Macrocyclic Peptides

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Butelase-Mediated Macrocyclization of D-Amino-Acid-Containing Peptides

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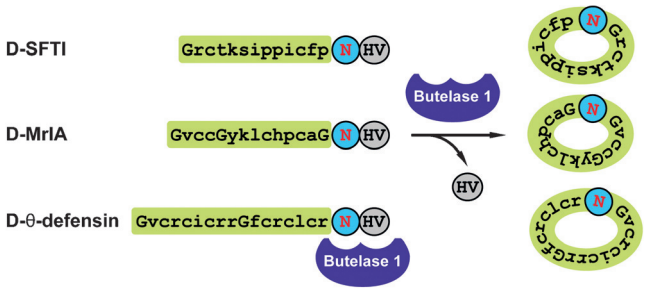
Abstract: *Macrocyclic compounds have received increasing attention in recent years. With their large surface area, they hold promise for inhibiting protein–protein interactions, a chemical space that was thought to be undruggable. Although many chemical methods have been developed for peptide macrocyclization, enzymatic methods have emerged as a promising new economical approach. Thus far, most enzymes have been shown to act on* L*-peptides; their ability to cyclize* D*-amino-acid-containing peptides has rarely been documented. Herein we show that macrocycles consisting of* D*-amino acids, except for the Asn residue at the ligating site, were efficiently synthesized by butelase 1, an Asn/Asp-specific ligase. Furthermore, by using a peptide-library approach, we show that butelase 1 tolerates most of the* D*-amino acid residues at the P1″ and P2″ positions.*

Macrocyclic peptides have emerged as an important class of molecules for drug discovery owing to their enhanced stability, binding affinity, and bioavailability.[1] A large number of natural macrocyclic peptides have been found in diverse organisms,[2] and many macrocyclic compounds are currently used as drugs.[3] Furthermore, macrocycles with their large surface areas and the potential for cell-membrane penetration are perceived to be suitable for inhibiting protein–protein interactions,[4] unlike conventional small molecules.[5] Despite the proven therapeutic potential of macrocyclic peptides, technical challenges in their synthesis, especially that of peptides without cysteine residues or with long chains (>100 amino acids), have hampered their application in biomedical research.

Butelase 1 fills this unique need with its ability to cyclize virtually any peptide from 10 to >200 residues in nearly quantitative yields and very short reaction times, generally within minutes.[6] It is the fastest known peptide ligase and was isolated from *Clitoria ternatea*, a common ornamental and medicinal plant in tropical areas.[6a,7] Butelase 1 enables the macrocyclization of peptides and proteins 20000–200000 times faster than sortase A, the most widely used enzyme for macrocyclization.[6b,8] It is a highly promiscuous enzyme and has been shown to cyclize a broad range of non-native peptides and proteins of various origins with high efficiency.[6b,9] It is C-terminal specific for Asn/Asp (Asx) at the P1 position and requires a C-terminal His-Val dipeptide at the P1′ and P2′ positions for substrate recognition (Scheme 1). Butelase 1 displays broad tolerance for the N-terminal residue at the P1″ position, accepting almost 20 natural amino acids (except for Pro).[6a] Interestingly, it has a more stringent specificity for the residue at the P2″ position, and highly favors aliphatic amino acids, such as Ile/Leu/Val and to some extent Cys residues.



Scheme 1. Butelase-mediated synthesis of D-amino-acid-containing analogues of SFTI, conotoxin MrIA, and θ-defensin. The upper-case letters indicate L-amino acids, and the lower-case letters indicate D-amino acids. Butelase 1 recognizes the C-terminal tripeptide motif NHV, from which HV is cleaved off upon cyclization. The resulted cyclic peptides consist of Gly and D-amino acids, except for Asn.

Thus far, butelase 1 has only been demonstrated to act on peptide substrates with natural L-amino acids. We therefore explored its ability to cyclize D-amino-acid-containing peptides. The incorporation of D-amino acids as building blocks would confer proteolytic resistance and greatly expand the diversity and flexibility in the design of peptide macrocycles as therapeutics.[10] Furthermore, D-peptides are weakly immunogenic and attractive candidates for oral peptide-based drugs.[11] We report herein the successful synthesis of three macrocyclic peptides with sequences derived from sunflower trypsin inhibitor (SFTI),[2e] conotoxin MrIA,[12] and θ-defensin[2c] (Scheme 1). These peptides consist of D-amino acids, except for the Asn residue, which serves as the recognition and cyclization site for butelase 1.

Our previous study showed that in an intermolecular ligation, butelase 1 highly favors Ile/Leu/Val/Cys residues at the P2″ position, while exhibiting broad specificity for the P1″ residue.[6a] We first explored whether this rule also applies to intramolecular cyclization. We designed two peptide libraries, one of which contained a favorable P2″ residue XLYRRGRLYRRN-HV (XL library, X is any of the 20 natural amino acids), and another with an unfavorable P2″ residue XRLYRGRLYRRN-HV (XR library). These peptide sequences were derived from a putative microbial-surface-

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recognition motif RLYR found in porcine antimicrobial protegrin peptides.[13] Their cyclization reactions were examined by MALDI-MS after 1 h by using an enzyme-to-peptide ratio of 1:1000 (see the Supporting Information).

Butelase 1 was markedly more efficient in cyclizing the XL than the XR peptide library. We observed an average 90% cyclization yield for all peptides in the XL library (Figure 1a; see also the Supporting Information). Interestingly, the cyclization was efficient even with Pro as the P1″ residue. In contrast, cyclization yields of less than 50% conversion were observed for the XR library, and were particularly low for hindered amino acids, such as I, V, and P (Figure 1b). We also observed hydrolyzed products for certain peptides in the XR library. When the incoming nucleophiles are not favorable, butelase 1 may function as an asparaginyl endopeptidase to hydrolyze the peptide bond between Asn and HisVal. These results confirm that butelase 1 prefers Leu over Arg at the P2″ position and accepts a broad range of P1″ residues for cyclization. Interestingly, two peptides starting with GR and HR in the XR library afforded >90% cyclization yields even though Arg is not a preferred amino acid at the P2″ position. The lack of a side chain may allow the fast diffusion of glycine into the enzyme active site to enable the cyclization. Histidine, on the other hand, is a conserved amino acid at the P1′ position in the cyclotide precursors, the natural substrates of butelase 1.[7,14] Its high sequence conservation indicates that butelase 1 may have a strong binding affinity for His as the incoming nucleophile. These factors could contribute to the efficient cyclization of GR and HR peptides.

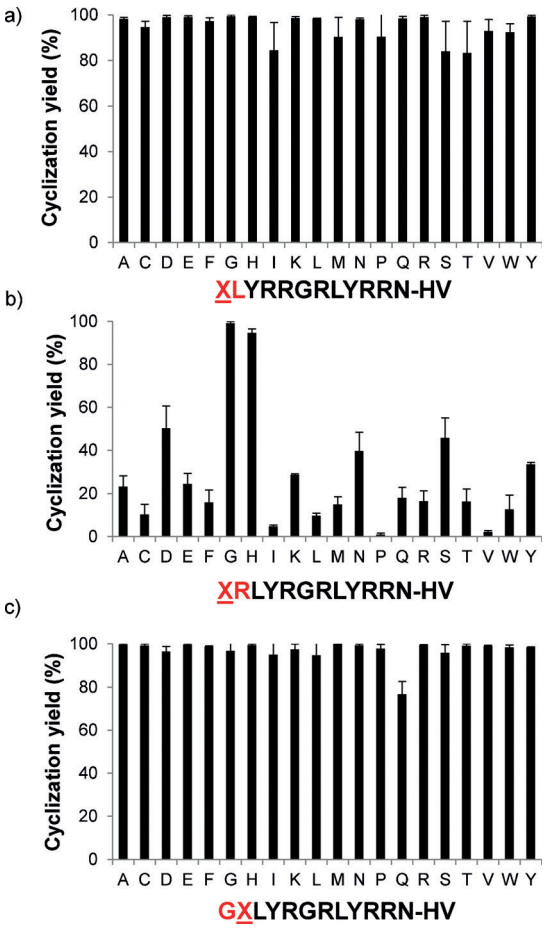

Figure 1. N-terminal substrate specificity for cyclization. a–c) Cyclization yields of three peptide libraries: XLYRRGRLYRRN-HV, XRLYRGRLYRRN-HV, and GXLYRGRLYRRN-HV. X is any of the 20 natural amino acids. Assays were performed with 50 nM butelase and 50 μM peptide at 42 °C for 1 h. The percentage yields are the mean values obtained from three replicates, and standard deviations for the measurements are indicated by the error bars.

To further confirm this observation, we synthesized and screened a third peptide library, GXLYRGRLYRRN-HV (GX library). MS analysis showed that butelase 1 cyclized all 20 peptide substrates in the GX library with excellent yields (Figure 1c; SI, Section I). This result suggests that when P2″ residue is not Ile/Leu/Val/Cys, P1″ residue is preferable to be Gly for efficient ligation. The HXLYRGRLYRRN-HV (HX) library remains to be tested. This finding provides new insight into the substrate specificity of butelase 1 and explanation for its ability to cyclize SFTI and insect antimicrobial peptide thanatin whose P2″ residues are Arg and Ser, respectively.[6a]

Next, we determined whether butelase 1 could recognize D-amino acids as incoming nucleophiles. We screened a fourth peptide library, xLYRRGRLYRRN-HV (xL library, x is a D-amino acid). MS analysis showed that butelase 1 tolerated the majority of D-amino acids as incoming nucleophiles, with yields ranging from 50 to 95% (Figure 2a; see also the Supporting Information). On the basis of cyclization efficiency, we could divide these amino acids into three groups: those producing high yields of 75–95% (a, c, h, m, n, q, s, w), moderate yields of 40–70% (d, e, f, k, r, t, y), and low yields of

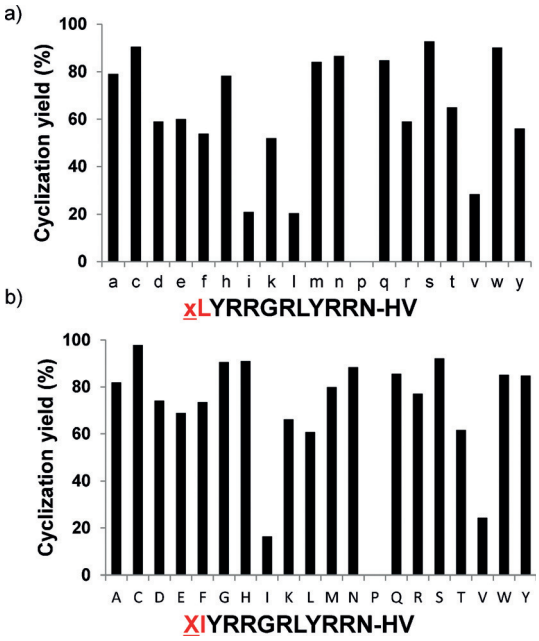

Figure 2. Substrate tolerance of butelase 1 for D-amino acids at the P1″ and P2″ positions. a, b) Cyclization yields of two peptide libraries: xLYRRGRLYRRN-HV and XlYRGRLYRRN-HV. X and x are L- and D-amino acids, respectively. Assays were performed with 200 nM butelase and 50 μM peptide at 42 °C for 2 h.

<20% (i, l, v, p). We observed the formation of hydrolyzed products in 10–60% yield for the latter two groups.

We further examined whether butelase 1 could tolerate a D-amino acid residue at the P2″ position by screening a fifth peptide library, XlYRRGRLYRRN-HV (Xl library, X is an L-amino acid, l is D-leucine at the P2″ position). We observed cyclization yields of 60–90% for all residues except for Pro and β-branched amino acids (I, V; Figure 2b; see also the Supporting Information). This result suggests that butelase 1 can tolerate D-Leu at the P2″ position.

We then explored the ability of butelase 1 to cyclize D-amino-acid-containing bioactive peptides. We synthesized D-amino acid versions of peptides with sequences derived from sunflower trypsin inhibitor, conotoxin MrIA, and θ-defensin. To allow cyclization, we modified these peptides with a C-terminal tripeptide NHV for butelase recognition. As the HV tail will be cleaved off upon cyclization, the resulting cyclized peptides will consist of D-amino acids, except for Gly and Asn at the ligating junction. The cyclization reactions were performed under reducing conditions and monitored by MS every 5 or 10 min. MS analysis showed cyclization yields above 95% for all three peptides (Figure 3; see also the Supporting Information), which demonstrated the synthetic utility of butelase 1 for D-amino-acid-containing peptides.

To gain further understanding of the substrate tolerance of butelase 1 for D-amino-acid-containing peptides, we synthesized and cyclized the L forms of these peptides for comparison. There were marked differences in cyclization efficiency between D- and L-peptides. Our results showed that butelase 1 cyclized L-peptides faster than D-peptides. However, the cyclization rates differed among the three pairs of peptides studied. Under the same experimental conditions (50 μM peptide and 0.5 μM enzyme), the cyclization of L-SFTI, (N_{14})-D-SFTI, L-conotoxin, (N_{15})-D-conotoxin, L-θ-defensin, and (N_{18})-D-θ-defensin was complete in 15, 60, 1, 15, 1, and 60 min, respectively (Table 1). The cyclization rate was about four times lower for D-SFTI than for L-SFTI. However, there were marked differences in cyclization efficiency between the D and L forms of conotoxin and θ-defensin. The cyclization of D-conotoxin was about 15 times slower than that of L-conotoxin, and the cyclization of D-θ-defensin was about 60 times slower than that of L-θ-defensin. The differences in the cyclization rates are partly attributed to the specificity of butelase 1 for P2″ residues. Whereas SFTI contains an Arg residue at this position, a Val residue is present at this position in both conotoxin and θ-defensin. As Arg is not a preferred amino acid for butelase 1 at the P2″ position, the difference between D- and L-Arg is smaller than the difference between the D and L forms of Val. Nevertheless, the high catalytic efficiency of butelase 1 still leads to a high conversion yield of D-amino-acid-containing peptides within an acceptable time frame of 15–60 min with 0.01 molar equivalents of the enzyme. To our knowledge, peptide macrocycles consisting of mostly D-amino acids have not been synthesized previously by an enzymatic method.

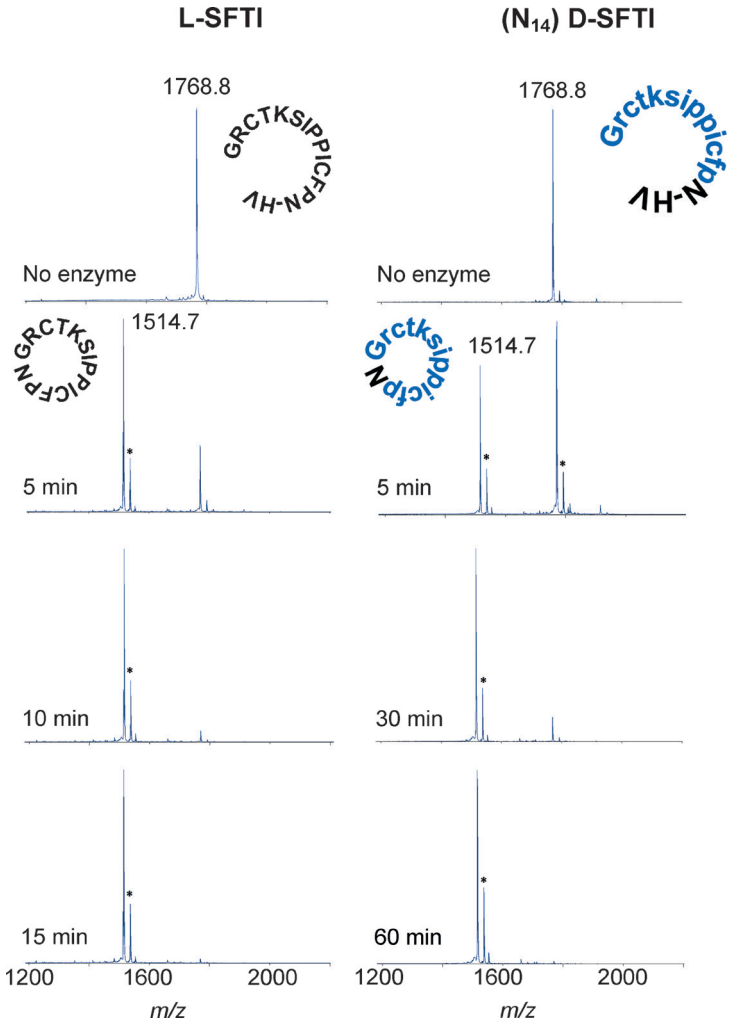

Figure 3. MS profiles of the cyclization of L- and D-SFTI over a time period of 60 min. The linear precursors had an *m/z* value of 1768.8, which changed to 1514.7 upon cyclization. The assays were performed at 42 °C in the presence of butelase 1 (0.5 μM) and the peptide substrate (50 μM). Peaks labeled with * indicate the sodium-binding adducts.

Table 1: Cyclization of D- and L-peptide substrates by butelase 1.

Peptide	Sequence[a]	t[b] [min]	Yield[c] [%]
L-SFTI	GRCTKSIPPICFPN-HV	15	>95
(N_{14}) D-SFTI	GrctksippicfpN-HV	60	>95
L-conotoxin MrIA	GVCCGYKLCHPCAGN-HV	1	>95
(N_{15}) D-conotoxin MrIA	GvccGyklchpcaGN-HV	15	>95
L-θ-defensin	GVCRCICRRGFCRCLCRN-HV	1	>95
(N_{18}) D-θ-defensin	GvcrcicrrGfcrclcrN-HV	60	>95

[a] The lower-case letters indicate D-amino acids. [b] Time required for the reaction to reach completion. All cyclization assays were performed at 42 °C in the presence of the peptide substrate (50 μM), the enzyme (0.5 μM), DTT (1 mM), and phosphate buffer (20 mM) at pH 6.0. [c] Cyclization yield.

To demonstrate the biological activity of the synthetic peptides, we oxidized the reduced and cyclized D- and L-θ-defensin with dimethyl sulfoxide under basic conditions to obtain the native form with three pairs of disulfide linkages.[15] The antimicrobial activity of the resulting peptides was assayed against *Escherichia coli* and *Staphylococcus aureus* in a radial-diffusion assay. To simulate physiological conditions, we performed all assays under high-salt conditions (10 mM sodium phosphate, 100 mM NaCl, pH 7.4). Both peptides were equally active with MIC values at low micromolar concentrations (Table 2). This result is consistent with

Table 2: Antimicrobial activity of D- and L-θ-defensin.

Bacterial strain	Antibiotic resistance	MIC [μM]	
		D form	L form
E. coli	nonresistance	2.1	2.9
S. aureus	nonresistance	6.2	5.3
S. aureus DB55850	methicillin	8.4	7.5
S. aureus DB14329	methicillin	4.7	9.8
E. coli DU09777	carbapenem	1.9	2.6
E.coli DM 04604	carbapenem	3.3	1.8
E. cloacae DM16303	carbapenem	1.7	3.2
E. cloacae DM15118	carbapenem	1.7	4.8
K. sp DR13779	not determined	2.8	3.2
K. sp DU07702	not determined	2.3	3.4
A. baumannii DM18905	carbapenem	2.2	3.6
A. baumannii TTS 6023688355	carbapenem	3.5	3.5

the hypothesis that θ-defensins act by binding to bacterial membranes without the involvement of a chiral receptor.[16] We also evaluated their antimicrobial activity against clinically isolated strains of drug-resistant bacteria,[17] including *Klebsiella*, methicillin-resistant *S. aureus* (MRSA), and carbapenem-resistant strains of *E. coli, Acinetobacter baumannii*, and *Enterobacter cloacae*. Both peptides were broadly active and displayed comparable MIC values to those observed for non-drug-resistant bacteria. Interestingly, D-θ-defensin was slightly more active against several strains. Our results are consistent with a previous study, which showed that θ-defensin composed exclusively of D-amino acids is more active than its all L-counterpart against HIV.[18] These findings highlight the potential of θ-defensin as a novel antimicrobial agent to tackle the global issue of antibiotic resistance.

In conclusion, we have provided new insight into the substrate specificity of butelase 1 by a peptide-library approach. Furthermore, we demonstrated that butelase 1 is a highly promiscuous enzyme with a unique ability to cyclize peptides containing almost exclusively D-amino acids. Its broad tolerance for both natural and unnatural amino acids is highly desirable and should greatly extend its synthetic utility for preparing large diverse peptide libraries for drug discovery.

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