Peptide Cyclization Methodologies Amenable to in Vitro Display

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https://doi.org/10.51167/acm00018

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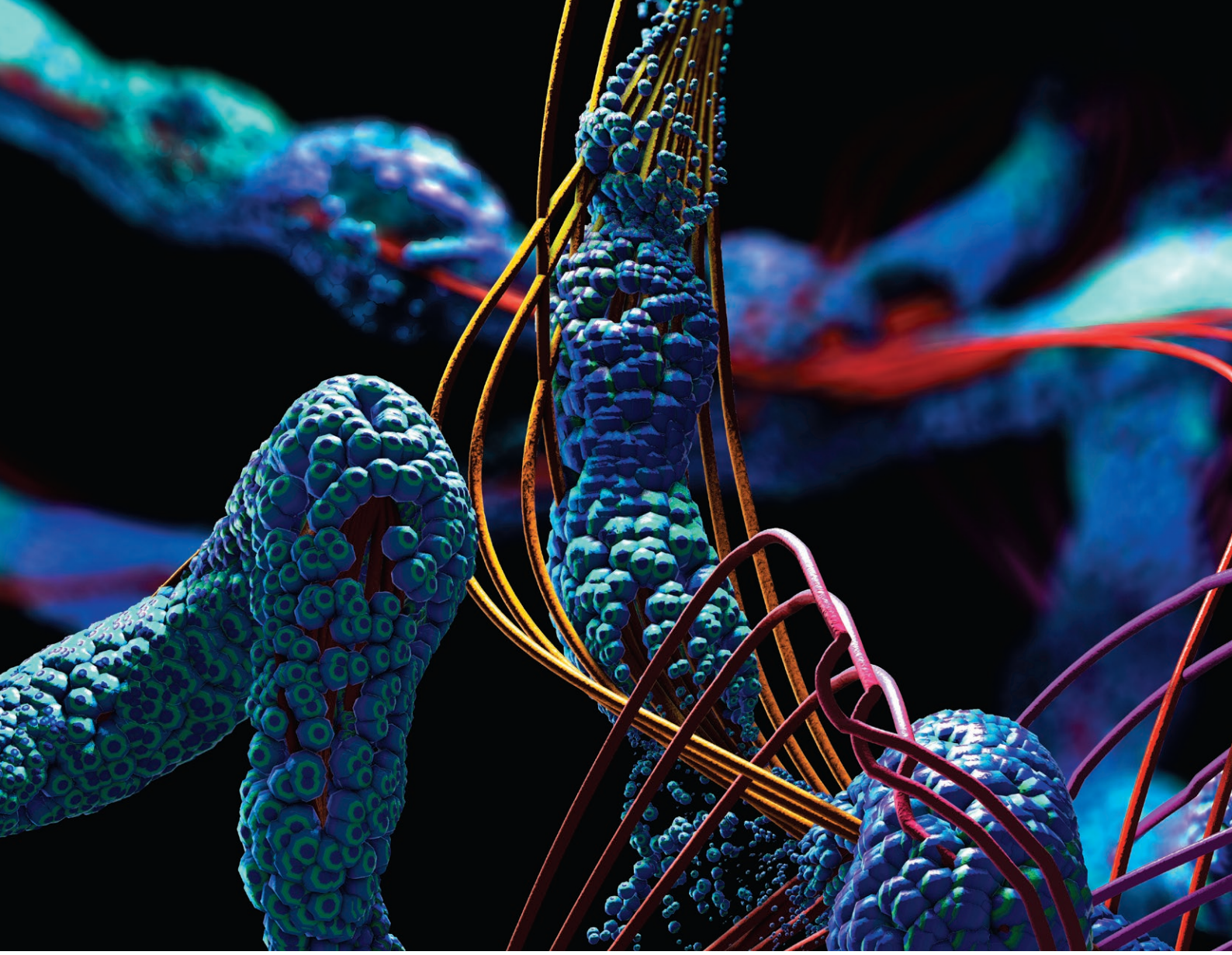
Display technology platforms offer their own unique set of challenges for chemical transformations, at the heart of which lies peptide macrocyclization. The amenable reactions for peptide macrocyclization on this platform need to meet a number of criteria like high reactivity, selectivity, mild conditions, irreversibility and in many cases, a unique requirement to be assimilated into the translation machinery. Skillful utilization of these reactions has led to the formation of huge macrocyclic peptide libraries with varied linkages and topographies which have in turn led to the discovery of a number of hits for purposes such as drug discovery and others. Herein, we review those reactions which have mainly been applied in mRNA and phage display and discuss their technical characteristics and significance.

GENETICALLY ENCODED LIBRARIES of peptides are an inexhaustible repertoire of therapeutic entities. They, however, generally work better when cyclized. Cyclic peptides are known to have two major advantages over their linear counterparts. Firstly, they are more resistant to proteases[1] and hence have longer half-lives and better bioavailability[2] for application as drugs etc. Secondly, they are more compact, have lesser degrees of freedom and fewer available conformations due to which they bind more tightly to the target protein by saving on entropy cost.[3] Moreover, they are indicated to possibly have better cell permeability than their linear counterparts.

The development of methodologies applicable to peptide cyclization under mild conditions constitutes an important and active area of research. Such methodologies must fulfil the requirement of application to not only diverse sequences but



also structures consisting of one, two, three or even more cyclic motifs. Cyclization reactions become more complicated and challenging due to the presence of various reactive sidechains on proteinogenic amino acids.

Even though there are various techniques for chemical synthesis of cyclic peptides on solid support based on traditional protection-deprotection chemistries[4] and/or metal-catalyzed reactions,[5] most of these reactions are not suitable for the use on display platforms because of the following reasons: they must be compatible to physiological-like conditions (*e.g.* at near-neutral pH) and high chemoselective to the aiming functional groups. This review deals with techniques of peptide cyclization as applied to in vitro display techniques, represented by the phage and mRNA displays.

Challenges

Display technologies[6, 7] rely on the translation machinery consisting of ribosome, protein translation factors, various enzymes including aminoacyl-tRNA synthetases, amino acids, tRNAs, mRNAs, energy sources, and others. Thus, the cyclization chemistry needs to selectively work for the aimed peptides in the presence of all these bio- and small-molecules. Even a harder challenge is that their chemistry must efficiently take place regardless of peptide sequences originating from huge mRNA libraries and vast tertiary structures originating from the diverse peptide sequences.

For the phage display, a classical and general method for generating cyclic peptides is disulfide bond formation via two cysteine (Cys) residues. This is simply because their genotype of mRNA or DNA sequence is packaged in the bacteriophage, the easiest way to cyclize the peptide sequences is to use the naturally occurring crosslinking bond(s) of disulfide. However, disulfide bond is a reducible bond, and therefore in consideration for physiological conditions this bond is not necessarily ideal for drug use. Even though such a disulfide bond can be elaborated to an alternative bond, but in such a case the activity of the parental peptide is often diminished. Thus, it is important to develop an alternative approach to produce macrocyclic peptides closed by a more physiologically stable bond from the initial library.

For the mRNA display, the respective peptides are directly attached to the genotype sequences of mRNA via puromycin molecule. Occasionally, the mRNA sequence is reverse transcribed to cDNA sequence forming the noncovalent annealing pair. This means that the peptide-mRNA/cDNA fusion contains not only the peptide motif but also 'naked' nucleic acids, and thereby the chemistry for cyclization is even more challenging than the phage case, where the cyclization must take place without unwanted reactions with sidechains of peptide nor with nucleic acid's nucleobases/phosphates.

Cyclization strategies

Traditionally, peptide cyclization has been categorized as taking place between two ends of the peptide (head-to-end), two sidechains (sidechain-to-sidechain) or one end to a sidechain (head-to-sidechain and sidechain-to-end). However, for the sake of this review which deals mainly with those methods applied to display technologies, we will broadly categorize the strategies in two, *i.e.*, cyclization without using genetic code manipulation and cyclization using genetic code manipulation.

Cyclization via chemical crosslinking

This strategy usually takes advantage of inherent reactivity of a native amino acid side chain and an external organic motif. Majority of groups have exploited nucleophilicity of thiol groups of

Cys or amino groups of lysine (Lys) ε-sidechain (or occasionally N-terminus), which are present at fixed positions in the translated peptide that conjugate with a small organic motif added after translation. Thus, this strategy has been applied for the majority of phage display works.

Thioether bond formation

This has been a popular strategy due to its simplicity and the ability to yield macrocyclic peptides with more than one loop. Cys thiols at fixed positions react with organohalides forming thioether bonds in a S_N2 reaction. The libraries have vast diversities consisting of proteinogenic amino acids only.

Using bis/tris/tetrakis (bromomethyl) benzenes

Inspired by some naturally occurring peptides with multiple fused rings and loops and having interesting biological activities,[8-10] many groups have tried to develop methods to create peptide libraries having similar topologies. Beginning of this was the report of Timmerman (Figure 1) that treating di-, tri-, and tetra-Cys containing peptides with bis-, tris-, and tetrakis(bromomethyl) benzene derivatives in aqueous ACN results in fast, one-step chemical synthesis of single-, double-, and triple loop peptides.[11]

In 2009, this reaction was later utilized by Winter *et al.*[12] to produce bicyclic peptide libraries for phage display. They designed peptide libraries with three reactive Cys residues, each separated by several random amino acids and conjugated with tris(bromomethyl)benzene (TBMB) in aqueous solvents (Figure 2A). The conjugation reaction however posed several challenges including cross reactivity of TBMB with the disulfide bridges D1 and D2 domain in the phage PIII and a loss in phage infectivity due, probably, to the crosslinking of the phage coat protein through lysine side chains. The problems were, however, solved by using a disulfide free gene-3-protein phage and using low concentration of TBMB. The phage display selection was successfully carried out to find an inhibitor ligand to human plasma kallikrein. This elegant approach represents that the appropriate engineering of the phage system allows to control selective crosslinking of Cys residues only appeared in the random library of displayed peptides.

In 2012, Szostak group also utilized a similar strategy to cyclize highly modified peptides having two flanking cysteine residues using dibromoxylene.[13] The peptide libraries having several non-proteinogenic amino acids were used for in vitro selection based on mRNA display against the target protease thrombin with successful isolation of binders with low nanomolar affinity.

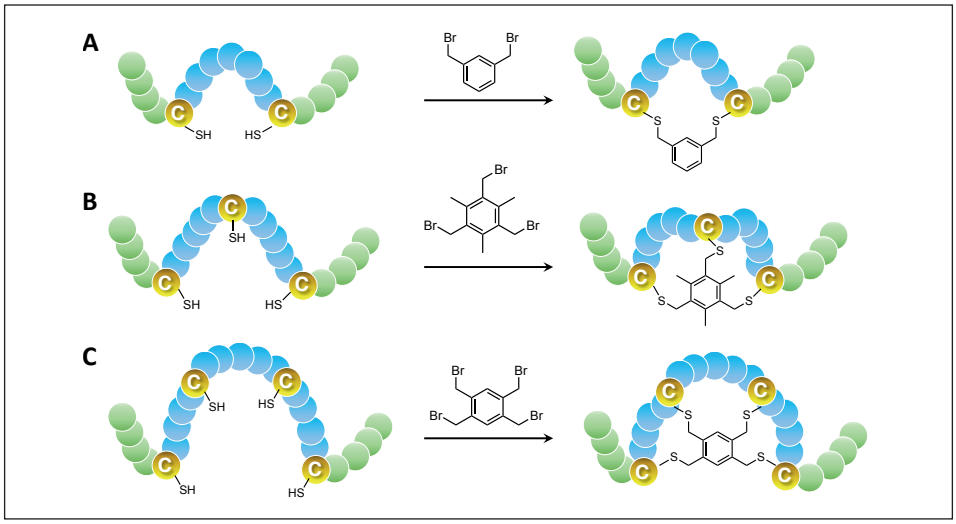


Figure 1. Formation of peptide loops by reacting Cys-containing peptides with di-, tri-, or tetra-Cys reacting to bis-, tris-, or tetrakis-(bromomethyl)benzene as a crosslinking agent.

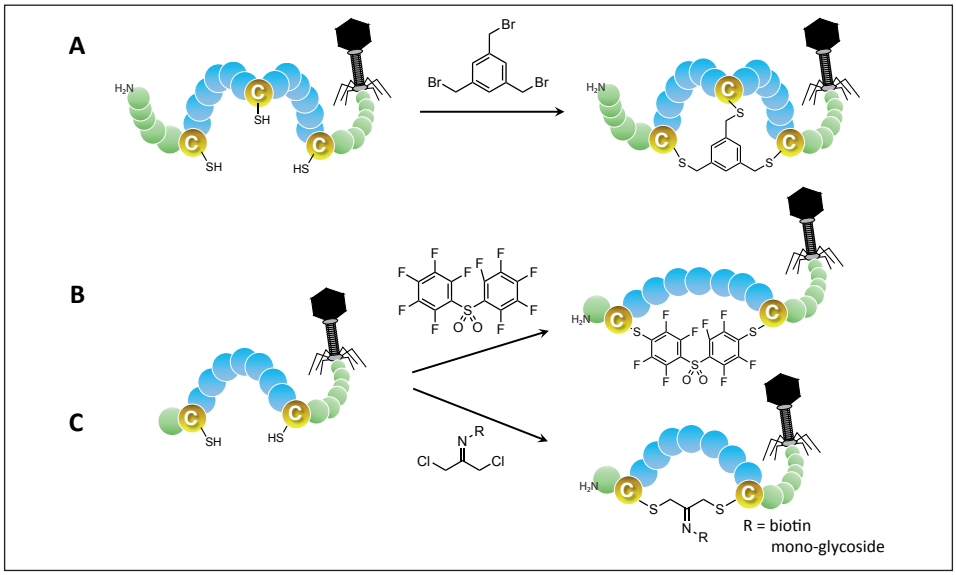


Figure 2. Examples of peptide cyclizations using bromomethyl benzenes, amenable to display technologies. (A) Bicyclic peptide library using 1,3,5-tris(bromomethyl) benzene reported by Winter group. (B) Decafluoro-diphenylsulfone (DFS) cyclizaton and (C) Dichloro-oxime cyclization reported by Derda group.

Using perfluoroarenes

Perfluoroarenes react with a reactive thiol in peptide via nucleophilic aromatic substitution reaction SNAr, which has been used extensively for polymer arylation and bioconjugation.[14-18] Derda *et al.*[19] used decafluoro-diphenylsulfone (DFS) to crosslink Cys thiols yielding cyclic peptides in one of the fastest Cys conjugation reactions (Figure 2B). They improved the previously reported S_NAr reagents such as 1-chloro-2,4-dinitrobenzene,[20] perfluorobenzene[21, 22] and perfluorobiphenyl[22] which show low reactivity and poor solubility in aqueous systems. The group has demonstrated this reaction to be biocompatible and faster than most Cys conjugation reactions with the reaction rates up to 180 $M^{-1}S^{-1}$, although the rate is largely sequence dependent; *e.g.* positively charged residues such as arginine accelerated it while negatively charged aspartate supressed the rate. This unique reaction is fairly selective for Cys, but with large excess and prolonged exposure to DFS showed some cross-reactivity with amine groups. As for applicability of the reaction in phage display, a clone of M13 phage could be 60–70% modified with DFS in 5% DMF as cosolvent. The modification efficiency was decreased to 35% when a whole library containing 109 peptides was used. Interestingly, the crosslinked peptides generally exhibit higher oxidative resistance compared with the traditional α,α'-dibromo-meta-xylene.

Using Dichloro-oxime

In 2015, Dawson *et al.* reported side chain linking of cysteine or homocysteine thiols using dichloroacetone (DCA) to give stapled (macrocyclic) peptide with an acetone bridge.[23] This linking not only stabilized the secondary structure of the peptides but also provided a ketone moiety to link various molecular tags through oxime ligation. Building further on this concept, Derda *et al.* used pre-formed dichloro-oxime (DCO) derivatives (Figure 2C) to cyclize phage displayed glycopeptide libraries.[24] Reaction went on to completion giving approximately

85% adduct in 3 hrs with a rate constant of 1.1 $M^{-1}S^{-1}$. Interestingly, it was found, unlike the reports of Heinis and Winter,[12] that DCO modification did not result in losing phage infectivity and more than 80% of phage remained viable after modification. This suggests that crosslinking of phage coat protein is negligible with DCO.

Amide bond formation

In one of the first reports, Robert *et al.* reported a general route for post-translational cyclization of mRNA display libraries by treating translated peptide with disuccinimidyl glutarate (DSG) at pH 8.[25] DSG reacted near-quantitively with N-terminal amine and an internal Lys ε-amino group crosslinked via two amide bonds. The same group then demonstrated mRNA display of DSG-linked library against Gαi1, successfully discovering a strong cyclic peptide binder with $K_d = 2.1$ nM.[26]

Disufide-rich loop formation

Disulfide bond formation was one of the first approaches developed to cyclize linear peptides displayed on phage but due to the instability of disulfide bond in reducing cellular environment, this approach finds little practical value for in vivo applications. However, plant-based cyclotides are a unique class of peptides having multiple loops in the form of cysteine knots. Their remarkable thermal and proteolytic stability and a wide range of biological activities make them ideal macrocycles to be screened as ligands for target proteins. There are several reports of selection of cyclotides with novel function using in vitro displays.[27-30]

As a recent demonstration, Wenyu *et al.* reported mRNA-display of a cyclotide library derived from *Momordica cochinchinensis* trypsin inhibitor-II (MCoTI-II), in which two loops, 1 and 5, were randomized. The selection campaign against human Factor XIIa (hFXIIa) successfully yielded an extraordinary potent and selective variant, referred to as MCoFx1, giving K_i of 0.37 nM to hFXIIa that is greater than three orders of magnitude selective over trypsin and other related proteases.[31]

Cyclization using genetic code reprogramming

Genetic code reprogramming is a powerful technique which enables incorporation of non-proteinogenic amino acids in translated polypeptides via codon reassignment[32] or expansion.[33,34] The technique has evolved and matured over the years (for recent reviews see these references[35,36]) in which task of reprogramming is achieved through a combination of an *Escherichia coli* reconstituted cell-free translation system and pre-aminoacylated tRNA with various nonproteinogenic amino acids facilitated by flexizymes. This system, referred to as FIT (Flexible In-vitro Translation), enables for devising many unique methods for macrocyclization of peptides discussed in the following sections.

Thioether Bond Formation

Thioether bond formation by nucleophilic substitution

Unlike the aforementioned strategy of adding an external organic moiety with multiple halogens, this strategy results in the formation of one thioether bond per cycle. The halo part is incorporated at the initiator position or at a suitable side chain through genetic code reprogramming.[37] An intramolecular substitution reaction by a downstream Cys thiol results in the formation of a physiologically stable thioether linkage. Suga group has explored, evolved and exploited this technique thoroughly, resulting in a number of interesting macrocyclic libraries and successful selections against various targets (for recent representative examples see references[37-45]).

In 2008, Goto *et al.* have used a methionine-depleted FIT system where the initiation codon AUG becomes vacant, and engineered the initiation event. To this sytem is added an aminoacyl-$tRNA^{fMet}_{CAU}$ charged with N-chloroacetylated amino acid, such as tryptophan (ClAc-Trp) or tyrosine (ClAc-Tyr), prepared by a flexizyme (eFx).[37] The ClAc-Trp-$tRNA^{fMet}_{CAU}$ was set as an initiator, for example, for the peptide expression, ribosome elongates amino acids starting from the ClAc-Trp according to mRNA template sequence, followed by a Cys residue at a downstream position. When the peptide synthesis is completed, the Cys thiol spontaneously reacts with the ClAc group to yield a thioether linked macrocyclic peptide (Figure 3A). It should be noted that other haloAc group, such as BrAc and IAc, yielded many byproducts originating from adducts of thiols present in the translation system, *e.g.* mercaptoethanol, DTT, and Cys. Thus, the ClAc group was the perfect reactivity toward the Cys thiol in peptide chain that effectively promotes the desired intramolecular reaction over undesired intermolecular reaction. This strategy has been applied to constructing mass libraries (over trillion members) of thioether macrocycles in combination with genetic code reprogramming for the incorporation of exotic amino acids[46-48] including N-methyl-L-amino acids[49,50], D-amino acids[51-53], and β-amino acids[54,55], etc. Suga group has integrated this strategy with mRNA display, referred to as RaPID (Random nonstandard Peptides Integrated Discovery) system, and enabled the 'rapid' discovery of various potent macrocycles[56] against extracellular and intracellular proteins and has reported more than 35 successful selection outcomes with a range of low nM to pM K_D values in the period of a decade.[57-84]

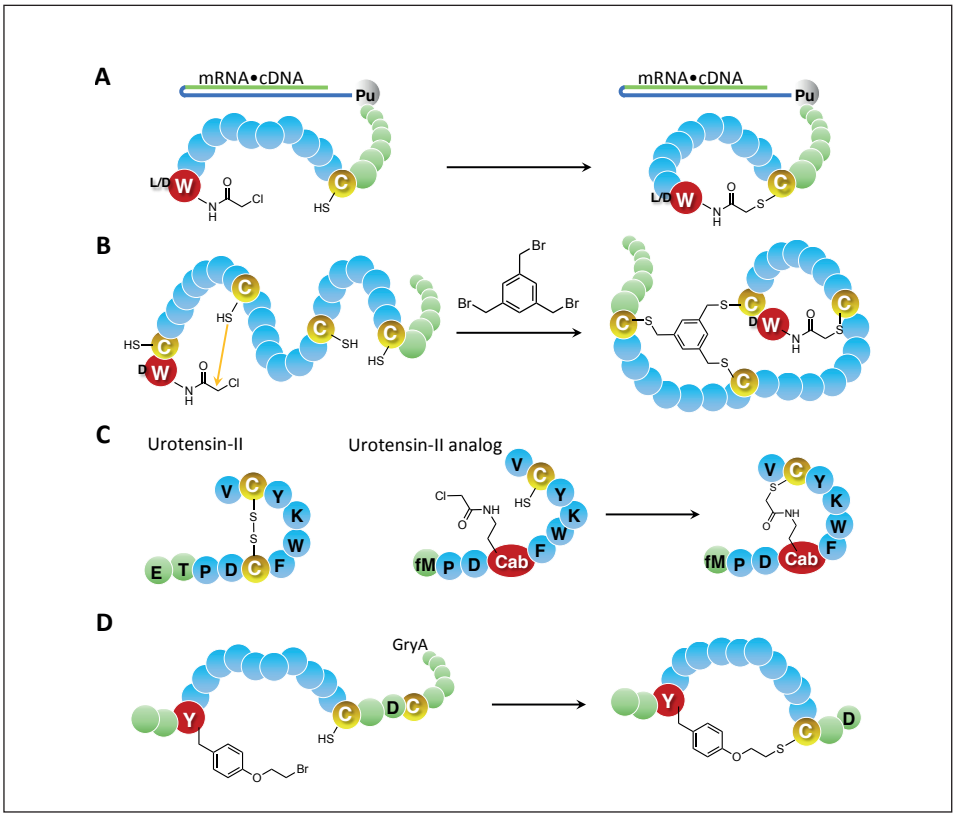


Figure 3. Ribosomal synthesis of macrocycles closed by a thioether bond via nucleophilic substitution. (A) N-terminal ClAc-Trp installed by the genetic code reprogramming reacts with a downstream Cys. (B) Tricyclic peptide synthesis of the intramolecular N-terminal ClAc with the second downstream Cys in the combination with TBMB that crosslinks three remaining Cys residues. (C) The ClAc group on the sidechain of Cab installed by the genetic code reprogramming reacts with a downstream Cys. The sequence represents a sequence of human urotensin II. (D) Macrocyclization using thioether bond formation by intramolecular reaction between non-proteinogenic amino acid O2beY and Cys inside living bacterial cells via intein-based protein splicing.

Interestingly, the N-terminal ClAc group reacts with Cys thiol at almost any position, except for Cys at the adjacent downstream position to ClAc-initiator (*i.e.* at the second position). This is simply because Cys cannot sterically reach to the ClAc group. Thus, when there is a Cys residue at the second position, arbitrary sequence and length of peptide followed by a downstream Cys residue, the latter Cys thiol (generally the second Cys residue) selectively reacts with the N-terminal ClAc group to form thioether-macrocycle. This fact has allowed to build a strategy for ribosomal synthesis of tricyclic peptides (Figure 3B). In this scheme, a peptide contains a total of four Cys residues, where ClAc-Trp is followed by Cys and then the rest of peptide sequence has three Cys residues at various position. The second Cys spontaneously reacts with the N-ClAc group to afford a monocycle. Then, the treatment of TBMB crosslinks the remaining three Cys residues to form a topologically complex tricyclic peptide.

This ClAc thioether strategy can be expanded to inter-sidechain cyclization by incorporating an Nγ-ClAc-α,γ-diaminobutylic acid (ClAc-Cab).[85] Again, a downstream cysteine thiol reacts with the ClAc group to afford a macrocycle closed by the thioether bond. Application of this methodology was demonstrated by translating a known biologically active peptide human urotensin II which is a potent vasoconstrictor. Single disulfide bond between cysteine residues at position 5 and 10 was replaced with a thioether bridge between Cab at position 5 and a cysteine at position 10 (Figure 3C). The resulting peptide was shown to retain biological activity and remarkable stability towards proteinase K under reducing conditions.[85]

In 2014, Fasan *et al.* developed a strategy of producing thioether linked macrocyclic peptides inside living bacterial cells (*E.coli*) which can be utilized on phage display platform (Figure 3D).[86, 87] In order to supress cross reactivity with many other nucleophiles in the cellular environment, they ribosomally incorporated a rather slow reacting nonproteinogenic amino acid (O-(2-bromoethyl)-tyrosine) termed O2beY. For proteolytic release of the cyclized peptide, they also incorporated an intein-based protein splicing element. Both features combined together, resulted in ribosomal production of a linear precursor peptide having a cysteine reactive nonproteinogenic amino acid O2beY and an intein splicing element. Remarkably, another cysteine present in the intein element did not show any reactivity towards cyclization reaction due to being partially buried within the active site. Yet, the practice of this approach for the disovery of de novo macrocyclic peptides has not been reported.

Michael Addition

Nucleophilicity of thiolate can also be exploited in Michael type addition reactions to yield thioether linkage. In fact, many biologically active natural lanthipeptides utilize this strategy for cyclization. For such ribosomally synthesized and posttranslationally modified peptides, dehydratase enzymes recognize the N-terminus of the precursor leader peptide and convert serine and threonine residues in the core peptide to dehydroalanine (Dha) and dehydrobutyrine (Dhb) respectively. The α,β-unsaturated moieties in Dha and Dhb acts as the electrophile where enzyme assisted Michael addition reaction by cysteine thiol generates a thioether linkage. The most extreme case observed in natural products is biosynthesis of nisin.

Inspired by this chemistry, Goto *et al.*[88] used genetic code reprogramming to incorporate vinylglycine in translated peptides which was isomerized to dehydrobutyrine by simply heating the peptide at 95°C for 30 minutes. This was followed by spontaneous Michael addition by a cysteine thiol to give methyllanthionine containing macrocyclic peptide. They later demonstrated the applicability of this reaction by synthesizing two ring segments of the natural bioactive peptide nisin (Figure 4). Due to high temprature requirement of this cyclization step, this approach is inapplicable to the display system; therefore, a better alternative approach is needed.

Oxidative Coupling

Genetic code reprogramming allows for incorporation of various nonproteinogenic amino acids including those with orthogonal reactive handles to accomplish click type ligation (*vide infra*). A practically useful application of this methodology was incorporation of benzylamine and 5-hydroxyindole.[89] These functional groups are known to react instantly under oxidative conditions to yield a fluorescent heterocyclic moiety. This methodology (Figure 5), although not used for display technology yet, seems to offer immense practical utility and potential for application in display-based selection.

Azide-Alkyne Coupling

Copper catalyzed Azide-Alkyne Click (CuAAC) reaction[90, 91] needs no introduction and remains one of the most versatile and practically useful bioconjugation reaction (for some reviews see[92-97]). It has been exploited widely for peptide cyclization in solid phase[98] and solution phase peptide synthesis.[99-101] Its underutilization in macrocyclization of peptides for display technologies, however, is, in part, due to the lack of compatibility with nucleotides[102-104] (with RNA in particular). RNA is susceptible to oxidation and degrades quickly in presence of Cu in aqueous medium.[105] Use of acetonitrile as cosolvent, Cu stabilizing ligands and degassing buffer solutions are some of the ways to prevent mRNA degradation when using CuAAC reaction. Additionally, since double incorporation of both azide and alkyne bearing unnatural amino acids is rather tedious and low yielding, the use of this strategy for preparing monocyclic peptide

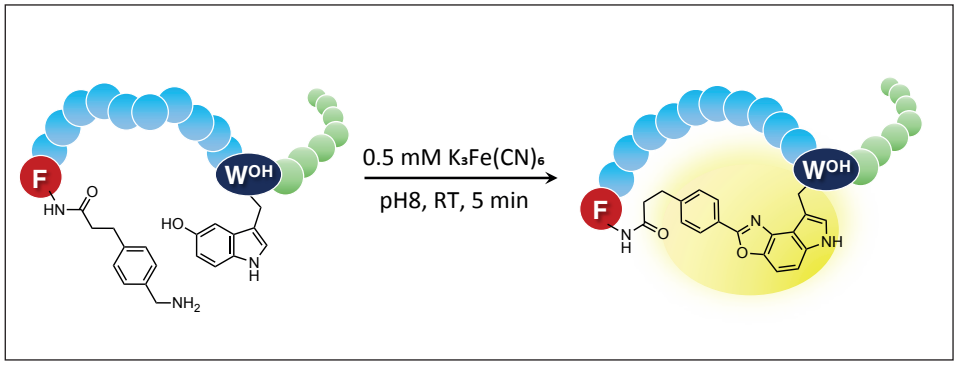


Figure 4. Benzyl amine and hydroxyindole incorporated in translated peptides react rapidly under oxidative conditions, yielding a unique fluorogenic aromatic linkage.

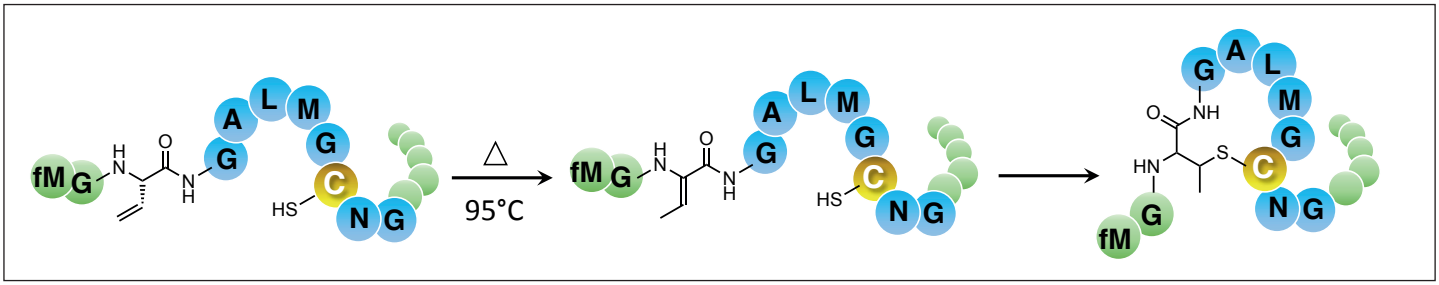


Figure 5. Cyclization via Michaels addition. Model peptide with vinylglycine isomerising to dehydrobutyrine on heating to 95°C and subsequent intramolecular Michael addition by cysteine thiol to give the macrocycle.

libraries is virtually unreported. This strategy has been proven to be valuable for producing bicyclic libraries in particular. Suga lab in 2008 reported first double incorporation of azide and alkyne[106] bearing unnatural amino acids azidohomoalanine (Aha) and propargylglycine (Pgl) respectively using Leu codon CUC for Aha and Thr codon ACC for Pgl. This orthogonal pair was expressed along with another reacting pair 4-(2-Chloroacetyl)aminobutyric acid (Cab) and cysteine to generate a bicyclic peptide scaffold (Figure 6).

Hartman's group utilized the CuAAC reaction generating bicyclic peptide library for mRNA display.[107] β-azidohomoalanine (AzHA) and p-ethynyl phenylalanine (F-yne) were incorporated in place of methionine and phenylalanine, respectively. The second cycle was formed by two cysteine thiols reacting with dibromoxylene. They further carried out a competitive mRNA selection on streptavidin target using a library of linear, monocyclic and bicyclic peptides to investigate the effect of different ring sizes and topologies on selection results and they found all the selection winners were linear peptides. This raised the question as to why all selection winners were linear peptides with only μM K_D values even though some cyclic peptides capable of exhibiting nM K_D values were known. For a detailed discussion on this see.[108]

Backbone Cyclization

Formation of backbone cyclized peptide libraries for in vitro display technologies coupled with ribosomal translation is not possible directly because the C-terminus of the peptide is involved in genotype-phenotype linkage and is not available for cyclization reaction. This intrinsic limitation had hindered devising a display strategy of backbone cyclized peptide. This means that a new strategy is required to covalently trap peptide phynotype to the cognate genotype via non-C-terminus.

To break this technical hinderance, Takatsuji *et al.* has devised a two-step rearrangement strategy by utilizing genetic code reprogramming to incorporate three nonproteinogenic amino acids in the peptide.[109] Peptide is expressed with a thiazolidine-Cys (Thz-Cys) dipeptide initiator charged onto $tRNA^{fMet}_{CAU}$ and ClAc-Cab are installed in the N-terminal region of peptide by the genetic code reprogramming (Figure 7). Continuing the elongation of arbitrary sequence of peptide (p2-W8-$^{S}F^{Cl}$9), an a-thio-*p*-chlorophenyl-lactic acid ($^{HS}F^{Cl}$) is installed to form a thioester in the backbone and Cys residue at a downstream position (generally dipeptide, *e.g.* Ile-Gly, are inserted between $^{HS}F^{Cl}$ and Cys). Upon the completion of ribosomal peptide synthesis, the Cys thiol spontaneously exchanges with the thioester bond of $^{HS}F^{Cl}$, to yield an intermediate (p2-W8-$_S$C12). The thiol group of $^{HS}F^{Cl}$ then reacts with the ClAc group on Cab to give a covalent thioether linkage (tcp2-W8-$_S$C12). In the second step, mild deprotection of the Thz group on the initiator Cys gives an N-methyl-Cys residue at the N-terminus (tcp2-W8-$_S$C12:deprptected), whose thiol sidechain immediately undergoes intramolecular thioester exchange followed by transfer to the N-methyl-amino group on Cys (similar to native chemical ligation) to yield backbone-cyclized peptide (bcp2-W8). Most importantly, it was demonstrated that this entire process allows to maintain the C-terminal region of peptide covalently attaching to the backbone-cyclized peptide via the thioether linkage between ClAc-Cab and $^{HS}F^{Cl}$ groups (Figure 7). Since this leaves C-terminal peptide region remaining as carboxyl group, the strategy is compatible to the RaPID display via the puromycin molecule attached to cognate mRNA, as demonstrated in this work.[109]

Macrocyclic Depsipeptide Formation

Cyclic depsipeptides (CDPs) are a class of naturally occurring peptides which contain one or more ester bonds and exhibit wide range of biological activities.[110] The O-acyl isopeptide bond, usually formed between the hydroxyl sidechain of serine or threonine residues and the carboxyl group of the C-terminus amino acid is stable towards esterases and proteases. Because of biological significance of CDPs, several methods for the chemical synthesis of CDPs have been developed, but none of them is applicable to the conditions required for translation where the chemistry must work at near neutral pH and mild temperature.

Nagano *et al.* conducted a selection campaign for self-esterifying peptide species from random peptide libraries using a thioester acyl-donor, and discovered a peptide containing a short SerProCysGly (SPCG) motif that can effectively esterify on the Ser residue. It turns out that trans-thioesterification between the acyl-donor and thiol sidechain of Cys residue in the SPCG motif firstly takes place, and the resulting acyl group on Cys rapidly transfers to the hydroxyl group of Ser (Figure 8A).[111] This unique chemistry was then applied for CDP synthesis. Linear peptide is expressed, where the SPCG motif in the N-terminal region, arbitrary peptide sequence,

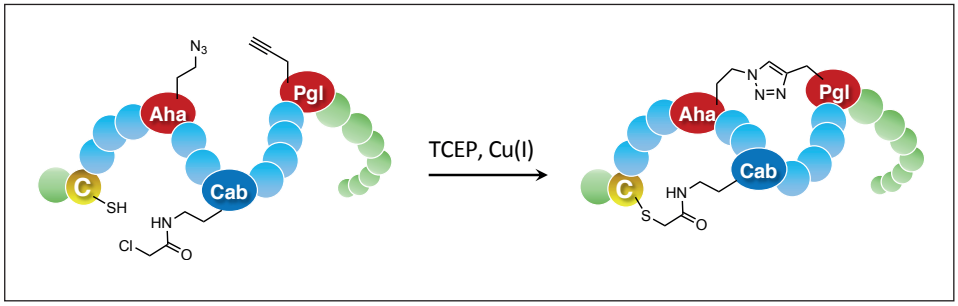


Figure 6. Cyclization of peptide via azide-alkyne coupling. Double incorporation of both azide and alkyne in translated peptides via genetic code reprogramming by Suga group and subsequent formation of bicyclic peptide in conjugation with thioether bond formation.

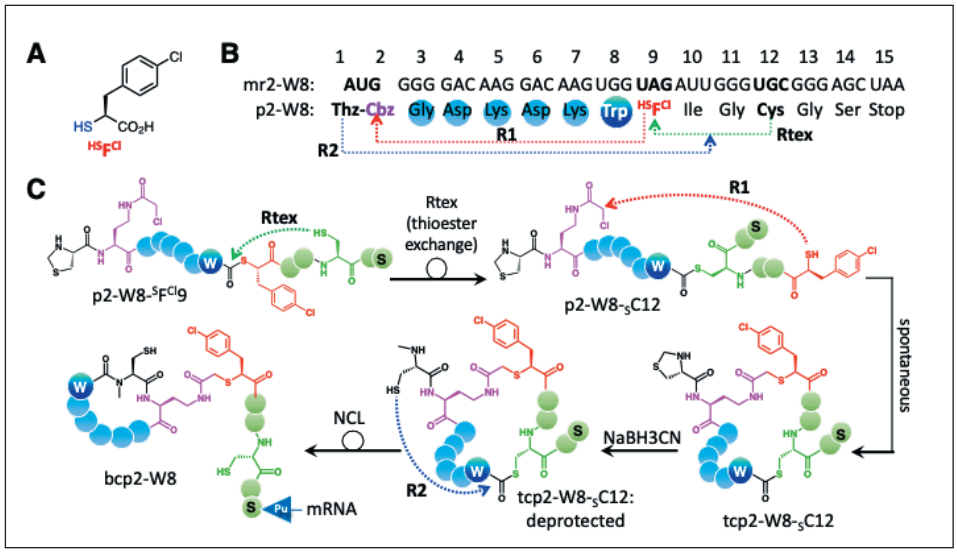
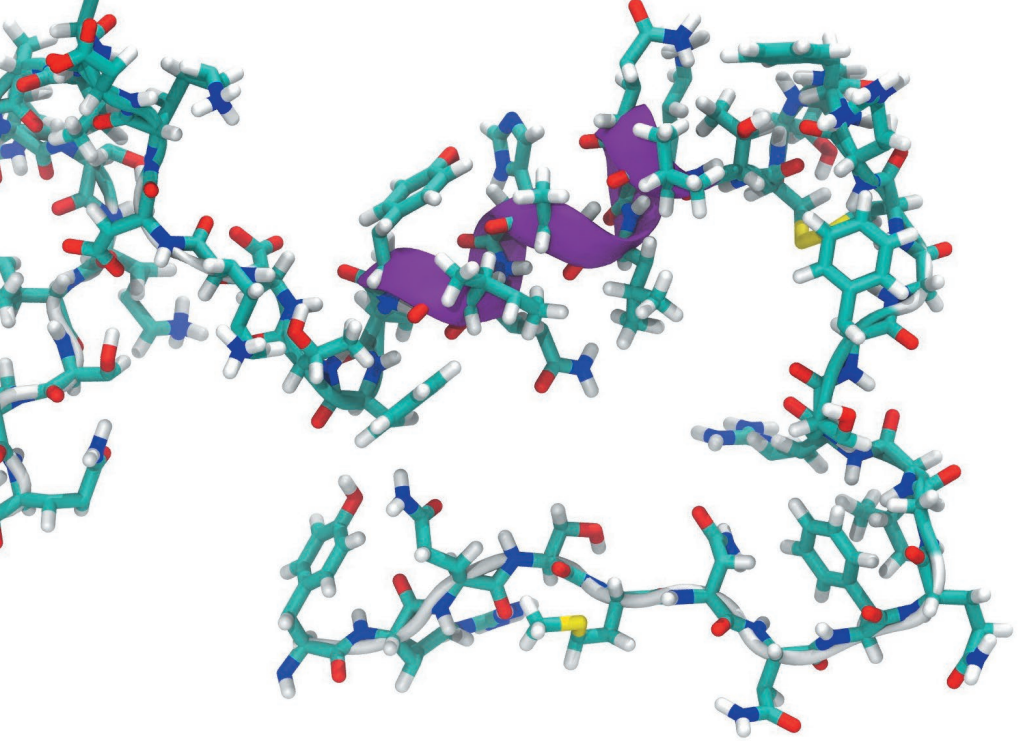


Figure 7. Backbone cyclization amenable to the RaPID display. (A) Non-proteinogenic thioacid HSFCl. (B) mRNA sequence coding the arbitrary peptide p2-W8. (C) Thioester exchange followed by thioether bond formation between ClAc-Cab and HSFCl groups keeps the C-terminal peptide region to the sidechain of backbone-macrocyclic peptide. Deprotection of Thz-Cys followed by spontaneous thiol-thioester exchange leads to native chemical ligation which yields the backbone cyclized peptide attached to its genotype.



and $^{HS}F^{Cl}$ are installed by the genetic code reprogramming in the FIT system. Since the thioester bond originating from the incorporation of $^{HS}F^{Cl}$ acts as a thioester donor, the intramolecular trans-thioesterification occurs at the Cys residue in SPCG to afford an intermediate of cyclic thiolactone; and then rapidly rearranges into the CDP (Figure 8B). In-depth studies on mutants of the SPCG motif have revealed that this motif can be relaxed to SXCX (X can be nearly any amino acids). Since this method of CDP synthesis can proceed in one-pot and also on a wide variety of peptide sequences between the SPCG and $^{HS}F^{Cl}$, it is applicable to the RaPID system to screen bioactive CDPs against protein targets of interest.

Conclusions

It becomes evident that so far, majority of chemical space for peptide macrocyclization on display platform is occupied by either bi/tri/tetra functional crosslinker induced cyclization or thioether bond formation by incorporating N-chloroacetyl group at the initiator position using genetic code reprogramming. The later one, in particular, has been tremendously successful in producing selection results against a variety of interesting targets yielding extremely strong binding macrocyclic peptide ligands against a wide range of intracellular and extracellular targets. The success of FIT and other genetic code reprogramming approaches have empowered researchers to explore newer and more challenging approaches for peptide macrocyclization in order to diversify the field and break through various barriers and limitations. Many valuable bioconjugation reactions like Michael addition to α,β-unsaturated systems and various 'click' type reactions remain underutilized on this platform and offer great opportunity for expanding the scope and pushing boundaries of this active field. ◆

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research S (26220204) and Specially Promoted Research (20H05618), and Japan Agency for Medical Research and Development (AMED), Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research, JP19am0101090) to H.S.

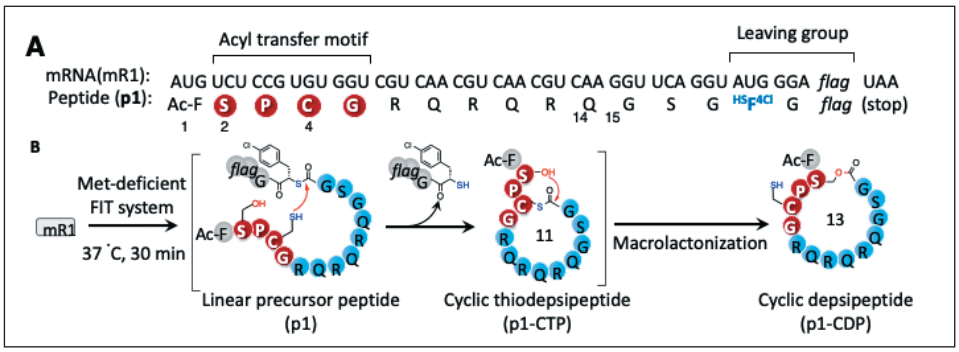


Figure 8. One-pot ribosomal synthesis of cyclic depsipeptides (CDPs). (A) Discovery of the SPCG motif that rapidly proceed the intramolecular S-to-O acyl-transfer reaction. (B) One-pot synthesis of CDP via intramolecular trans-thioesterification followed by the intramolecular S-to-O acyl-transfer reaction.

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