Bioorthogonal Peptide Macrocyclization using Oxime Ligation

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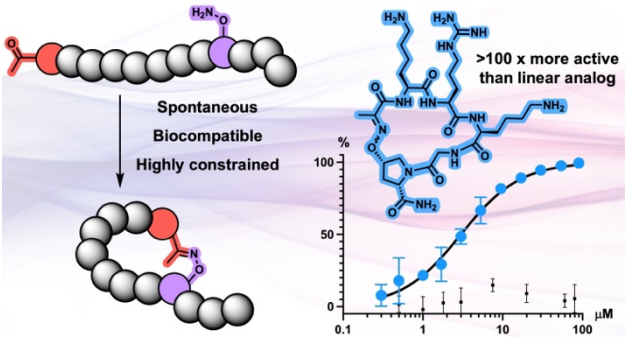
Supporting Information Placeholder



ABSTRACT: The biocompatible synthesis of constrained peptides is challenging. Oxime ligation is a bioorthogonal technique frequently used for protein bioconjugation. We report a straightforward method to install N-terminal ketones and aminooxy side chains during standard solid-phase peptide synthesis. Cyclization occurs spontaneously after acidic cleavage or in aqueous buffer. We demonstrate the facile synthesis of protease inhibitors with varying conformational constraint. The most constrained peptide displayed an activity two orders of magnitude higher than its linear analog.

Macrocyclic peptides are of rapidly growing interest for drug developers in academia and pharmaceutical industries.[1] Constraining peptides into macrocycles can prevent their breakdown through proteolysis and hence increase their metabolic stability. Cyclization further prearranges peptides into their active conformation, thus increasing their affinity due to a reduced entropic barrier compared to their linear analogs.[2] However, chemoselective modification of peptides, such as macrocyclization, remains a major challenge.[3] Biocompatible methods are particularly valuable, as they allow chemoselective modifications of peptides and proteins under physiological conditions.

Oxime ligation is a bioorthogonal technique that is frequently used for protein conjugation, but currently neglected for peptide macrocyclization.[4–8] Oximes are formed from the biocompatible reaction between ketones or aldehydes and alkoxyamines.[9] The resulting ligation product consists of only three heavy atoms (C=N-O). It is this small size that allows the moiety to be incorporated into peptides without disturbing the natural properties.[10]

In this study we present a bioorthogonal one-pot macrocyclization technique that uses oxime formation between an N-terminal α-ketoamide and an alkoxyamine amino acid (Figure 1). While N-terminal α-ketoamides in peptides and proteins can be indirectly accessed through a biomimetic transaminase reaction using pyridoxal 5′-phosphate (PLP) under biocompatible conditions,[11–14] the coupling of α-ketoacids to the N-terminus offers a direct and sequence-independent route to α-ketoamides with various substituents. Our technique generates macrocyclic peptides with little to no impurities and can be applied across a variety of amino acid sequences. The strategy capitalizes on solid-phase peptide synthesis (SPPS) using standard building blocks and is hence fully amenable to automatization (Figure 1).

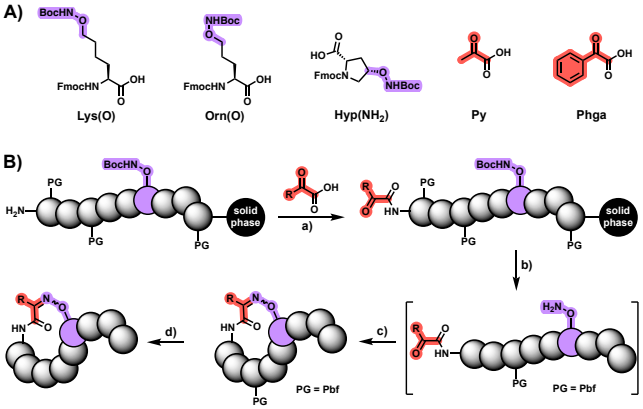


Figure 1. (A) Protected alkoxyamine amino acids and α-ketoacids used in this study: (*S*)-2-(amino)-6-(aminoxy)hexanoic acid, Lys(O); (*S*)-2-(amino)-5-(aminoxy)pentanoic acid, Orn(O); (*S,S*)-4-(aminooxy)proline, Hyp(NH_2); pyruvic acid, Py; phenyl glyoxylic acid, Phga. (B) General scheme for the synthesis of macrocyclic peptides using oxime ligation (PG = protective group). (a) HBTU, HOBt, DIPEA, DMF; (b) HFIP, HCl (aq.), TIPS (92.5:2.5:5); (c) spontaneous or ammonium acetate buffer pH 4.5; (d) TFA, H_2O, TIPS (92:4:4); only necessary if PG = Pbf.

Few orthogonally protected amino acids bearing alkoxyamines in the side chain for SPPS are commercially available (Figure 1A). However, the installation of N-terminal α-ketoamides during SPPS has proven challenging in the past, mainly due to acid-catalyzed hydrolysis of the amide bond adjacent to the ketone.[15] Indeed, when we attempted to couple pyruvic acid to the peptide N-terminus using standard SPPS conditions and subsequent TFA cleavage, we obtained mixtures of the desired and truncated peptides (data not shown). To exclude the possibility of incomplete pyruvate coupling, we added an acetylation (capping) step and still observed truncation, proving TFA cleavage conditions as the cause of truncation (data not shown). Attempts to optimize the contents of the TFA cleavage cocktail were unsuccessful to fully suppress hydrolysis (data not shown).

Presuming that the α-oxime amide product is less prone to hydrolysis than the α-ketoamide intermediate, we set out to identify more benign (TFA-free) conditions that allow cleavage of the solid support and Boc-deprotection of the alkoxyamine to facilitate oxime formation prior to final TFA exposure (Figure 1). After optimization (data not shown), we successfully identified a cleavage cocktail containing HFIP, HCl (aq.), and TIPS (92.5:2.5:5) that is broadly applicable to a range of peptide sequences (Table 1) without any evidence of hydrolysis. To our surprise, this cleavage cocktail also resulted in spontaneous oxime ligation and peptide macrocyclization. Only peptide **5a** required additional treatment with ammonium acetate buffer at pH 4.5 to trigger cyclisation to **5b**. The only protective group that remained on peptides following this treatment was the arginine-protective group Pbf. To ensure full deprotection of any given peptide sequence, we routinely applied a final cleavage mixture of TFA, H_2O, and TIPS (92:4:4) after macrocyclization. Using these general conditions, we obtained macrocyclic peptides **1b**-**14b** in 83–98% HPLC purity (Table 1).

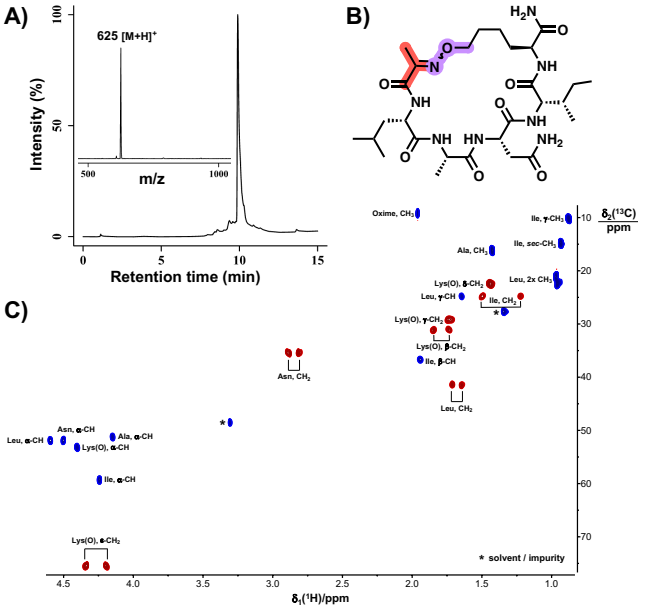
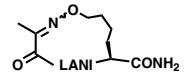
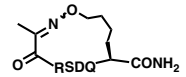
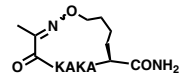
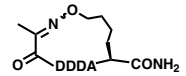
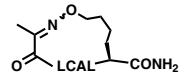
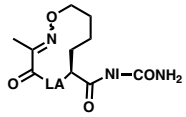
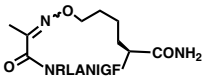
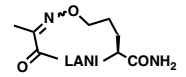
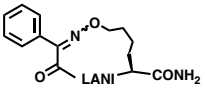
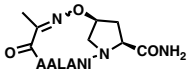
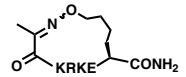
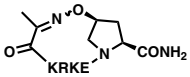
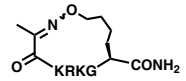
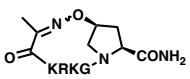


Figure 2. Characterization of model peptide **1b**. (A) LC-MS chromatogram (254 nm) and corresponding mass spectrum of the crude reaction product indicating 95% of **1b**. (B) Structure of macrocycle **1b**. (C) [^{1}H,^{13}C]-HSQC spectrum of **1b** in D_2O/methanol-d_4 (1:1). Assignments for all cross peaks are indicated (CH_2, red; CH and CH_3, blue).

Table 1: Peptide Sequences and Macrocyclization Purities

Linear peptide[a]		Cyclic peptide		
Cpd.	Sequence	Cpd.	Sequence	%[b]
1a	Py-LANILys(O)	**1b**		95
2a	Py-RSQDLys(O)	**2b**		94
3a	Py-KAKALys(O)	**3b**		98
4a	Py-DDDALys(O)	**4b**		96
5a	Py-LCALLys(O)	**5b**		98
6a	Py-LALys(O)NI	**6b**		91
7a	Py-NRLANIGFLys(O)	**7b**		87
8a	Py-LANIOrn(O)	**8b**		92
9a	Phga-LANILys(O)	**9b**		83
10a	Py-AALANIHyp(NH_2)	**10b**		98
11a	Py-KRKELys(O)	**11b**		92
12a	Py-KRKEHyp(NH_2)	**12b**		88
13a	Py-KRKGLys(O)	**13b**		93
14a	Py-KRKGHyp(NH_2)	**14b**		98

[a]Peptides synthesized by Fmoc solid-phase peptide synthesis using Rink amide resin. [b]Proportion of cyclic peptide in mixture according to LC-MS (purity by HPLC).

We used a simple neutral, hydrophobic model peptide (**1a**) comprised of four amino acids between the N-terminal pyruvate and the C-terminal alkoxyamine amino acid Lys(O) to further analyze the macrocyclization reaction. The high specificity of this transformation is demonstrated by the LC-MS chromatogram displaying only one major peak; indicating the absence of isomers such as *E* and *Z* which commonly occur in oximes (Figure 2A). HPLC purity of **1b** at various wavelengths ranged from 89% to 96% (Figure S1). We further purified cyclic peptide **1b** (Figure 2B) using standard HPLC conditions (solvents with 0.1% TFA; 25% absolute isolated yield) and recorded NMR spectra in D_2O/methanol-d_4 (1:1). We fully

assigned the [^{1}H,^{13}C]-HSQC spectrum (Figure 2C), which also clearly indicates the absence of major isomers. For example, the chemical shifts of the methyl group adjacent to the oxime double bond should be sensitive to *E* and *Z* isomers; however, we only observe a single cross peak for this methyl group. Although we failed to clearly assign the *E* or *Z* isomer by NMR, we assume that the acidic cleavage conditions catalyze oxime isomerization and shift the equilibrium toward the thermodynamically more stable isomer. We found further evidence for such a process, for example, in peptide **7b** which over a duration of 2 hours shifted from an equimolar ratio of isomers toward a 9:1 ratio (Figure S21). Other peptides, e.g., **5b**, **9b**, **14b**, display two major species of identical mass in LC-MS after 2 hours of TFA exposure, indicating equally populated *E* and *Z* isomers.

To explore the full scope of this macrocyclization strategy, we examined 14 peptides of varying sequence with three different alkoxyamine amino acids and two α-ketoacids (Table 1). With sequences between five and nine amino acids long, and cycles containing two to eight amino acids, we demonstrate the application of this technique to a range of macrocyclic structures. Using the amino acid Lys(O) with a rather flexible tether, the cyclization succeeds well with shorter (**6b**) or longer (**7b**) sequences. More constrained cycles can be generated with the hydroxyproline-based amino acid Hyp(O), which was used to generate macrocycles comprising four to six amino acids (**10b**, **12b**, **14b**). We further proved the orthogonality of the cyclization reaction by including a wide range of potentially reactive amino acids (Table 1). Highly positively charged (**3b**), highly negatively charged (**4b**), and even supercharged (**11b, 12b**) sequences did not affect the cyclisation yield. Of particular importance is the tolerance for cysteine (**5b**), as this enables further orthogonal bioconjugation.[16–22] Similarly, the incorporation of a C-terminal tail (**6b**) was well tolerated.

In addition, we investigated phenyl glyoxylic acid (Phga) as an alternative α-ketoacid. Standard amide coupling afforded peptide **9a**, and subsequent cyclisation to **9b** proceeded in 83% overall purity determined by HPLC, proving that aromatic and aliphatic α-ketoacids are equally well tolerated. Hence, this method steps beyond the substrate scope that is accessible through the biomimetic transaminase reaction, which only tolerates a limited number of N-terminal amino acids, particularly Ala, Gly, Asp, Glu, and Asn.[13,14] It must be noted that earlier transamination protocols expand this scope.[23] Attempts to install glyoxylic acid at the N-terminus of peptides using our SPPS approach proved unsuccessful (data not shown), likely due to the increased α-carbonyl reactivity.

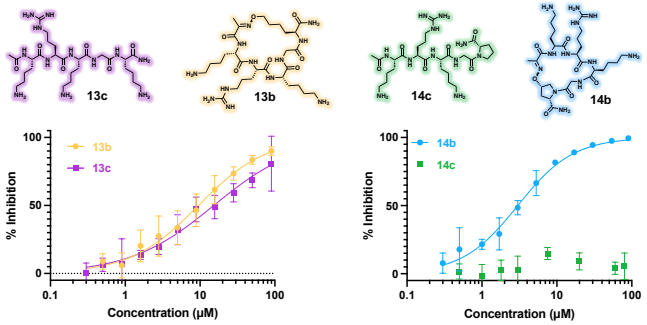


Figure 3. Linear and cyclic peptides and their dose-response curves against the Zika virus protease NS2B-NS3. Compound **14c** showed no inhibition at the highest tested concentration. **13c**, IC_{50} = 15.3 ± 3.7 μM; **13b**, IC_{50} = 9.5 ± 1.8 μM; **14b**, IC_{50} = 3.0 ± 0.4 μM.

To demonstrate the benefit of our oxime macrocyclization strategy for the generation of peptide-based drug candidates, we synthesized linear and cyclic analogs of the peptide substrate of the Zika virus protease NS2B-NS3 (ZiPro). Infections with the Zika virus are linked to an increased risk of the autoimmune disease Guillain-Barré syndrome and, if infected while pregnant, associated with fetal loss and microencephaly.[24,25] The NS2B-NS3 protease is responsible for processing viral polyproteins that are essential for viral replication, making it an important drug target.[26,27] We designed substrate analogs containing the Lys-Arg-Lys-Gly recognition sequence (P_3-P_1' according to Schechter-Berger)[28–30] between the N-terminal α-ketoamide and the C-terminal alkoxyamine amino acid (Figure 3). We deliberately compared Lys(O) with Hyp(O) in macrocycles **13b** and **14b**, respectively, to directly assess the impact of cycle constraint on biological activity. We further constructed two linear control peptides, **13c** and **14c**, which lack the ketone and alkoxyamine functional groups. Dose-response curves indicate only a marginal difference in activity between the rather flexible macrocycle **13b** and its linear analog **13c**; however, the more constrained peptide **14b** is over three times more active than **13b** and its linear analog **14c** shows no activity at the highest measured concentration (Figure 3). These effects are likely related to higher constraint of **14b**, resulting in a lower entropic barrier of binding to NS2B-NS3. We have previously demonstrated that the linker length of cyclic peptides is an important parameter to optimize inhibition of ZiPro.[28,31]

In summary, we present a bioorthogonal technique to cyclize peptides rapidly and selectively through intramolecular oxime formation. This process produces macrocyclic peptides in high purity and is fully amenable to automatization. In addition, we provided a facile synthetic route to access α-ketoamides directly during SPPS, which may have important implications for the design of covalent enzyme inhibitors.[32–34] We demonstrate that highly constrained peptides with superior enzyme inhibition can be generated.

ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

Experimental details and supporting data for peptide synthesis, reaction conditions, inhibition assays, NMR data, Table S1, Figures S1−S21. The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

C.N. gratefully acknowledges funding from the Australian Research Council for a Future Fellowship (FT220100010). L.J.D. and L.M.S. acknowledge PhD and Honours scholarships from The Medical Advances Without Animals Trust.

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