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Site-Selective Peptide Macrocyclization

Rachel Wills,[a] Victor Adebomi,[a] and Monika Raj*[a]

Cyclized peptides have seen a rise in popularity in the pharmaceutical industry as drug molecules. As such, new macrocyclization methodologies have become abundant in the last several decades. However, efficient methods of cyclization without the formation of side products remain a great challenge. Herein, we review cyclization approaches that focus on site-selective chemistry. Site selectivity in macrocyclization decreases the generation of side products, leading to a greater yield of the desired peptide macrocycles. We will also take an in-depth look at the new exclusively intramolecular N-terminal site-selective CyClick strategy for the synthesis of cyclic peptides. The CyClick method uses imine formation between an aldehyde and the N terminus. The imine is then trapped by a nucleophilic attack from the second amidic nitrogen in an irreversible site-selective fashion.

1. Introduction

Cyclic peptides have been known to display a wide range of biological activities in nature.[1–7] As such, they play an important role as pharmaceuticals because they merge the specificity of proteins with the stability of small molecules. They also have the ability to inhibit protein-protein interactions (PPIs) and can bind to extended interfaces of protein targets which have been previously referred to as undruggable.[8–17] Macrocyclic peptides tend to be very resistant to proteolytic degradation and exhibit high binding affinity to their targets as compared to their linear counterparts due to the low entropy cost of binding.[18–19] These qualities have made macrocyclic peptides desirable therapeutic agents and increased the demand for efficient cyclization techniques. In the past three decades, there has been an abundance of new methodologies for the generation of cyclic peptides.[20,21] However, the generation of side products and the limited yield of the cyclized peptides limit their application for the preparative synthesis of peptide macrocycles. One approach known to diminish the creation of side products is the use of site-selective chemistry for the macrocyclization of peptides.

Site selectivity is a form of chemoselectivity that allows for a reaction to occur on a specific location even in the presence of similar functional groups (Figure 1). Chemoselective reactions that are not site selective have a tendency to generate multiple products and are not a part of this review (Figure 1). By exploiting the nature of this chemistry, cyclization can occur at a single location within the complex linear peptide, thus minimizing the side reactions and increasing the desired cyclic product yields.

The site-selective peptide macrocyclization strategies are the main focus of this article. Those strategies have been categories into two major groups for the purpose of this review:

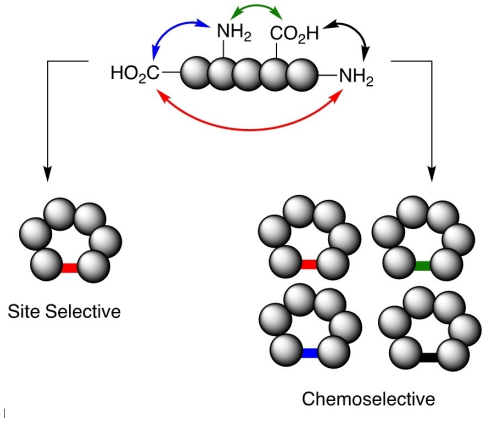

Figure 1. Site selectivity is a type of chemoselectivity that demonstrates the ability of macrocyclization to occur at a single specific locale. Chemoselectivity illustrates the use of functional groups to allow cyclization at various positions.

enzyme-mediated macrocyclization and chemically facilitated macrocyclization.

2. Enzyme-Mediated Macrocyclization

Enzyme-catalyzed cyclization of peptides is intriguing because enzymes can cyclize peptides efficiently at a low enzyme to substrate ratio.[22,23] Several enzymes responsible for mediating peptide cyclization are obtained from nature and catalyze the reaction through ribosomal or nonribosomal pathways.[24,25] There are several recent methods reported that allow the synthesis of cyclic peptide libraries through the use of enzymes in a site-selective manner.[26–28] Thioesterase domains, peptiligase, asparaginyl endopeptidases, sortase, glutathione S-transferase, split intein method, and several other examples of enzyme-mediated macrocyclization will be explored in herein. This review focuses on the different mechanistic pathways followed by the enzymes to catalyze site-selective peptide macrocyclization.

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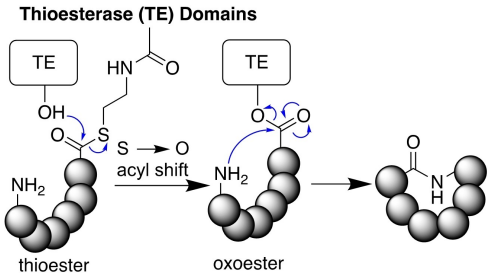
2.1. Thioesterase (TE) domains

The site specific biosynthesis of many non-ribosomal peptide macrocycles are catalyzed by multidomain mega synthetases containing a downstream thioesterase domain. The multidomain mega synthetases are large multifunctional proteins organized into sets of functional domains called modules.[29] The order of the modules corresponds directly to the sequence of monomers in the cyclic products. Linear peptide intermediates are covalently tethered by thioester linkages to a carrier domain (S-phosphophantheine) in each module (Scheme 1).[29] The final step in the synthesis of the cyclic peptides involves the S to O acyl transfer of the linear peptide to the serine active site of thioesterase. This is followed by a reaction with the N terminus to release the macrocyclic peptide (Scheme 1).[29] As per references [30–32], several groups were able to show that the thioesterase (TE) domain of *tyrocidine synthetase* can independently catalyze the cyclization of linear peptides in a site specific manner,[30–32] with 59 turnovers per minute.[29] These groups cyclized peptides with 9–11 residues containing an S-acetyl cysteine instead of the traditional S-phosphophatetheine.[29] However, any kind of mutations to the D-Phe at the C terminus or L-Orn adjacent to the C terminus abolishes the cyclization.[29] Moreover, thioesterase allows for a chemoenzymatic approach of cyclization because the TE domain can recognize peptidyl oxoesters created on resin or in solution.[31] The use of TE domains has further been explored for the generation of glycopeptide antibiotics by macrocyclization followed by ligation of glycan groups using click chemistry.[32] Another interesting subtilisin-like protease, PatG, cyclizes peptides by following a similar pathway with recognition sequences instead of thioesters.[33] The amide bond next to the heterocycle Thz/Oxz/Pro in the peptide sequence is cleaved and forms an activated ester with the serine at the active site. The activated ester then reacts with the N terminus to generate the cyclic product.[33]



Scheme 1. Thioesterase-mediated peptide cyclization allows terminal cyclization through the use of an S to O acyl transfer and cleavage from the participating enzyme by N-terminal attack on an activated oxoester.

2.2. Peptiligase

Peptiligase is an enzyme known for chemoenzymatic peptide synthesis. The enzyme was first obtained by point mutations of Ser212Cys and Pro216Ala in the proteolytic enzyme subtilisin.[34] The mutations enable peptiligase to carry out covalently mediated amide bond formations. The active site of the peptiligase is thought to have a catalytic triad of Asp, His, and Cys. Based on the mechanism of subtilisin, we believe that Asp forms a H-bond with His, which in turn forms a H-bond with Cys, thus activating it for a nucleophilic attack to form a thioester between the linear peptide and the enzyme (Scheme 2A). The resulting enzyme-acyl complex is susceptible to nucleophilic attack by the N terminus, generating a cyclic peptide with a high synthesis over hydrolysis (S/H) ratio.[34] Peptiligase has been successfully used to cyclize a long peptide, microcin J25, with up to 21 amino acids and a yield of 82%[35]. This enzyme can tolerate organic solvents and cyclization can be performed in the presence of dimethyl sulfoxide and dimethyl formamide. The use of these solvents allows for the solubilization and synthesis of highly hydrophobic peptide macrocycles.[34] An interesting feature of peptiligase is that it

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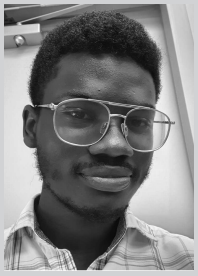

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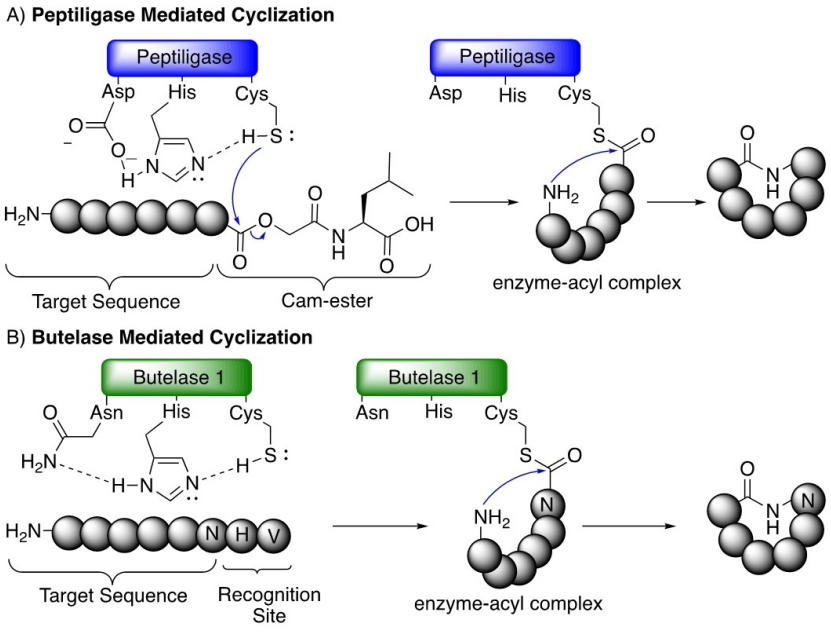

Scheme 2. Role of the catalytic triad in enzymes for synthesis of peptide macrocycles. The Cys in the triad binds with the target sequence and is later cleaved by an S to N acyl transfer initiated by the N terminus. A) Peptiligase cyclizes peptides independent of recognition sequence by using Cam esters as targets. B) Butelase 1 requires a specific recognition sequences (HV) and a terminal Asn for efficient peptide cyclization.

does not require a particular recognition motif making it broadly applicable.[34]

A well-known variant of peptiligase is omniligase. This enzyme has been applied for the synthesis of a wide variety of cyclic peptides, including those containing unnatural moieties such as polyethylene glycol, isopeptide bonds, and D-amino acids.[36] It was also used for the synthesis of a highly constrained tricyclic peptide and a class of multicyclic peptides called cyclotides. Cyclotides are cyclic peptides rich in disulfide bonds.[37] Their unique features often cause them to be referred to as cyclic cystine knots and they have been reported to have important biological activities.[37] Nuijens and co-workers were able to synthesize a cyclotide, MCoTI-II, using chemoenzymatic peptide synthesis (CEPS) in the presence of omniligase.[36] Despite the unique features of omniligase, its wide range application for cyclic peptide synthesis is hampered by the fact that there is a reduced yield associated with short peptide length. A peptide containing a minimum of 13 amino acids is required for efficient cyclization (>85%) without high amounts of dimerization due to intermolecular reactions. 11-mer peptides led to a 25% conversion to cyclic peptides due to competing intermolecular reactions.[36] To enhance cyclization efficiency, the introduction of a well-known turn inducing sequence, D-Pro/L-Pro, was attempted at various positions, but the cyclization efficiency could not be improved over 40%.[36]

2.3. Asparaginyl endopeptidases

Asparaginyl endopeptidases are a class of proteolytic enzymes that hydrolyze amide bonds at the C terminus of asparagine residues.[38] This is accomplished through the use of thiol groups present in their cysteine active site. Several asparaginyl endopeptidases have been involved in the synthesis of peptide macrocycles. A very common example is Butelase 1, which is a peptide ligase that was first isolated from *C. ternatea* by Tam and co-workers.[39] This protein is involved in the ribosomal synthesis of cyclic peptides and is capable of cyclizing a wide range of linear peptides (10 to 200 amino acid residues) at an extremely fast rate with turnover numbers of up to 17 s^{-1} and high catalytic efficiency of 542000 $M^{-1}s^{-1}$.[39] The cyclization by Butelase 1 is highly sequence dependent as it requires Asn/Asp residues at the C terminus. A dipeptide, His-Val (HV), acts as a substrate recognition unit necessary for cyclization. HV is later cleaved from the chain during the cyclization process (Scheme 2B).[38] The rate of the Butelase 1-catalyzed cyclization increases when the amino acid adjacent to the N terminus is aliphatic in nature, such as Leu, Ile, Cys, and Val. Conversion rates averaging 90% were obtained.[38] The replacement of this position with charged residues like Arg lead to significant decreases in the rate of cyclization and the conversions proved to be less than 50%. In addition, hindered amino acids at the N-terminal reduce the yields of peptide cyclization. Yields lower than 10% were reported when the N terminus contained Ile, Val, or Pro and the adjacent amino acid was a positively charged Arg residue.[38] The mechanism of action for Butelase 1 is not yet fully understood. However, it is thought to be mediated by covalent catalysis similar to peptiligase.[39] This occurs via a catalytic triad of Asn, His, and Cys which binds loosely to the cleaved substrate resulting in the formation of an enzyme-acyl complex via Cys, thus preventing it from premature hydrolysis (Scheme 2B).[39] This is followed by the nucleo-

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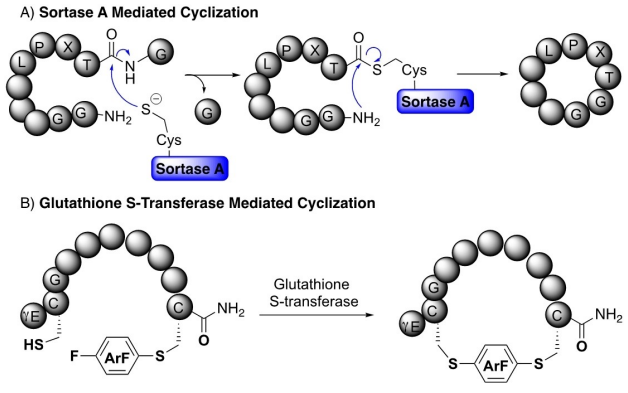
philic attack from the N terminus to the enzyme–acyl complex, generating the cyclic peptide. The disadvantage of using Butelase 1 is the requirement of specific recognition sequence motifs for effective cyclization.[39]

Another asparaginyl endopeptidase that has been implicated in peptide cyclization is rOaAEP1$_b$, a cyclase first isolated from the cyclotide producing plant, *Oldenlandia affinis*.[40] Anderson and co-workers were able to unequivocally show that rOaAEP1$_b$ is involved in the synthesis of cyclotides.[40] They also used this enzyme for the cyclization of a range of bioactive peptides. In contrast to Butelase 1 which requires a NHV recognition sequence, rOaAEP1$_b$ requires a NGL sequence at the peptide's C terminus for cyclization to proceed efficiently.[40]

2.4. Sortase A

Sortase A, an enzyme commonly used for transpeptidation reactions, has also been applied for the synthesis of cyclic peptides. For cyclization, the C-terminal region must have a specific recognition sequence (also known as sorting sequence) LPXTG where X is any amino acid.[41] Additionally, the N terminus must contain oligo glycine residues (Scheme 3A). A cysteine residue on the active site cleaves the bond between Gly and Thr on the sorting signal to form a thioester bond. This bond is then attacked by the amino terminal of the linear peptide to form a cyclic peptide product (Scheme 3A). A major drawback to the use of sortase A is the requirement of 15 amino acids long peptide for efficient cyclization. The need for longer peptide is due to the competing intermolecular reactions.[41] Another major disadvantage of sortase A is the need for the specific recognition sequences at both termini. These factors greatly limit the variety of cyclic peptides that can be made by using this method.



Scheme 3. Mechanistic pathway of sortase A and glutathione S-transferase-mediated cyclization. A) Sortase A is selective for the LPXT sequence at the C terminus and GG at the N terminus, and catalyzes the cyclization by cysteine residue at the active site of enzyme. B) Glutathione S-transferase-mediated cyclization occurs in an S_NAr reaction between cysteine and perflouroaromatic moieties (ArF) on the linear peptides and is selective for γECG sequence at the N terminus.

2.5. Glutathione S-transferase

Glutathione S-transferase is well known for the conjugation of glutathione to drug molecules. It is being applied for peptide cyclization. Glutathione S-transferase-mediated cyclization occurs by an S_NAr reaction between cysteine and perflouroaromatic moieties (ArF) on the linear peptides to yield the corresponding cyclic peptides (Scheme 3B).[42] The specificity of the reaction stems from the fact that it requires an N-terminal γ-Glu-Cys-Gly (γ-E-C-G) tag for the cyclization to occur.[42] Notably this cyclization procedure was applied for the synthesis of cyclic peptides with long chain lengths of up to 40 amino acids.[42]

A major advantage of this cyclization method over many other enzyme-mediated cyclizations is that the cyclization does not yield unwanted dimers and oligomers seen in other enzymatic cyclization method even at concentration up to 10 mM.[42]

2.6. Split intein method

Split intein-mediated circular ligation of peptides and proteins (SICLOPPS) is an interesting technology that allows access to libraries of cyclic peptides from ribosomal synthesized precursors.[43–46] During SICLOPPS, amino acid residues with an N-terminal intein fragment (I_N) and a C-terminal part intein (I_C) fragment are interconnected by a target peptide sequences that is genetically encoded.[43–46] The autocatalytic splicing of the intein fragments results in the head to tail cyclization of linear targets to generate cyclic products. The mechanism for cyclization is a five-step process (Scheme 4).[46] First, fusion occurs between the N-terminal intein fragment I_N and the C-terminal intein fragment I_C to generate an active ligase. Second, the enzyme catalyzes an N to S acyl shift at the I_N junction to create a thioester. In the third step, the thioester undergoes esterification/thioesterification with a nucleophile at the I_C junction to generate a lactone/thioester lariat (Scheme 4). The fourth step involves the cleavage of the lactone/thioester lariat from the fused intein, followed by the fifth step which leads to an O/S to N acyl transfer. This transfer creates a cyclic lactam.[46] Based on this mechanism, the amino acid adjacent to the C-terminal intein (I_c) is involved in cyclization, limiting it to serine, cysteine, or threonine residues. The residue located adjacent to the I_N junction also affects cyclization. For example, sequences with glutamic acid, proline, and asparagine adjacent to the I_N junction do not produce cyclic products.[47]

2.7. Oxime intein-mediated ligation of organo-peptides

Oxime-mediated ring closing is used to create macrocyclic organopeptides through a chemo-biosynthetic pathway. One of the precursors is genetically encoded to generate a *p*-acetylphenylalanine residue at the N terminus, followed by a target sequence of varying lengths and a mini-intein at the carboxy terminal. The intein undergoes transthioesterification similar to

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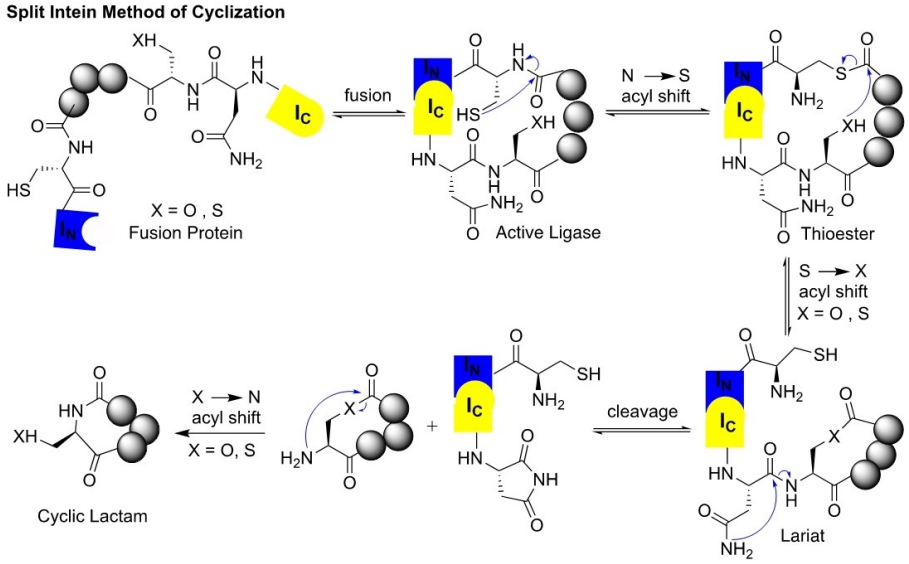
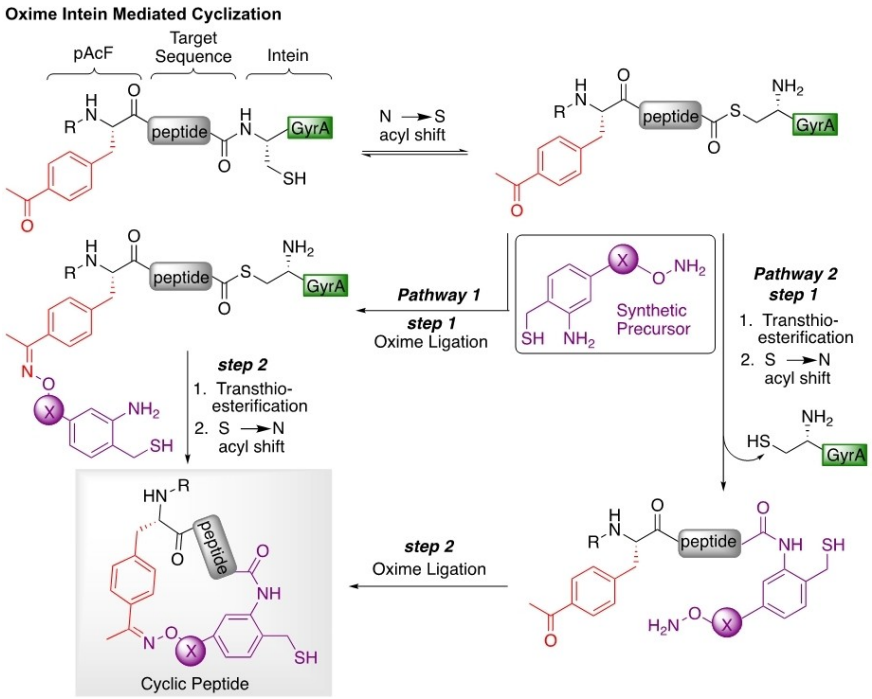


Scheme 4. Mechanistic pathway of split-intein-mediated circular ligation of peptides and proteins (SICLOPPS) for the synthesis of cyclic peptides. A target sequence is located within the molecule that contains inteins at the termini. Cyclization occurs upon fusion of the inteins, and a series of acyl shifts allows for cleavage of the cyclic product from the enzyme precursor.

the split intein method to generate an active thioester at the C terminus (Scheme 5).[48] The addition of a synthetic precursor oxyamine/aminothiol arene to the activated ester leads to the macrocyclization of a biosynthetic peptide through two possible pathways (Scheme 5). Pathway one follows the formation of an oxime between the synthetic precursor oxyamine/aminothiol arene and the *p*-acetyl phenylalanine residue of the biosynthetic peptide followed by the transthioesterification with the C-terminal thioester and an S to N acyl exchange that generates an amide bond at the site of cyclization.[48] The



Scheme 5. Two possible pathways for the synthesis of cyclic peptides by using oxime-intein-mediated cyclization. Pathway 1 involves the oxime ligation of the synthetic precursor (purple) to the aryl ketone (red). This is followed by transthioesterification and a S to N acyl shift for the removal of the GyrA protein (green). The second pathway uses transthioesterification and S to N acyl shift to replace the GyrA protein with the synthetic precursor. Cyclization then occurs through oxime ligation.

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second pathway involves the transthioesterification with the synthetic precursor oxyamine/aminothiol arene followed by an S to N acyl shift to form an amide bond at the N terminus. The cyclization occurs in the second step by the formation of an oxime between the *p*-acetyl phenylalanine residue and the oxyamine/aminothiol arene at the C terminus (Scheme 5). As in the split intein method, not all the amino acids are tolerated at the position adjacent to the intein. The cyclization product was not observed with 8 natural amino acid residues (P, H, G, D, V, K, L, and I). The use of an oxime for a ring closing strategy makes this method a novel chemo-biosynthetic macrocyclization technique.[48]

3. Site-Selective Chemical-Mediated Macrocyclization

Macrocyclization through chemical means has become more prevalent in the field of peptide cyclization because it allows for more flexibility within the amino acid sequence, including the incorporation of unnatural amino acids. However, unlike enzyme-mediated cyclization, site selectivity with unprotected peptides has rarely been achieved by chemical methods. For the purpose of this review, we have split site-selective chemical methods into two categories, N-terminal specific macrocyclization and head to tail selective macrocyclization.

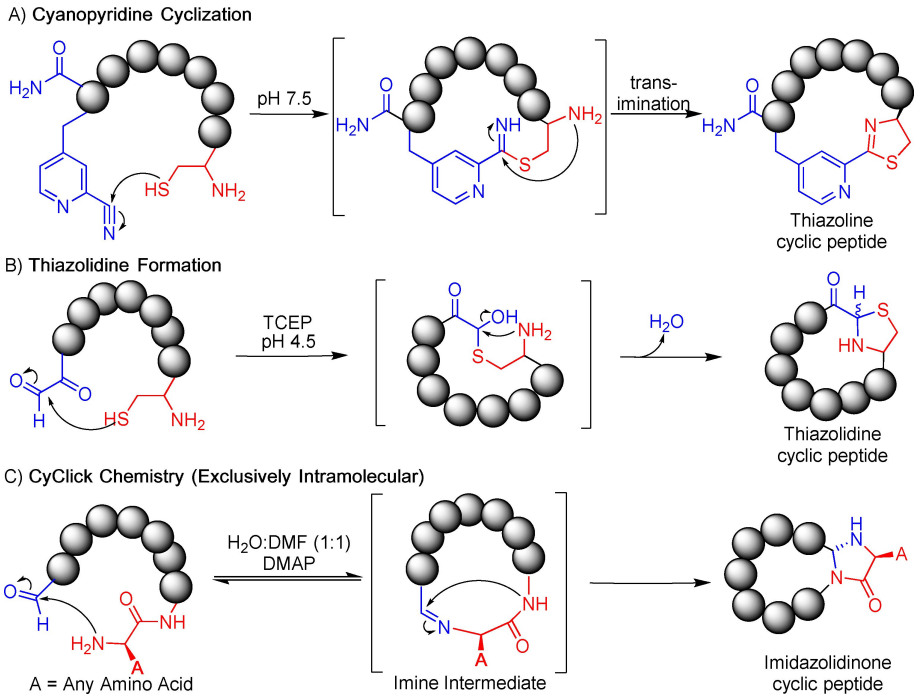
3.1 N-Terminal Macrocyclization

3.1.1 Cyanopyridine cyclization

A novel site-selective cyclization technique that employs a condensation reaction between the N-terminal cysteine and 2-cyanopyridine was published by Huber and co-workers (Scheme 6A).[49] The method proved to be site selective for the N terminus even in the presence of internal cysteine residues.[49] The group cyclized peptides containing five-seven amino acid residues with high yields (90–98%). In addition, the reaction proceeded spontaneously in aqueous solution at neutral pH in less than one hour without any evidence of side products or oligomers. The group also developed a facile method for the incorporation of the 3-(2-cyano-4-pyridyl)-alanine directly on the C terminus utilizing solid support.[49] However, this method is limited due to the requirement of cysteine at the N terminus. It would also be intriguing to see the effect of branched amino acids positioned adjacent to both the termini in macrocyclization process.[49]

3.1.2. Thiazolidine formation

The N-terminal site specific cyclization, known as thiazolidine formation, was created by Tam and co-workers.[50] This method also requires the use of an N-terminal cysteine residue like the cyanopyridine method mentioned above. The reaction occurs between the terminal cysteine and an aldehyde to create a



Scheme 6. N-terminal site-selective methods for cyclization of peptides leading to the formation of heterocycles at the site of macrocyclization. A) N-terminal cysteine cyanopyridine-selective peptide macrocyclization with the formation of thiazoline at the site of cyclization. B) N-terminal cysteine aldehyde-selective peptide macrocyclization with the formation of thiazolidine at the site of cyclization. C) N-terminal aldehyde-selective, exclusively intramolecular CyClick method for peptide macrocyclization forming 4-imidazolidinone at the site of cyclization.

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thiazolidine ring at the point of cyclization (Scheme 6B). A ketoaldehyde, glyoxylaldehyde, was implemented on amino acid side chains to facilitate the cyclization.[50] Glyoxylaldehyde was generated by the oxidation of a serine residue linked to a lysine sidechain, effectively allowing the cyclization between the N terminus and the ε nitrogen of lysine residue. Only one peptide was cyclized as an example of the method. The peptide, C(S*t*Bu)GIGPGRAFGK(Ser)-βAla, gave the cyclic product in 95% yield in sodium acetate buffer at pH 4.5. The group did not show any examples containing branched amino acids or multiple lysine residues.[50] It would be interesting to see how cyclization proceeds with such examples.

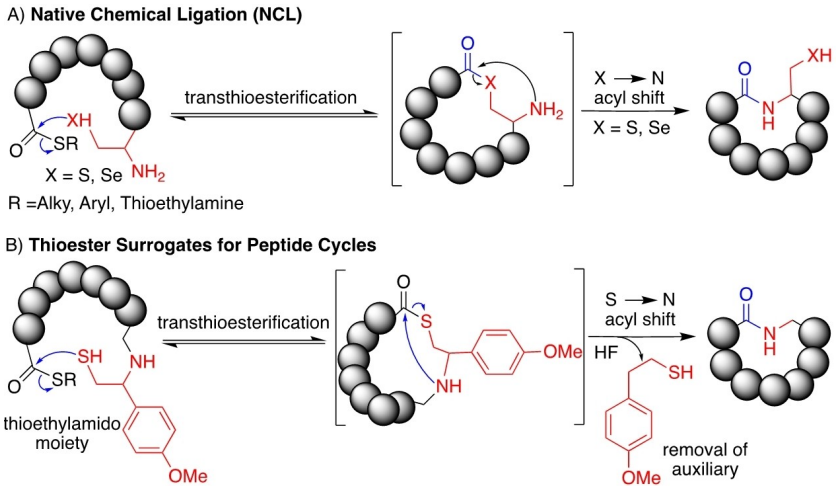
3.1.3. CyClick chemistry

CyClick chemistry is a recent methodology that leverages the highly chemoselective reaction between the N-terminal amine and a peptide aldehyde for cyclization.[51] The reaction between the two moieties creates a reversible cyclic imine intermediate that is trapped by the amidic nitrogen at the second position, creating a stable 4-imidazolidinone cyclic peptide (Scheme 6C). This technique circumvents the N-terminal residue specificity needed in other methods because the reaction takes place with the peptide backbone instead of an amino acid side chain. The reaction occurs in an exclusively intramolecular fashion and tolerates all reactive amino acids.[51] Peptides with various N-terminal residues were cyclized at room temperature in a 1:1 ratio of DMF/H_2O over 16 h. Peptides containing reactive side chains such as Lys, Ser, Asn, Asp, His, Tyr, Cys, and Gln gave the 4-imidazolidinone cyclic products with good yields (45–84%). However, a peptide containing branched amino acid at the N terminus, like valine, resulted in lower yield of the cyclized product (37%).[51] These reactions were examined to reveal only the intended macrocyclic product with no evidence of side products. Additionally, the reaction generated a new chiral center in the imidazolidinone ring that proved to be stereospecific (*de* >99%). A substrate scope with 30+ examples of various ring sizes (12–23 atoms) and sequences was performed with moderate to high yields. The cyclization occurs in both head to tail or side to tail manner, depending on the aldehyde position. The exclusive intramolecular nature, stereospecificity, and N-terminal site selectivity makes this technique attractive for the synthesis of cyclic peptide libraries. However, it would be interesting to see the application of this method in the synthesis of large peptide macrocycles (>17 amino acid residues) and strained macrocycles (<12 atoms).[51]

3.2. Head-to-Tail Macrocyclization

3.2.1. Native chemical ligation and similar methods

Native chemical ligation (NCL) is a site-selective method for macrocyclization that utilizes a thioester at the carboxy terminal and a cysteine residue at the N terminus. The two react by transthioesterification followed by an S to N acyl shift to form a native amide bond at the site of cyclization, much like the split intein method (Scheme 7A).[52] Other cysteines in the amino acid sequence are unable to form the cycle because they lack the necessary free amine. The popularity of this method has led to its expansion by several other groups.[50,53–57] Tam and co-workers introduced novel thioester surrogates for cyclization that are compatible with Fmoc chemistry for facile synthesis.[53] They used a thioethylamido moiety as a surrogate to create a thiolactone ring expansion method of cyclization.[53] Botti and co-workers followed this trend by creating a removable N^{α}-(1-(4-methoxyphenyl)-2-mercaptoethyl) auxiliary group (Scheme 7B).[55] The benefit of this method is that the auxiliary contains a cysteine like moiety adjacent to the terminal amine precluding the need for cysteine in the amino acid chain. However, a standard HF cleavage was required for the removal



Scheme 7. Native chemical ligation (NCL) and its variants for the N-terminal site-selective peptide cyclization. A) NCL approach for cyclization between an N-terminal cysteine and a C-terminal thioester. B) The N-terminal thioester surrogate is used for the synthesis of cyclic peptides with C-terminal thioesters. The auxiliary is later removed with HF to yield the target product.

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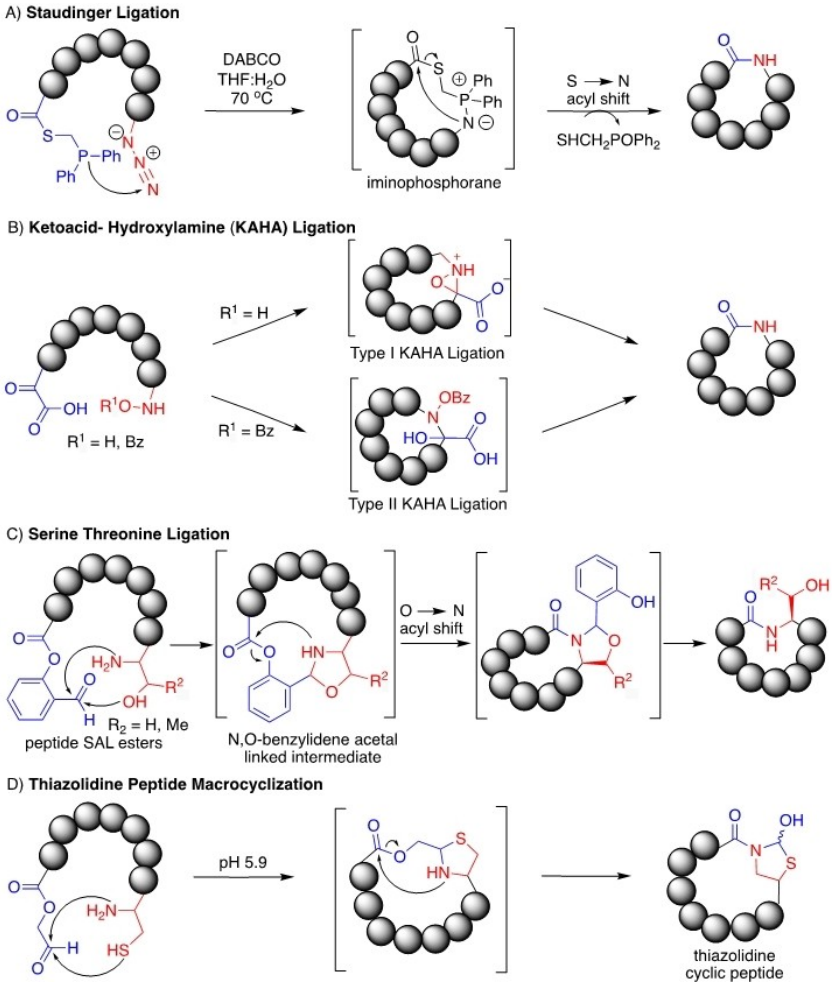
of the auxiliary group.[55] In a similar fashion, Hilvert and Quaderer created a selenocysteine-mediated method.[57] The added advantage of this technique is that selenols have high nucleophilicity[58–60] and low pK_a[61] so they can act as a handle for further selective modification of the cyclic peptide. Since the inception of the technique, modifications have been made to circumvent the terminal cysteine limitation. Desulfurization methods and cysteine surrogates have been created by several groups other than those mentioned above. There is a recent review that details the use of cysteine surrogates in peptide ligation methods that have potential for peptide cyclization.[62]

3.2.2. Staudinger Ligation

Staudinger ligation uses the reactivity of an azide at the N terminus with a phosphine functionalized thioester. This reaction leads to the creation of an iminophosphorane.[63] The iminophosphorane then reacts intramolecularly with the thioester to form a native amide bond at the site of macrocyclization (Scheme 8A).[63–65] The two moieties are highly specific for each other, leading to the cyclization of unprotected peptides in a site-selective manner.[63–65] The method is attractive for peptide cyclization because it allows for the macrocyclization of unprotected peptides without the requirement of any specific amino acid as in the native chemical ligation method. The reaction is often termed traceless because the phosphane-containing auxiliary and the azide are removed in a ring contraction step to create a natural amide bond at the site of cyclization. However, the installation of the reactive groups at the termini requires multiple steps and has proved to be a major challenge for this technique. The method has been applied to the cyclization of dipeptides to generate diketopiperazine.[63] Van Maarseveen and co-workers cyclized dipeptides at 70 °C in the presence of DABCO. Cyclization occurred with various yields (29–84%) with bulky side chains greatly reducing the yields of the cyclic product.[63]



Scheme 8. N- and C-terminal site-selective methods for the cyclization of peptides. A) Staudinger ligation requires an N-terminal azide and C-terminal phosphine functionalized thioester for cyclization. An S to N acyl shift removes the terminal groups for the formation of a native amide bond. B) KAHA ligation requires C-terminal α-ketoacid and N-terminal hydroxylamine for cyclization. C) Serine threonine ligation requires C-terminal peptide salicylaldehyde (SAL) esters and a serine or a threonine residue at the N terminus for selective cyclization. D) The thiazolidine method utilizes a glycoaldehyde at the C terminus and an N-terminal cysteine for synthesis of cyclic peptides.

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Katritzky et al. also generated diketopiperazines in high yields ranging from 72% to 82% by carrying out reactions in organic solvents at 50 °C for 5 min.[64] Staudinger ligation has also been applied for the cyclization of larger peptides.[65] Hackenerger and Kleineweishede utilized a more soluble phosphinothioester for the cyclization of 11-residue linear peptides at room temperature with DIPEA. All peptides contained glycine residues at both the termini and showed moderate yields ranging from 20 to 36%.[65] It would be interesting to see the effect of other amino acids at the terminal positions. The formation of a natural amide bond and the site selectivity of the reaction makes this method highly attractive for dipeptide macrocyclization but has some challenges with larger peptides. The major limitation of this method is the solubility of peptides containing phosphinothioesters and azides, as well as the formation of a side product due to the oxidation of the phosphinothioester. These limitations result in poor yields of cyclic peptides.

3.2.3. KAHA ligation

α-Ketoacid-hydroxylamine amide ligation (KAHA) is another method that requires the installation of reactive groups on the termini of the peptide. The C terminus must bear an α-ketoacid and the N terminus must have a hydroxylamine for cyclization to occur. The two reactive groups undergo a site-selective reaction to yield an amide bond at the point of cyclization (Scheme 8B).[66] Two different mechanistic pathways have been proposed for the cyclization of peptides. These are termed Type I and Type II, based on the nature of R group attached to the hydroxylamine (Scheme 8B).[67] This method is widely used, but a major challenge is the addition of the reactive moieties. In an effort to make this technique more accessible, several groups have developed methods for simplistic installation of terminal groups. Bode et al. initially reported the use of a sulfur ylide linker on solid support that gave rise to the α-ketoacid after cleavage from resin.[68] The group also reported the synthesis of N-terminal hydroxylamines as protected N-benzylidene nitrones.[68] However, alanine and glycine derived nitrones proved difficult to hydrolyze for the formation of hydroxylamines. The group cyclized natural cyclic products like gramicidin S, tyrocidine A, hymenamide B, and stylostatin A with yields of 13, 36, 15, and 13%, respectively. The reactions were carried out at 40 °C over 40 h.[68] In a subsequent paper by the group, they proposed the use of (*S*)-5-oxaproline as a masked hydroxylamine. Bode et al. showed that (*S*)-5-oxaproline generated a homocysteine at the cyclization site.[69] The new methodology allows for easy installation of the reactive groups during solid phase synthesis with cyclization occurring immediately after deprotection and cleavage from the resin. A substrate scope of 24 linear peptides with various amino acids and lengths were successfully cyclized with or without turn inducers.[69] The presence of turn inducers has a large impact on cyclization efficiency. Yields were significantly impacted when turn-inducers like proline were removed from the peptide sequence, in some cases it lowered the product formation from 54 to 11%. Peptides with bulky amino acids showed good yields (60–83%) but required the presence of turn inducers. Furthermore, epimerization ranging from 4.7–5.6% was observed with peptides containing leucine at the N terminus.[69] Regardless, KAHA ligation is an appealing method of cyclization because it lacks the need for specific amino acids. Further research for the easy installation of reactive moieties would prove beneficial for this method.

3.2.4. Serine threonine ligation

Serine and threonine ligation is a site-selective technique that was developed by Li and co-workers.[70,71] The method utilizes the reactivity of peptide salicylaldehyde (SAL) esters at the C terminus with a serine or threonine residue at the N terminus. When the two reactive groups are placed within the same peptide, an imine-induced ring contraction occurs to facilitate head to tail cyclization (Scheme 8C).[71] The reaction proceeds through a N,O-benzylidene acetal–linked intermediate followed by an O to N acyl transfer to produce a natural amide bond.[71] The method has been further explored by Li to include the synthesis of natural cyclic products like Daptomycin in 4 h with a yield of 67% and no evidence of other side products.[71] The reaction was also used by the group to cyclize a variety of strained cyclic tetrapeptides with 12 atoms.[72] All examples contained the obligatory serine or threonine residue at the N terminus and either an alanine, leucine, or phenylalanine residue at the C terminus. However, dimerization was observed in all examples with ratios as high as 3:2, monomer to dimer.[72] Nevertheless, it would be fascinating to know the effectiveness of this methods in making cyclic tetrapeptides that contained other branched residues at the C terminus. One of the major challenges of this technique is the synthesis of C-terminal SAL esters. The Liu group expanded on this chemistry by creating a method for the addition of C-terminal SAL esters directly on solid support which also limits the racemization of the linear peptide.[73] The Liu group utilized this ligation technique to cyclize eight linear peptides of varying lengths (4–9 residues) and sequences with product yields ranging from 20 to 65%. The addition of bulky residues, such as leucine and isoleucine, at the C terminus decreased the yield of the cyclized product (20%).[73]

3.2.5. Head to tail thiazolidine formation

The paper by Tam et al. mentioned in Section 3.1.2 also contained a methodology for the site-selective head to tail cyclization of peptides. A glycoaldehyde was placed at the C terminus which reacted with the N-terminal cysteine to form a thiazolidine ring at the site of cyclization (Scheme 8D). This method resulted in the formation of the thiazolidine ring with a new chiral center but in a racemic mixture. The technique was tested on different sequences varying in lengths (5–26) and amino acids.[49] Reactive amino acids like lysine and threonine were fully tolerated without any formation of side products.

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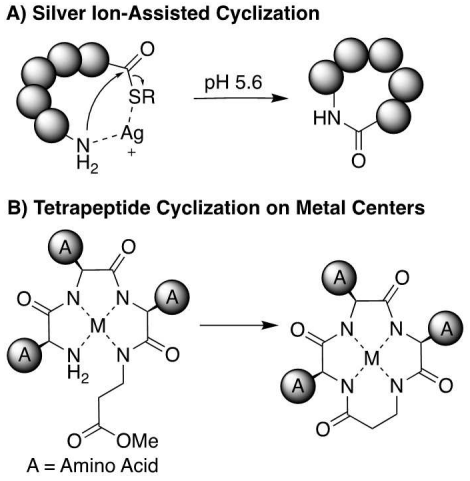
However, peptide length seemed to have heavy impact on the yield. A pentapeptide afforded 25% yield in more than 100 h at 52 °C while a 12-mer peptide cyclized to 96% in 20 h and a 26-mer peptide was cyclized to 75% over 14 h.[49] The requirement of cysteine amino acid at the N terminus, like many other techniques in this review, limits the use of this method.

3.2.6. Metal-assisted cyclization

The use of metal ions in cyclic peptide synthesis is wide spread.[74–79] However, the ability to execute these methods in a site-selective manner is limited. An interesting methodology for the synthesis of cyclic peptides by silver ion assisted cyclization was published by Tam et al.[80] Silver ion coordination of peptide thioesters allowed the close proximity of C terminus with free amines (Scheme 9A). Site selectivity in this reaction is dependent upon the pH of the solution. Site-selective macrocyclization of the N terminus occurs when the pH ranges from 5.0–5.7 because the ε amine of the lysine side chain is protonated.[80] A substrate scope of more than 17 peptides was conducted with successful cyclization in 20% DMSO at pH 5.7, in the presence of silver ions. However, the rate of macrocyclization is highly dependent on the peptide sequence. Peptides ranging from five to ten residues were cyclized in good yields (79–94%). Nonetheless, these examples were limited to glycine or serine at the N terminus.[80] The use of other amino acids at the N terminus would be an interesting addition to this methodology.

Another fascinating site-selective technique that employs metal ions was published by Beck and associates for the cyclization of tetrapeptide esters.[81] The site-selectivity of this method occurs because of the preorganization of the molecule, not through chemical reactivity. The four amidic nitrogens in the backbone of the peptide coordinate to the metal (Ni, Cu, or Pd) to form a planar ring structure (Scheme 9B). The conformation of the molecule brings the C-terminal ester in close proximity to the N-terminal amine for cyclization. However, this method can only be employed for the cyclization of four residue peptides or dipeptides. The reaction proceeds at 65 °C over 24 h.[81] Dipeptide esters showed cyclization yields of 17 to 96% and the single tetrapeptide example cyclized with 45% yield. This method is novel in that it does not require protected amino acids at any stage, nor does it need activating groups, coupling reagents, or any derivatization of the C or N terminus.[81]



Scheme 9. Metal-assisted site-selective methods for peptide cyclization. A) Coordination with the silver ion brings the N terminus and the C-terminal thioester into close proximity for peptide macrocyclization. B) Metal ion coordination of the backbone amide bonds brings the N terminus and the C-terminal ester into close proximity for macrocyclization.

4. Conclusion

Over the past several decades the demand for cyclized peptides has greatly increased as pharmaceutical targets. As such, the published methods for cyclization have also increased. However, site selectivity within these techniques is rare. Site selectivity is an important feature for macrocyclization because it reduces the number of protecting and deprotecting steps needed and the reaction can be carried out with completely unprotected peptides. Furthermore, yields of cyclopeptides created with a site-selective method show greatly diminished side product formation. Described above are methods currently known to employ site-selective cyclization. Major limitations in known chemical techniques include the requirement of unnatural functional groups or the addition of reactive molecules at the termini for macrocyclization to occur. In addition, many of the techniques described above are ligation methods that induce the production of dimers and oligomers at high concentration, which are known to decrease product yields. The CyClick method mentioned above solves this problem by carrying out cyclization in an exclusively intramolecular fashion thus eliminating side products due to dimerization or oligomerization, even at high concentrations (100 mM). Also, the CyClick technique is not dependent on the nature of the N-terminal amino acid, which is one of the major limitations of the other N-terminal site-selective chemical macrocyclization strategies. However, the method does require the addition of an aldehyde group within the peptide for cyclization to occur. The development of a method that does not require modification of the native peptide chain and is applicable to universal sequences with a high reaction rate, site selectivity, exclusively intramolecular nature, and lack of racemization would be very beneficial for the chemical synthesis of cyclic peptides and increase their applicability as therapeutics.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: chemoselectivity · cyclic peptides · enzyme mediation · macrocyclization · site selectivity

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