; Harvey, P. J.; Cheneval, O.; Buchko, G. W.; Pulavarti, S. V. S. R. K.; Kaas, Q.; Eletsky, A.; Huang, P.-S.; Johnsen, W. A.; Greisen, P. J.; Rocklin, G. J.; Song, Y.; Linsky, T. W.; Watkins, A.; Rettie, S. A.; Xu, X.; Carter, L. P.; Bonneau, R.; Olson, J. M.; Coutsiaris, E.; Correnti, C. E.; Szyperski, T.; Craik, D. J.; Baker, D. Accurate *de novo* Design of Hyperstable Constrained Peptides. *Nature* **2016**, *538*, 329–335.
- (2) Sawyer, T. K. Renaissance in Peptide Drug Discovery: The Third Wave. In *Peptide-based Drug Discovery: Challenges and New Therapeutics*; The Royal Society of Chemistry, 2017.
- (3) Lau, J. L.; Dunn, M. K. Therapeutic Peptides: Historical Perspectives, Current Development Trends, and Future Directions. *Bioorg. Med. Chem.* **2018**, *26*, 2700–2707.
- (4) Luther, A.; Bisang, C.; Obrecht, D. Advances in Macrocyclic Peptide-Based Antibiotics. *Bioorg. Med. Chem.* **2018**, *26*, 2850–2858.
- (5) Conibear, A. C.; Chaousis, S.; Durek, T.; Rosengren, K. J.; Craik, D. J.; Schroeder, C. I. Approaches to the Stabilisation of Bioactive Epitopes by Grafting and Peptide Cyclisation. *Biopolymers* **2016**, *106*, 89–100.
- (6) Craik, D. J.; Conibear, A. C. The Chemistry of Cyclotides. *J. Org. Chem.* **2011**, *76*, 4805–4817.
- (7) Wang, C. K.; Craik, D. J. Designing Macrocyclic Disulfide-Rich Peptides for Biotechnological Applications. *Nat. Chem. Biol.* **2018**, *14*, 417–427.
- (8) Nicolaou, K. C.; Mitchell, H. J.; Jain, N. F.; Winssinger, N.; Hughes, R.; Bando, T. Total Synthesis of Vancomycin. *Angew. Chem., Int. Ed.* **1999**, *38*, 240–244.
- (9) Brambell, F. W. R. The Transmission of Immunity from Mother to Young and The Catabolism of Immunoglobulins. *Lancet* **1966**, *288*, 1087–1093.
- (10) Malins, L. R.; Degruyter, J. N.; Robbins, K. J.; Scola, P. M.; Eastgate, M. D.; Ghadiri, M. R.; Baran, P. S. Peptide Macrocyclization Inspired by Non-Ribosomal Imine Natural Products. *J. Am. Chem. Soc.* **2017**, *139*, 5233–5241.
- (11) Schmidt, M.; Toplak, A.; Quaedflieg, P. J. L. M.; Ippel, H.; Richelle, G. J. J.; Hackeng, T. M.; Van Maarseveen, J. H.; Nuijens, T. Omniligase-1: A Powerful Tool for peptide Head-to-Tail Cyclization. *Adv. Synth. Catal.* **2017**, *359*, 2050–2055.
- (12) Nguyen, G. K. T.; Kam, A.; Loo, S.; Jansson, A. E.; Pan, L. X.; Tam, J. P. Butelase 1: a Versatile Ligase for Peptide and Protein Macrocyclization. *J. Am. Chem. Soc.* **2015**, *137*, 15398–15401.
- (13) Jia, X.; Kwon, S.; Wang, C.-I. A.; Huang, Y.-H.; Chan, L. Y.; Tan, C. C.; Rosengren, K. J.; Mulvenna, J. P.; Schroeder, C. I.; Craik, D. J. Semi-enzymatic Cyclization of Disulfide-Rich Peptides Using Sortase A. *J. Biol. Chem.* **2014**, *289*, 6627–6638.
- (14) Schmidt, M.; Toplak, A.; Quaedflieg, P. J. L. M.; van Maarseveen, J. H.; Nuijens, T. Enzyme-Catalyzed Peptide Cyclization. *Drug Discovery Today: Technol.* **2017**, *26*, 11–16.

- (15) Nuijens, T.; Toplak, A.; Van De Meulenreek, M. B. A. C.; Schmidt, M.; Goldbach, M.; Quaedflieg, P. J. L. M. Improved Solid Phase Synthesis of Peptide Carboxyamidomethyl (Cam) Esters for Enzymatic Segment Condensation. *Tetrahedron Lett.* **2016**, *57*, 3635–3638.
- (16) Timmerman, P.; Beld, J.; Puijk, W. C.; Meloen, R. H. Rapid and Quantitative Cyclization of Multiple Peptide Loops onto Synthetic Scaffolds for Structural Mimicry of Protein Surfaces. *ChemBioChem* **2005**, *6*, 821–824.
- (17) Heinis, C.; Rutherford, T.; Freund, S.; Winter, G. Phage-Encoded Combinatorial Chemical Libraries Based on Bicyclic Peptides. *Nat. Chem. Biol.* **2009**, *5*, 502–507.
- (18) Angelini, A.; Cendron, L.; Chen, S.; Touati, J.; Winter, G.; Zanotti, G.; Heinis, C. Bicyclic Peptide Inhibitor Reveals Large Contact Interface with a Protease Target. *ACS Chem. Biol.* **2012**, *7*, 817–821.
- (19) Bashiruddin, N. K.; Nagano, M.; Suga, H. Synthesis of Fused Tricyclic Peptides Using a Reprogrammed Translation System and Chemical Modification. *Bioorg. Chem.* **2015**, *61*, 45–50.
- (20) Liu, W.; Zheng, Y.; Kong, X.; Heinis, C.; Zhao, Y.; Wu, C. Precisely Regulated and Efficient Locking of Linear Peptides into Stable Multicyclic Topologies through a One-Pot Reaction. *Angew. Chem., Int. Ed.* **2017**, *56*, 4458–4563.
- (21) Richelle, G. J. J.; Schmidt, M.; Ippel, H.; Hackeng, T. M.; van Maarseveen, J. H.; Nuijens, T.; Timmerman, P. A One-Pot “Triple-C” Multicyclization Methodology for the Synthesis of Highly Constrained Isomerically Pure Tetracyclic Peptides. *ChemBioChem* **2018**, *19*, 1934–1938.
- (22) Ulrich, S.; Boturyn, D.; Marra, A.; Renaudet, O.; Dumy, P. Oxime Ligation: A Chemoselective Click-Type Reaction for Accessing Multifunctional Biomolecular Constructs. *Chem. - Eur. J.* **2014**, *20*, 34–41.
- (23) Assem, N.; Ferreira, D. J.; Wolan, D. W.; Dawson, P. E. Acetone-Linked Peptides: A Convergent Approach for Peptide Macrocyclization and Labeling. *Angew. Chem., Int. Ed.* **2015**, *54*, 8665–8668.
- (24) Dirksen, A.; Dawson, P. E. Rapid Oxime and Hydrazone Ligations with Aromatic Aldehydes for Biomolecular Labeling. *Bioconjugate Chem.* **2008**, *19*, 2543–2548.
- (25) Smeenk, L. E. J.; Timmers-Parohi, D.; Benschop, J. J.; Puijk, W. C.; Hiemstra, H.; van Maarseveen, J. H.; Timmerman, P. Reconstructing the Discontinuous and Conformational b1/b3-Loop Binding Site on hFSH/hCG by Using Highly Constrained Multicyclic Peptides. *ChemBioChem* **2015**, *16*, 91–99.
- (26) Haney, C. M.; Loch, M. T.; Horne, W. S. Promoting Peptide α -Helix Formation with Dynamic Covalent Oxime Side-Chain Cross-Links. *Chem. Commun.* **2011**, *47*, 10915–10917.
- (27) Smeenk, L. E. J.; Dailly, N.; Hiemstra, H.; Van Maarseveen, J. H.; Timmerman, P. Synthesis of Water-Soluble Scaffolds for Peptide Cyclization, Labeling, and Ligation. *Org. Lett.* **2012**, *14*, 1194–1197.
- (28) Chen, S.; Bertoldo, D.; Angelini, A.; Pojer, F.; Heinis, C. Peptide Ligands Stabilized by Small Molecules. *Angew. Chem., Int. Ed.* **2014**, *53*, 1602–1606.
- (29) Tian, F.; Tsao, M.-L.; Schultz, P. G. A Phage Display System with Unnatural Amino Acids. *J. Am. Chem. Soc.* **2004**, *126*, 15962–15963.
- (30) Takasu, A.; Kondo, S.; Ito, A.; Furukawa, Y.; Higuchi, M.; Kinoshita, T.; Kwon, I. Artificial Extracellular Matrix Proteins Containing Phenylalanine Analogues Biosynthesized in Bacteria Using T7 Expression System and the PEGylation. *Biomacromolecules* **2011**, *12*, 3444–3452.
- (31) Satyanarayana, M.; Vitali, F.; Frost, J. R.; Fasan, R. Diverse Organo-peptide Macrocycles via a Fast and Catalyst-free Oxime/intein-mediated Dual Ligation. *Chem. Commun.* **2012**, *48*, 1461–1463.
- (32) Frost, J. R.; Vitali, F.; Jacob, N. T.; Brown, M. D.; Fasan, R. Macrocyclization of Organo-Peptide Hybrids through a Dual Bio-orthogonal Ligation: Insights from Structure-Reactivity Studies. *ChemBioChem* **2013**, *14*, 147–160.
- (33) Smith, J. M.; Hill, N. C.; Krasniak, P. J.; Fasan, R. Synthesis of Bicyclic Organo-peptide Hybrids via Oxime/intein-mediated Macrocyclization Followed by Disulfide Bond Formation. *Org. Biomol. Chem.* **2014**, *12*, 1135–1142.
- (34) Liu, F.; Thomas, J.; Burke, T., Jr. Synthesis of a Homologous Series of Side-Chain-Extended Orthogonally Protected Amino-oxo-Containing Amino Acids. *Synthesis* **2008**, *2008*, 2432–2438.
- (35) Berger, A.; Schechter, I. Mapping the Active Site of Papain with the Aid of Peptide Substrates and Inhibitors. *Philos. Trans. R. Soc., B* **1970**, *257*, 249–264.
- (36) Richelle, G. J. J.; Ori, S.; Hiemstra, H.; van Maarseveen, J. H.; Timmerman, P. General and Facile Route to Isomerically Pure Tricyclic Peptides Based on Templated Tandem CLIPS/CuAAC Cyclizations. *Angew. Chem., Int. Ed.* **2018**, *57*, 501–505.