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Peptide Cyclization at High Concentration

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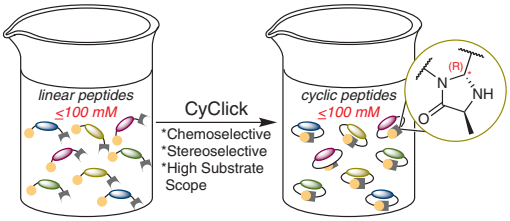

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Abstract The emergence of cyclic peptides as pharmaceuticals has led to an eruption of new methodologies for macrocyclization. However, the cyclization of peptides at high concentrations presents a challenge due to the production of side products like dimers and oligomers. This factor is more pronounced with the cyclization of peptides composed of fewer than seven amino acids, thus has created a need for a new synthetic strategy. Herein, we will elucidate a new chemoselective method termed 'CyClick' that works in an exclusively intramolecular fashion preventing the formation of commonly occurring side products such as dimers and oligomers, even at relatively high concentration.

Key words peptides, macrocycles, high concentration, oligomerization, CyClick chemistry, stereoselectivity

Monika Raj (middle) received her Ph.D. from the Indian Institute of Technology, Kanpur, carried out postdoctoral research at University of Pennsylvania and New York University, USA. She joined as Assistant Professor at Seton Hall University in 2014. Since 2017, she has been Assistant Professor in the Department of Chemistry and Biochemistry at Auburn University where her group develops new chemical tools for detecting posttranslational modifications responsible for diseases, and new methods for synthesis of libraries of cyclic peptides for various biological targets. She supervises second year graduate student, **Rachel Wills** (left) and third year graduate student, **Victor Adebomi** (right).

1 Introduction

There has been an abundance of new methodologies for peptide cyclization in recent years because of the pharmaceutical interest in macrocycles. Macrocyclic peptides have found a niche as therapeutics because they combine the enzymatic stability and membrane permeability of small molecules with the target specificity of biologics.[1] Additionally, they adopt particular secondary structures that allow the efficient inhibition of protein–protein interactions (PPIs).[2,3] However, the utilization of cyclic peptides in the pharmaceutical industry has proven difficult because of the challenges presented in synthetic production. Nature has been known to create peptide cycles by joining ends of linear peptides in a side to side, side to tail, and head to tail manner.[3,4] Researchers have followed this pathway and use commonly known strategies for the macrocyclization of peptides like lactamization, lactonization, and disulfide bridges.[5,6] The issues with these methods include a general lack of site selectivity, stereoselectivity, and perhaps, most importantly, their unsuitability for application on a large scale. At large scale, intermolecular reactions lead to the formation of dimers and oligomers, resulting in low yields of the desired cyclic products. Several current macrocyclization methodologies encounter such side products when cyclization is performed at larger than pseudo dilute conditions (>1 mM conc.).[6–9] High dilution also results in long reaction times, which can lead to epimerization.[10] There are different solutions to circumvent oligomerization at elevated concentrations (Figure 1). These include the use of (A) conformationally induced cyclization via turn-induc-

ers or two-step reactions, (B) entropically driven reactions, (C) carbon–hydrogen functionalization, (D) slow addition of peptides in the reaction mixture, and (E) exclusive intramolecular method.

2 Known Methodologies

A Conformationally Induced Macrocyclization

The introduction of turn inducers into linear peptides has allowed researchers to limit the number of side products formed during the cyclization.[11] Proline is commonly used as a turn-inducer because of its ability to exist in a cis-oid conformation in the peptide, which brings the termini within proximity leading to the formation of a macrocycle.[12] However, the use of well-known turn inducers like Pro-Gly or Asn-Gly[13] create the need for specific amino acids, which limits the applicability to peptide sequences. Moreover, proline creates a hydrophobic area within the surface of the turn, thus decreasing the solubility of the peptide.[14] It has also been shown to restrict biological application and physiochemical modulation[13d] of the peptide chain. Some of these challenges were tackled in an interesting method developed by Katrina Jolliffe.[15,16] The technique utilizes one to three pseudo prolines as turn inducers that are later cleaved during deprotection to form serine or threonine residues[15] (Figure 1, A1). This enables cyclization at higher than pseudo dilute conditions (5 mM conc.) without dimerization, in one pot. Pseudoprolines, which undergo acidic deprotection to generate free cysteines, have also been used to enhance peptide cyclization.[16] However, these methods require specific amino acids like threonine, serine or cysteine as a part of the peptide for macrocyclization to occur.

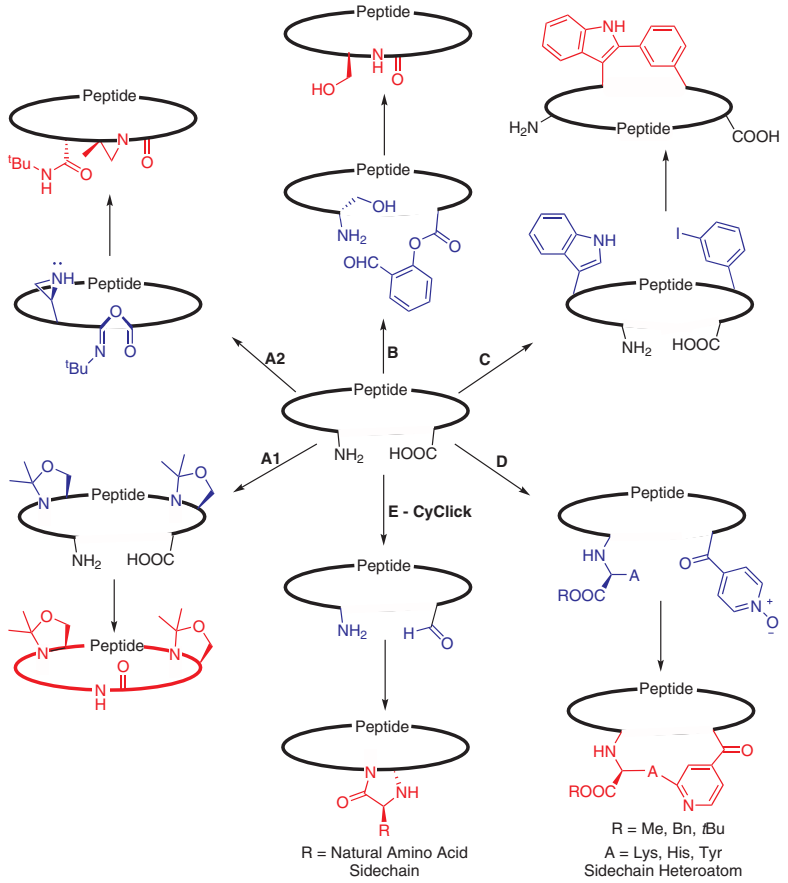

Figure 1 Macrocyclization Methods: (A1) Jolliffe's conformationally induced peptide cyclization facilitated through the use of pseudo-proline turn inducers. (A2) The addition of an aziridine aldehyde and *tert*-butyl isocyanide to the linear peptide by Yudin allowed the formation of a cyclic piperazinone product. (B) Serine/threonine ligation method developed by Xuechen Li uses an imine-induced ring-closing contraction for cyclization. (C) Albericio and Lavilla functionalized the C–H bond of Trp to react with iodo-phenylalanine or iodo-tyrosine to complete the cyclization. (D) Piotrowski utilized Lys, Tyr, and His side chains to react with a pyridine-N-oxide-carboxamide to create the cyclic product. (E) CyClick exploits the chemoselective nature of amine aldehyde reactions to create an imine intermediate that is then trapped by a nucleophilic attack from amidic nitrogen at the second position to form a 4-imidazolidinone macrocycle.

Other turn inducers that are known to promote cyclization include oxetane[17] and dihydrophenylalanine.[18] However, side products from dimerization were observed through the use of these methods.

The introduction of specific groups to increase the flexibility of the linear peptide is also known. Glycine and non-canonical amino acids like β-alanine and γ-aminobutyric acid[19] have been shown to favor the formation of intramolecular cyclization. However, intermolecular reactions that lead to dimerization still occur at high concentrations. Other conformational methodologies for the cyclization of peptides at high concentrations have been created to work in a two-step manner. These procedures apply an initial reversible step to restrict the peptide, allowing the termini to be in close proximity, followed by a second irreversible step resulting in cyclization. This was demonstrated by Yudin through the use of amphoteric amino aldehydes.[20] They exploited trifluoroethanol as a solvent for stabilization of the peptide and promotion of polar interactions. The peptide was reacted with an amphoteric amino aldehyde followed by the addition of isocyanide for the formation of a macrocycle by a four-component Ugi reaction (Figure 1, A2). This procedure afforded reactions at a high concentration of 200 mM.[20] However, the reaction requires free carboxylic acid and amine groups for cyclization, thus amino acids like Asp, Glu, and Lys need to be protected to preclude the formation of unwanted side products.

B Entropically Driven Reactions

Entropy poses a major challenge in terms of the cyclization of peptides. Generally, the creation of a ring from an acyclic precursor decreases the entropy, making it an energetically unfavorable reaction. Xuechen Li employed an imine-induced ring-closing strategy to overcome the high energy barrier of cyclization. The group was able to cyclize Daptomycin[21] at a concentration of 5 mM by leveraging the increase in enthalpy in amide bond formation to allow for an intramolecular ring contraction performed by O to N acyl transfer (Figure 1, B).[22] The group also applied this chemistry for the cyclization of tetrapeptides, but only achieved considerable yields of cyclic peptides under pseudo dilute conditions (1 mM) along with the formation of cyclodimers.[22] This method requires peptide salicylaldehyde esters at the C-terminus and Ser/Thr amino acid at the N-terminus for the ring-closing reaction to occur, thus requiring additional modification and specific amino acid sequences.

C Carbon–Hydrogen Functionalization

The functionalization of a C–H bond for the creation of new carbon–carbon and carbon–heteroatom bonds has been applied for peptide cyclization in recent years. However, the use of this chemistry requires specific methods to facilitate site selectivity because of the numerous C–H bonds in complex peptides. Some of the approaches include the use of bidentate directing groups, innate directing groups, or targeting aromatic amino acids with weaker C(sp^2)-H bonds.[23–26] Albericio and Lavilla achieved this by reacting iodo-phenylalanine or iodo-tyrosine with tryptophan in the presence of palladium as a C–H activation catalyst (Figure 1, C).[27] They were able to achieve cyclization at a concentration of 3 mM with low levels of cyclodimerization. However, the nature of this reaction requires that additional tryptophan residues be protected to prevent side reactions.

D Controlled Addition of Peptides

By carrying out the reaction under pseudo-dilute conditions, the formation of the dimers/oligomers can be avoided. Piotrowski and co-workers carried out the cyclization reaction at high concentration (20 mM) by the slow addition of a linear peptide in a reaction mixture/reagent to create an effective pseudo dilute conditions.[28] The linear peptides were slowly added over 15 min to a solution of PyBrop and iPr$_2$EtN in THF to limit the formation of dimers/oligomers.[28] Pendent pyridine-N-oxide-carboxamides at the N-terminus were reacted with side chains of tyrosine, lysine, and histidine at the C-terminus to afford the cyclic product (Figure 1, D).[28] Only trace amounts of oligomerization occurred in specific examples. However, the method is not chemoselective in nature, and thus requires the protection of reactive side chains of Tyr, Lys and His. This kind of effective pseudo dilute conditions works only for the methodologies with high reaction kinetics.

E Exclusively Intramolecular Method

Reactions, that occur in an intramolecular fashion only, can preclude the formation of dimers and oligomers at high concentrations. To achieve this goal, a new CyClick methodology was developed that involves the reaction between peptide aldehydes and the N-terminal amine (Figure 1, E).[29] The aldehyde reacts with the N-terminus to form a reversible cyclic imine intermediate. This promotes conformational preorganization followed by trapping of the cyclic imine with the amidic nitrogen at the second position to generate a stable 4-imidazolidinone cyclic peptide (Figure 2).[29]

The unique feature of this approach is that it works in an exclusively intramolecular fashion. This method was tested to prove its exclusive intramolecular nature with two different experiments. In the first experiment, the CyClick method was compared with a well-known reductive amination approach for macrocyclization[30] that also involves the formation of the cyclic imine intermediate. The macrocyclizations were carried out at high concentration (25 mM) and the formation of only the desired cyclic peptide

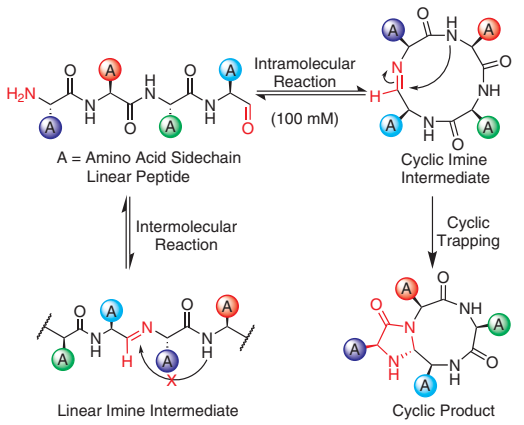

Figure 2 Exclusive Intramolecular Reaction: CyClick Chemistry undergoes an intramolecular imine formation followed by trapping with amidic nitrogen at the second position to form a 4-imidazolidinone cyclic peptide.

was observed by using the CyClick method (right HPLC trace, Figure 3A).[29] However, the reductive amination approach at 25 mM concentration resulted in both linear and cyclic dimers as byproducts (left HPLC trace, Figure 3A).

In the second approach, the CyClick method was attempted in an intermolecular fashion by carrying out a reaction between an aldehyde, pentanal, and the free amine of peptide FVA at high concentration (60 mM, Figure 3B). The formation of the 4-imidazolidinone conjugate product between the two fragments was not observed, even after 24 hours. The linear imine that was formed during the reaction was unable to undergo the second step of trapping by amidic nitrogen. This was confirmed by the reduction of the linear imine with sodium cyanoborohydride, $NaBH_3CN$.

The CyClick method was also carried out at higher concentrations (100 mM) without any evidence of oligomerization. The reaction between the N-terminal amine and the peptide aldehyde is highly chemoselective in nature, preventing the need for reactive amino acid side chains, including lysine, to be protected during cyclization. Overall, this method does not require harsh coupling reagents, removable directing groups, or the protection of side chains. Furthermore, the reaction has proved highly stereoselective. Peptide sequences that contained all L-amino acids resulted in the generation of a new *R*-configured stereocenter on the imidazolidinone ring (**A**, Figure 4). To determine the source of the high stereoselectivity, a peptide sequence was synthesized with D-Ala at the N-terminus but it again resulted in the formation of a new chiral center with *R*-configuration (**B**, Figure 4). A peptide with D-Ile at the second amino acid position generated the imidazolidinone ring with a new *S*-configured stereocenter (**C**, Figure 4). These studies showed that the chirality of the amino acid at the second position dictates the stereochemistry of the new chiral center.

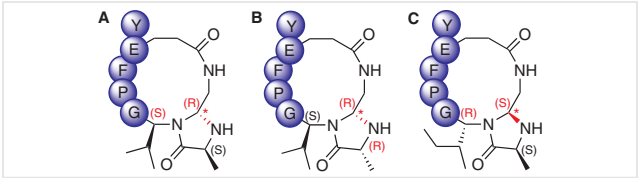

Figure 4 Origin of Stereochemistry: (A) Peptide sequence with all L-amino acids showed a new *R*-configured stereocenter. (B) The terminal amino acid was replaced with D-Ala and resulted in a consistent *R*-stereocenter. (C) The second amino acid position was replaced with D-Ile to afford a new stereocenter with *S*-configuration.

The CyClick method was tested on a wide range of linear peptide sequences to confirm the broad substrate scope of the method. Trials were performed on over 35 linear peptides with varying sequences. No evidence of unwanted byproducts was observed with unprotected linear peptides containing reactive amino acids such as Lys, Ser, Asp, Trp,

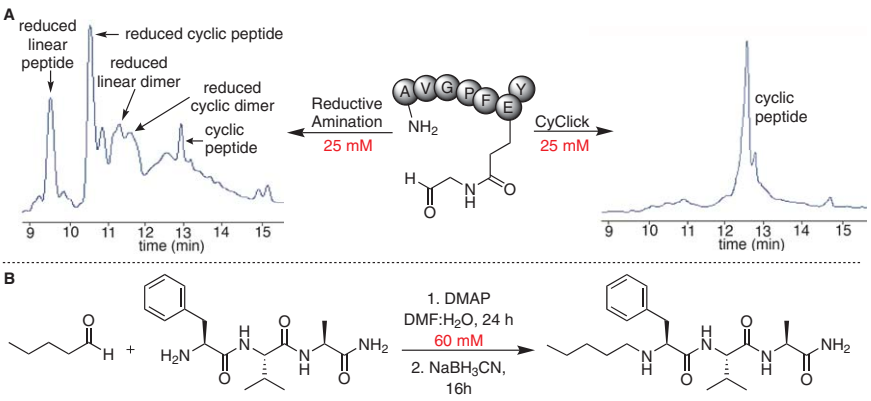

Figure 3 Exclusive Intramolecular Nature of CyClick Chemistry: (A) CyClick macrocyclization was carried out at 25 mM conc., DMF/H_2O (1:1), DMAP, at room temperature and showed only the cyclic product. Reductive animation was performed at 25 mM, DMF/H_2O (1:1), $NaBH_3CN$, at room temperature and gave evidence of oligomerization. (B) Intermolecular reaction of aldehyde, pentanal with peptide FVA did not produce 4-imidazolidinone cyclic peptide after 24 h. However, treatment with $NaBH_3CN$ generated a linear reduced imine dimer product.

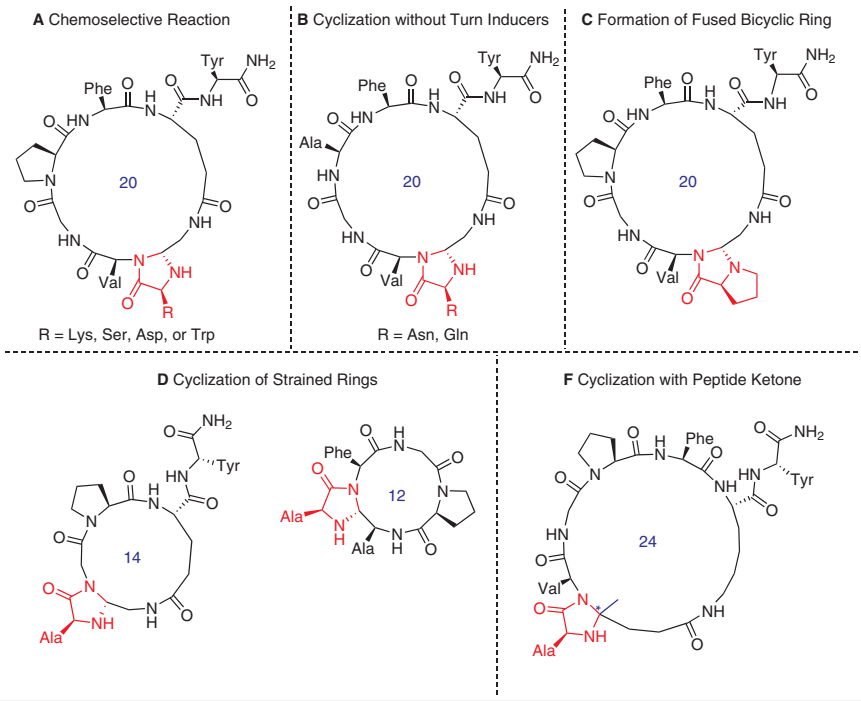

Figure 5 Chemoselectivity of CyClick Chemistry: (A) Amino acids with reactive side chains displayed good yields. Lys: 84%, Ser: 80%, Asp: 64%, Trp: 84%. (B) Peptides without turn inducers generated cyclic peptides in good yields, Asn: 65%, Gln: 53%. (C) The formation of a bicyclic ring with N-terminal proline. (D) AGPEY cyclized in a head-to-side manner in 88% yield. Head-to-tail cyclization with AFGPA gave a tetracyclic peptide with 71% yield. (F) CyClick reaction with peptide ketones generated cyclic peptides with a quaternary chiral center. Center numbers denote atoms within the cyclic ring.

and Tyr (Figure 5A), thus verifying the chemoselective nature of the method. Linear peptides lacking turn inducers were cyclized with good conversions (53–65%; Figure 5B). CyClick reaction with proline amino acid at the N-terminus generated a fused bicyclic 1*H*-pyrrolo[1,2-*c*]imidazole-1-one macrocycle (Figure 5C). CyClick chemistry generated strained head-to-tail and head-to-side chain cyclic peptides (14–12 atoms) in good yields (71–88%, Figure 5D).

CyClick chemistry also worked with a less reactive peptide ketone instead of a peptide aldehyde and generated the cyclic peptide with a quaternary chiral center at the site of cyclization (36%, Figure 5F). These reactions highlight the versatility of CyClick chemistry as a chemoselective, intramolecular strategy for the cyclization of peptides at high concentrations.

Conclusion and Outlook

The macrocyclization of peptides, as a field, has expanded rapidly in the last decade and new methodologies are being published often. One of the major challenges with the synthesis of cyclic peptides is the formation of byproducts such as dimers and oligomers at high concentrations due to intermolecular reactions. CyClick chemistry is one such method that has found a way to resolve this particular challenge. The methodology allows for the synthesis of cyclic peptides at high concentrations of 100 mM without the formation of dimers or oligomers. However, cyclization of larger peptide sequences over 17 amino acids and small peptide sequences under four amino acids has yet to be explored. The CyClick reaction also showed a drastic reduction in cyclic product yields when bulky amino acids, like valine, are located at the N-terminus. Moreover, the nature of the cyclization reaction prohibits proline from being placed at the second amide backbone position. A method that encompasses the features of CyClick chemistry and is applicable to all peptide sequences, regardless of length and position, could greatly enhance the field of cyclized peptides and extend their use in the pharmaceutical industry.

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