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Ligase-controlled Cyclo-oligomerization of Peptides

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Supporting Information Placeholder

ABSTRACT: A biomimetic, one-step ligase-catalyzed cyclo-oligomerization mediated by butelase 1, an Asn/Asp-specific ligase, is introduced, which is time, concentration, length and sequence dependent. This reaction yields cyclic mono-, di-, tri-, and tetra-mers from peptide precursors containing 3–15 amino acids ended with Asn and a His-Val tail. The cyclomonomers were favored when the peptide lengths were >9 amino acids. A turn-forming Pro residue at the P2 position favored the formation of higher-order cyclo-oligomers.

One-step catalyst-controlled oligomerizations are efficient and avoid the repetitive steps of coupling and purification. This approach has been successfully demonstrated in the synthesis of small molecules^{1, 2} as well as a collection of cyclic oligomeric depsipeptides.³ An enzymatic process using a ligase is timely and highly underdeveloped to provide a “green” approach for oligomeric peptides.^{4–6} Here, we report an efficient ligase-controlled cyclo-oligomerization using butelase 1, a recently isolated peptide ligase from a cyclotide-producing plant *Clitoria ternatea*.^{7, 8}

Butelase 1 belongs to C13 subfamily of Cys proteases represented by asparaginyl endopeptidase (AEP) but it acts as a ligase in near neutral conditions to mediate peptide-bond formation. Butelase 1 is linkage-specific and recognizes the C-terminal Asx-His-Val motif, forming an Asx-Xaa peptide bond either intra- or intermolecularly with broad tolerance for almost any N-terminal amino acids Xaa, including D-amino acids.⁹ Thus far, butelase 1 is the most efficient Asx-specific ligase, as it shows a catalytic efficiency up to $1.34 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$.¹⁰ In addition, butelase-mediated ligation is traceless and does not introduce an extrinsic recognition signal into the product. Thus, this enzyme is well suited for preparing naturally occurring^{10, 11} and modified peptides and proteins^{9, 12, 13} as well as for labeling live cells.¹⁴

Previously, we successfully designed an antimicrobial peptide (AMP) dendrimer containing four copies of RLYR motif¹⁵ that was derived from protegrin PG-1 (RGGRLCYCRRRFCVVCVGR)¹⁶ and tachyplesin TP-1 (KWCFRVCYRGICYRRCR).¹⁷ We also showed that the cyclic forms of tachyplesin- and protegrin-mimetics retain their antimicrobial activity and show improved stability.^{18, 19} Thus, we sought to prepare cyclo-oligomeric AMPs containing multiple RLYR motifs using butelase 1 in a one-pot reaction.

Four series of RLYR-containing peptide precursors (**1a–z**) were designed and prepared by Fmoc chemistry (Table S1). Series A precursors (**1a–m**) consisted of a core peptide with 3 to

15 amino acids ($n=3-15$) ended with the butelase-recognizing Asn followed by the HV dipeptide tail. We envisioned that butelase-controlled cyclo-oligomerization would first produce linear dimers **2**, trimers **3**, and tetramers **4** and then the cyclic forms of the dimers **c2**, trimers **c3**, and tetramers **c4**, all with their HV tail removed (Figure 1). Our recent work showed that butelase-mediated ligation with Pro at the P1' position (using the Schechter and Berger nomenclature²⁰) is slow, suggesting that a Pro moiety adjacent to the Asn P1 site could profoundly influence the catalytic ligation reaction.⁷ Accordingly, we incorporated Pro to study the sequence dependence of the butelase-mediated cyclo-oligomerization in the next three series.

Figure 1. Schematic illustration of butelase-controlled oligocyclization. *i*, sequential ligation of linear precursors **1(a–z)** resulted in the formation of linear oligomers; *ii*, head-to-tail cyclization to yield cyclic oligomers. Linear precursors **1(a–z)** were grouped in four series: A, RLYR-related peptide without proline; B, substrate with proline at different positions; C, substrate with proline at P2 position; D, RLYXN-HV with different residues at P2 position. *n*, length of the core peptides containing 3 to 15 amino acids. Designated compound numbers for linear oligomers: **2–4**; cyclic products: **c1**, cyclomonomer; **c2**, cyclodimer; **c3**, cyclotrimer; **c4**, cyclotetramer.

The precursors included peptides containing Pro at the P2, P3, P4 or other positions (series B, **1n–t**, $n=5-11$) for a pairwise

comparison with the peptides in series A to evaluate the effect of proline, peptides with different lengths and a Pro at the P2 position (series C, **1u-y** and **1n**, $n=4-9$), and pentapeptides RLYXN where X = R, P, A, C, Q and T (series D, **1c**, **1n**, and **1z1-4**).

The butelase-controlled cyclo-oligomerization was firstly examined with a representative precursor RLYRGAN-HV ($n = 7$) **1e** in series A at five time points (0 to 16 h) (Figure S1). At 0.5 h, cyclo-oligomers, cyclic dimer **c2e**, cyclic trimer **c3e** and cyclic tetramer **c4e**, were observed. Cyclodimer **c2e** eventually became the major product after 3 h and reached 77% yield at 16 h. Cyclotrimer **c3e** and cyclotetramer **c4e** increased in the first 3 h but were reduced at 16 h time point. This result suggested that the formation of the oligomers is reversible.

To determine the effect of the substrate concentrations, we compared another two peptides RLYRN-HV **1c** and RLYRGRLYRN-HV **1h** in series A, using a fixed enzyme:substrate molar ratio (1:500) and three different peptide concentrations $[S] = 0.01, 0.1$ and 1 mM (Figure S2). At 1 mM, pentapeptide **1c** gave 68% of cyclodimer **c2c** and 6% of cyclotrimer **c3c**. Reducing $[S]$ 100-fold to 0.01 mM resulted in a sluggish reaction, producing 33% of cyclodimer **c2c** with 63% of starting material **1c** remaining after 16 h. In contrast, decapeptide **1h** efficiently afforded cyclic monomer **c1h** as the major product (72-91%) within 12 min at all three concentrations, suggesting the length of the 10-residue RLYR-containing peptide is sufficient for forming a stable head-to-tail macrocycle. These results suggested that the intermolecular step(s) in the butelase-controlled cyclo-oligomerization are concentration-dependent and rate limiting, whereas the termination step involving the head-to-tail cyclization is fast.

Figure 2. Length effect of butelase-controlled cyclo-oligomerization with peptide precursors (**1a-m**, $n = 3-15$, see Table S1). Yield of each cyclic product was quantified using analytical HPLC. n : core peptide length.

A length-dependent effect on product distribution was observed by comparing all 13 peptides precursors in series A (Figure 2, Figure S3). Precursors consisting of peptides with ≥ 9 amino acids, rapidly gave cyclic monomers within minutes and often in yields $>80\%$, suggesting that the minimum core peptide length that favors macrocyclization by butelase 1 is nine amino acids. Precursors consisting of peptides with 5 to 8 amino acids,

preferentially formed cyclodimers with 10 to 16 amino acids, suggesting this is the ideal length for preparing natural cyclodimers that mimic natural cyclodimeric AMPs such as θ -defensins²¹ and gramicidin S.²² The most interesting results were found for short-chain peptide building blocks ($n = 3-4$) **1a** and **1b**. In a one-pot reaction, these precursors yielded cyclotrimers and cyclotetramers as the major cyclic products.

To show the sequence effect on product distribution, we used series B precursors containing Pro residues at different positions (**1n-t**, $n=5-11$) (Figure 3A). RLYPN-HV **1n** with Pro at

Figure 3. Butelase-controlled oligocyclization with peptide precursors containing proline. (A) Peptides (core peptide length $n = 5-11$, **1n-t**) with Pro at P2, P3, P4 or other positions. (B) Peptides ($n = 4-9$) with Pro at P2 position (**1u-y** and **1n** see Table 1). (C) Pentapeptides RLYXN-HV (X = A, C, P, Q, R or T, **1n**, **1o** and **1z1-4**).

P2 position yielded 33% of the cyclotrimer as the major cyclic product and $<1\%$ of the cyclodimer. In contrast, its Pro-free counterpart RLYRN-HV **1c** gave 68% of the cyclodimer and

Table 1. Antimicrobial activities of cyclic and linear peptide oligomers

Sequence ^c	AA ^d	MIC (μM)							
		<i>E. coli</i>		<i>S. aureus</i>		CREC ^a		MRSA ^b	
		lin ^e	cyc ^f	lin	cyc	lin	cyc	lin	cyc
Monomers	5-7	>100	- ^g	>100	-	>100	-	>100	-
(RLYRN) ₂	10	>25	0.7	>50	10.1	5.0	1.9	>100	14.9
(RLYRGN) ₂	12	9.6	4.2	>50	16.2	9.5	1.3	>100	21.0
(RLYRGAN) ₂	14	6.4	1.0	>50	17.3	3.6	1.0	>100	>25
(RLYRPN) ₂	12	>25	0.9	>50	6.3	12.0	1.0	>100	7.8
(RLYRPAN) ₂	14	>25	0.6	>50	17.0	5.5	0.5	>100	39.5
(RLYRN) ₃	15	1.4	1.8	1.8	2.7	2.1	2.1	0.7	2.1
(RLYRGN) ₃	18	0.9	0.7	4.7	2.7	1.6	0.8	0.5	2.8
(RLYRGAN) ₃	21	0.5	0.5	3.3	3.8	0.1	1.1	3.5	5.1
(RLYRPN) ₃	18	1.6	0.6	2.4	4.2	4.3	0.8	3.7	2.5
(RLYRPAN) ₃	21	0.5	0.8	3.2	2.8	0.2	0.9	4.6	4.2
TP-1	17	0.42	-	2.44	-	1.2	-	2.0	-

^aCREC: strain DR23975. ^bMRSA: strain DR15686. ^cMonomers: synthetic short peptide RLYRN, RLYRGN, RLYRPN, RLYRGAN and RLYRPAN **1(a-e)**. ^dAA: length of peptides. ^eLin: linear peptide without HV tail. ^fCyc: cyclic peptide. ^g:- data not applicable.

only 6% of the cyclotrimer. Other pairwise comparisons between **1o-t** and **1c-i** showed that there were no substantial differences in the product distribution patterns when Pro was in positions other than P2 (Figure S4). To confirm the findings from series B, the Pro residue was included at the P2 position in peptides with different lengths ranging from 4 to 9 amino acids (series C) and the increasing yields of the higher-order cyclo-oligomers were obtained as compared with the Pro-free counterparts in series A (Figure 3B, Figure S5). For example, the major products for RLPN-HV **1u**, RLYPN-HV **1n** and RLYRPN-HV **1v** were the cyclotrimers, while their Pro-free counterparts **1b-d** (n = 4-6) gave the cyclodimers as the major products. In the case of RLPYRPN-HV **1w** and RLYRLYPN-HV **1x**, approximately 30% of the cyclodimers and 18-24% of the cyclotrimers were produced, whereas their Pro-free counterparts **1e** and **1f** (n = 7-8) gave 42-77% of the cyclodimers and only 3-5% of the cyclotrimers. The most instructive precursor for the sequence-dependent cyclo-oligomerization was RLYRLYRPN-HV **1y**, which yielded 71% of the cyclodimer, while Pro-free **1g** gave predominantly the cyclomonomer (67%) and only 22% of the cyclodimer. Finally, using series D with RLYXN-HV (X = A, C, P, Q, R and T) as examples, we showed that the oligomeric product distribution of the cyclo-oligomerization was only influenced by Pro at the P2 position, and other tested amino acids did not display such influence (Figure 3C, Figure S6). These results suggested that proline at the P2 position profoundly influenced the product distribution of the cyclo-oligomerization. The steric hindrance of Pro could affect both the backbone flexibility, which controls the proximity of the two termini of our peptides, and the binding affinity towards butelase 1, leading to reduced reaction rates since Pro-Asn is known to form a tight turn in proteins.²³ For peptides (n > 9) that can undergo direct macrocyclization, the presence of Pro residues in the middle of a peptide did not seem to affect the yield of the cyclomonomers.

Next, we performed *in vitro* stability tests of the linear and cyclo-oligomers in human serum (Figure S7). The plasma half-lives of the cyclic peptides were up to 4-fold greater than those

of their linear counterparts and Pro-containing peptides were 2-4 fold more stable than their Pro-free counterparts. Results supported that the cyclo-oligomers were protected from proteolytic degradation by exopeptidase and Pro-containing peptides were more resistant to degradation by endopeptidase and protease.²⁴

The antimicrobial activities of the linear and cyclic oligomers were examined against four bacterial strains (Table 1). As negative controls, none of the monomers showed bactericidal activity at concentrations up to 0.1 mM. The linear dimers were effective against the Gram-negative bacteria *E. coli* and carbapenem-resistant *E. coli* (CREC) in the low μM range, but they were not potent enough to inhibit the growth of Gram-positive *S. aureus* and Methicilin-resistant *S. aureus* (MRSA). In contrast, the cyclodimers showed enhanced inhibitory activities against Gram-negative strains and inhibited the growth of Gram-positive strains with MIC values of 6.3-39.5 μM. All the linear and cyclic trimeric peptides containing six positive charges were able to effectively inhibit bacterial growth with MICs of 0.1-5.1 μM, which are comparable to that of the positive control TP-1. Our results suggested that the RLYR-containing cyclodimers and trimers are good mimics of natural AMPs. A cyclization mixture of cyclodimers and trimers thus could be used for antimicrobial assays directly without further purification. None of the cyclic oligomeric peptides were cytotoxic at concentrations up to 0.1 mM in the haemolytic assay against human erythrocytes, highlighting the selective membranolytic effects of these antimicrobial cyclo-oligomers. Cationic AMPs are known to exert wound healing effects.²⁵ Here, we also observed moderate wound healing-promoting effects of our antimicrobial cyclo-oligomers in the *in vitro* angiogenesis tube formation assays (Figure S8) and scratch assays (Figure S9). Cyclo-dimers and trimers showed two-to-four-fold greater tube formation compared with the negative control. All tested peptides enhanced wound closure by approximately 20% regardless of the backbone morphology or oligomerization status.

In summary, we report the application of butelase 1 for efficiently preparing AMPs via cyclo-oligomerization. This ligase-

controlled reaction is time-, concentration-, length-, and sequence-dependent. The RLYR-containing peptides with 3-8 residues have lengths suitable for preparing cyclodimers, cyclotrimers and cyclotetramers, particularly if they contain a Pro residue at the P2 position. For precursors containing 9 or more core residues, butelase 1 preferentially mediates direct macrocyclization to produce cyclic monomers. The ligase activity of butelase 1 is highly specific, and generally, the hydrolyzed products were not detectable under our experimental conditions. This approach does not require any side-chain protection. Overall, butelase-controlled cyclo-oligomerization is a low-cost and simple one-pot approach for preparing bioactive cyclic peptides with enhanced stability. Because of its compatibility with aqueous conditions, a ligase-controlled approach could be applied to yield, selectively or combinatorially, the cyclomonomers, cyclooligomers and crosslinking of long helical peptides, proteins, foldamers, nanoparticles, and hydrogels. Our approach also provides access to a collection of biased cyclic peptides that could be useful for structure-activity studies.

ASSOCIATED CONTENT

Supporting Information

Materials and Methods, summary table of product yields, HPLC profiles and wound healing assays data were included.

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The manuscript was written through contributions of all authors.

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