Development of Ribosome-Mediated Thioester Bond Formation and Its Application to Backbone Macrocyclization of Peptides

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学位論文（要約）

Development of Ribosome-Mediated Thioester Bond Formation and Its Application to Backbone Macrocyclization of Peptides

（リボソームによるチオエステル結合の形成と
ペプチド主鎖環化反応への応用）

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Abstract

In this thesis, I developed ribosome-mediated thioester bond formation and its applications. Enen though it has been shown that engineering of the translation apparatus allows for incorporating or even polymerizing analogs of α-amino acids, such as *N*-alkyl-amino acids and α-hydroxy acids (lactic acids), there had been no clear report of successful thioester bond formation in ribosome so far.

In chapter 2, I demonstrated the ribosome-mediated thioester bond formation using our Flexible In-vitro Translation (FIT) system, where the genetic code reassignment is facilitated by flexizymes and a reconstituted *E. coli* in-vitro translation system. This is the first clear report for thioester bond formation by ribosome. This technique was applied to native chemical ligation (NCL) of peptide and even protein that is not usually accessible by chemical synthesis. Here the semi-synthesis of a yellow fluorescent protein, VENUS was performed by NCL of the truncated VENUS-thioester expressed by in vitro translation to the synthetic peptide, demonstrating the utility of ribosome-mediated thioester bond formation.

In chapter 3, I applied the ribosome-mediated thioester bond formation for backbone macrocyclization of peptides. Although backbone-cyclized peptides are increasingly expected as a promising drug scaffold due to their significant therapeutic potential, in vitro display technologies allowing for the facile construction of polypeptide libraries consisting of hundreds of millions to over trillions mutants and the rapid screening of strong and selective binders to target proteins have not been compatible with backbone-cyclized peptides. This is because backbone macrocyclization would involve the disruption of indispensable linkage for in vitro displays between the genotype and the phenotype peptide. To circumvent this issue, I developed the synthesis of backbone-cyclized peptides compatible with in vitro display via a complex rearrangement strategy using ribosome-mediated thioester bond formation.

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Chapter 1

General Introduction

Flexible Catalytic Activity of Ribosome to Incorporate Non-Canonical Substrates

In all living organisms, ribosomes synthesize proteins by polymerizing 20 different proteinogenic amino acids using mRNA as a template in the translation system. The specificity of utilization of these 20 proteinogenic amino acids is determined by aminoacyl-tRNA synthetases (ARSs) [1] [2] [3]. ARSs recognize the correct pairs of amino acid and tRNA to afford aminoacyl tRNA, enabling the decoding of mRNA according to the genetic code and their accommodation into ribosome. Despite the strict control of fidelity between codons and encoding proteinogenic amino acids in translation, it is known that the ribosome does not strictly recognize the substrate bound on the tRNA [4] [5] [6] [7] [8] [9]. Therefore, ribosomes can incorporate or even polymerize analogs of α-amino acids by preparing appropriate substrate-tRNA. For example, it has been reported more than 40 years ago that ribosomes can incorporate α-hydroxy acid into polypeptide and form an ester bond by supplying tRNA charged with α-hydroxy acid, called hydroxyacyl tRNA, to the translation system [10]. In addition, the ribosomal incorporation of *N*-alkyl amino acids [11], D-amino acids [12], β-amino acids[13], hydrazino acids [14], etc. have been reported so far.

Recently our group has successfully demonstrated such events using our Flexible In vitro Translation (FIT) system [15] [16]. The FIT system is composed of two technologies, flexizymes and a reconstituted *E. coli* in vitro translation system [17]. Flexizymes are artificially evolved tRNA acylation ribozymes that facilitate the aminoacylation of tRNA using appropriately activated amino acid substrates by cyanomethyl ester, 3,5-dinitrobenzyl ester, etc. The flexizyme system is capable of charging not only a wide variety of amino acids but also various other substrates such as hydroxy acids, peptides, and so on. Acyl tRNAs prepared in this way are supplied to a custom-made reconstituted *E. coli* in vitro translation system, wherein some components are excluded in order to reassign the codons from proteinogenic to nonproteinogenic amino acids or other acid substrates. Thus the governance of the genetic code is

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arbitrarily reprogrammed and this strategy is referred to as genetic code reprogramming.

On the other hand, even though α-thio acid is similar to α-hydroxy acid, there had been no clear report of successful formation of thioester bond by ribosome. Although Schultz, P. G. *et al.* described ribosomal incorporation of the thiol analog of alanine in the early study of ribosomal incorporation of various substrates[18], they did not show any data indicating the ribosomal α-thio acid incorporation. In addition, no follow-up studies or applications on the ribosomal α-thio acid incorporation have been reported. Therefore it remains unclear whether or not ribosomal thioester bond formation is possible and compatible with applications.

Thioester Bond in Protein Chemistry and Chemical Biology

Thioester bond is an analogue of ester bond with the functional group R-COO-R' replaced by R-COS-R'. Thioester bond is more hydrolytically labile than the ester bond, but more importantly it is readily trans-thioesterified with other thiol group and the latter characteristic turns out to be useful for ligating two peptide fragments, so-called native chemical ligation (NCL) [19]. NCL is the most convenient and commonly used technique for selective ligation of unprotected polypeptide fragments under aqueous neutral conditions where the "native" peptide bond is formed via an intermediate of trans-thioesterified species (**Figure 1**). NCL was initially developed to solve the decades of problem on how to chemically synthesize proteins from smaller, synthetically accessible peptide building blocks [20] [21] and peptide-thioesters for use in NCL had been prepared solely by chemical synthesis. Thus NCL has extended the limitation of the total synthesis of proteins but chemical synthesis of larger proteins beyond 20 kDa is still challenging [22] [23].

To solve this problem, a semisyntheis version of NCL has been developed, called expressed protein ligation (EPL), where recombinant polypeptide-thioesters are used for ligation [24]. In EPL, recombinant polypeptide-thioesters are prepared by the
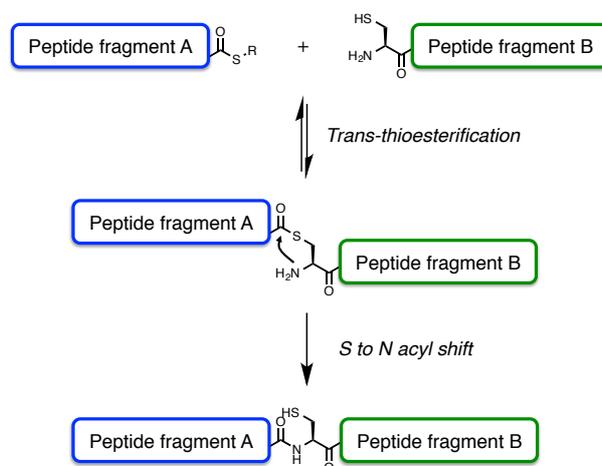
Figure 1 | Scheme of native chemical ligation. Peptide fragment A bearing C-terminal thioester undergoes trans-thioesterification with N-terminal cysteinyl peptide fragment B, followed by *S* to *N* acyl shift to afford the ligated peptide by native peptide bond.

intein-based approach utilizing a process termed protein splicing. This process is mediated by the autosplicing domain called intein[25] via the formation of thioester intermediates (**Figure 2**). Here the intermediates can be intercepted by using appropriate intein mutants, and further cleaved to afford the desired thioester forms through thioester exchanges [26] [27] (**Figure 3**). Thus EPL can bridge the gap between synthetic peptides and recombinant proteins and significantly increases the size and complexity of chemically synthesized proteins. This capability has been used for the production of various modified proteins bearing fluorophores [28] [29], isotopic labeling [30] [31] [32], cross-linkers [33], posttranslational modifications [34] [35], and so on.

Despite the success of the intein-based EPL in many protein systems, the generation of modified proteins via ligation can be still challenging because all proteins have their own sequence, structure, properties and behaviors, and thus an optimal ligation strategies are required on a case-by-case basis [36] [37] [38]. Therefore the development of new polypeptide-thioester preparation method that does not use the intein-based approach may expand protein targets and applications accessible by ligation.
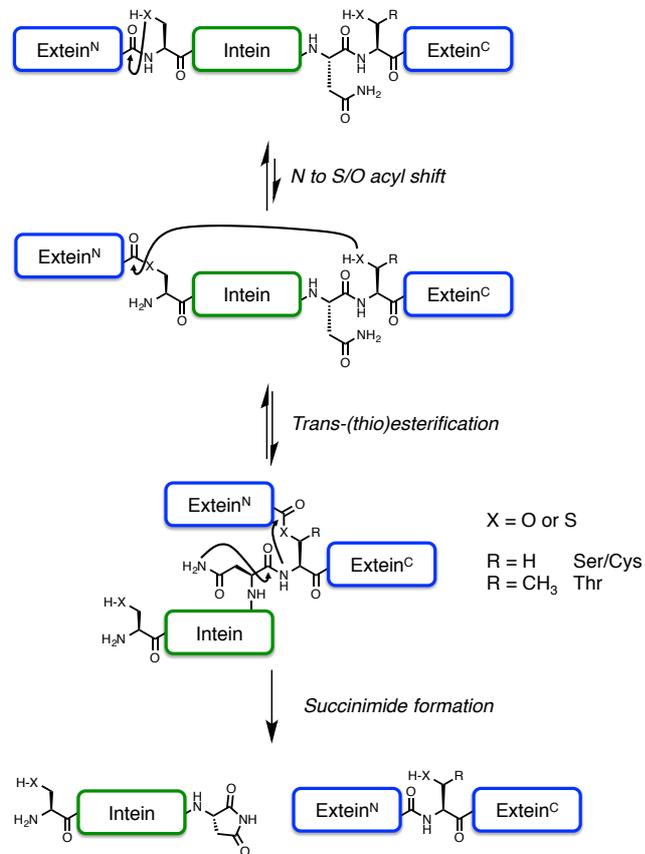
Figure 2 | Mechanism of protein splicing. In protein splicing, internal protein segment (called intein) is removed from precursor protein to cause ligation of C-terminal and N-terminal external proteins (called exteins) on both sides.
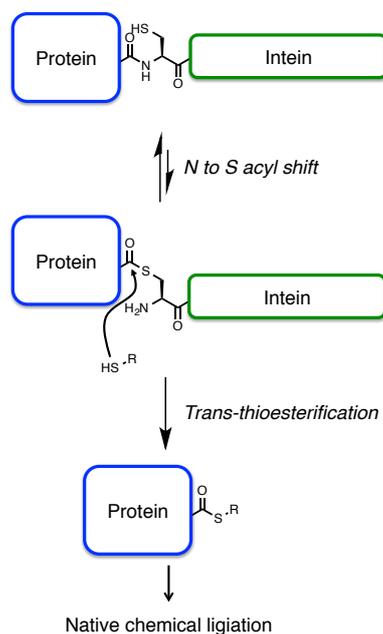
Figure 3 I Recombinant protein-thioester preparation for expressed protein ligation (EPL)

Backbone-Cyclized Peptides

   Macrocyclic peptides are increasingly expected as a promising drug scaffold

due to their significant therapeutic potential such as strong and selective binding affinity

to target proteins [39], proteolytic stability [40], and possible passive membrane permeability

conferred by intramolecular hydrogen bonds [41]. The macrocyclic structure can be formed

by linking one end of the peptide and the other with an amide bond, or other chemically

stable bonds such as lactone, ether, thioether, disulfide, and so on. Among them,

backbone cyclization (or head-to-tail cyclization) is amide bond formation between

amino and carboxyl termini. Backbone-cyclized peptide is of particular interest because a

number of biologically active macrocyclic peptides are formed this way [42]. Moreover,

several backbone-cyclized peptides found in nature are already used in clinic such as

gramicidin [43] and tyrocidine [44] with bactericidal activity, and cyclosporin A [45] with

immunosuppressive activity (**Figure 4**). In addition, cyclotides are a new emerging

family of large plant-derived backbone-cyclized peptides with a knotted arrangement of

three disulfide bonds [46] [47]. Due to their stability, biological activities like anti-HIV [48],

uterotonic [49] and insecticidal [50], and cell membrane permeability [51] [52], cyclotides are expected as a scaffold for novel peptide drugs.
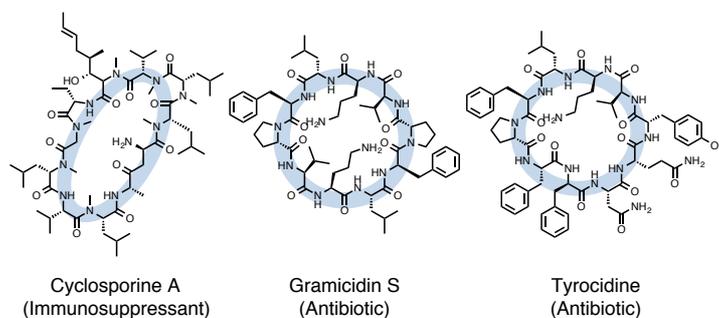

Figure 4 | Backbone-cyclized peptides used in clinic. In this figure, Backbone macrocyclic structure is highlighted.

To date, due to their potential as lead compounds for drugs, many efforts have been made to develop biologically active backbone-cyclized peptides with both synthetic and genetic approaches. Backbone-cyclized peptides can be synthetically prepared by solid phase peptide synthesis of linear peptides followed by macrocyclization using condensation reagents [53], enzymatic reactions [54], traceless Staudinger ligation [55], intramolecular NCL [56,57], and so on. Then synthetic backbone-cyclized library can be prepared in combination with split-and-pool synthesis [58]. However, this approach has a limitation and difficulty in terms of library size (up to ~10^7) [59] and the identification of hit compounds because conventional Edman degradation cannot be used for cyclic peptides once the free *N*-terminus disappears after the backbone macrocyclization. Although tandem mass spectrometry can be used to analyze peptide sequences, the analysis of cyclic peptide sequence is more difficult than that of linear peptide sequence[60-63].

On the other hand, the genetic approach can circumvent these problems. This approach consists of two technologies. The expression of backbone-cyclized peptides relies on the split-intein circuit ligation of peptides and proteins (SICLOPPS) system[64]. In the SICLOPPS, the *C*- and *N*-terminal intein fragments (I_C and I_N, respectively) come

together to form an active intein that splices to give a backbone-cyclized peptide (**Figure 5**). The other technology is the reverse two-hybrid system (RTHS) [65,66]. The RTHS can couple host cell growth to the disruption of protein-protein interaction and thereby permits the activity-based selection of protein-protein interaction inhibitors. Thus the combination of SICLOPPS with RTHS allowed for the diverse library construction and in vivo selection of bioactive backbone-cyclized peptides[67,68]. The diversity is typically around 10^8, larger than that of the synthetic libraries. In addition, straightforward identification of hit compounds can be performed by sequencing of host cell gene.

Although the genetic selection with SICLOPPS has been used for the discovery of inhibitors against a wide variety of targets, it has several limitations. First, the selection condition is not versatile, and restricted as set in the context of the disruption of protein-protein interaction. Second, the SICLOPPS is limited to using the
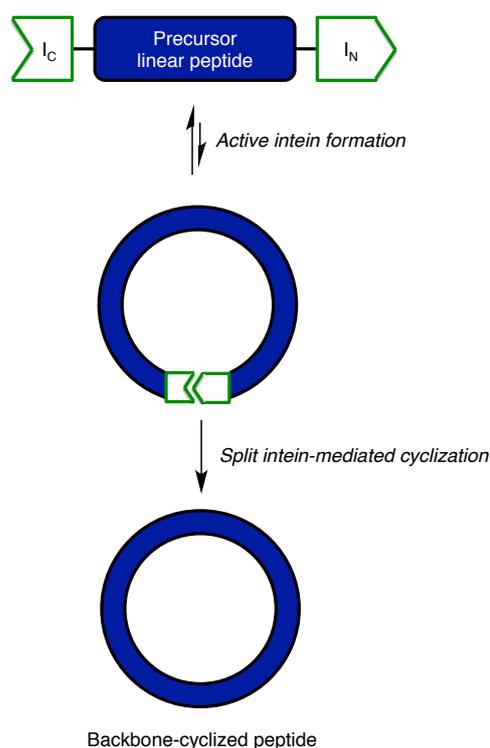

Figure 5 | Expression of backbone-cyclized peptide by SICLOPPS. An expressed fusion peptide folds to form an active intein. The following splicing results in backbone macrocyclization of the target peptide.

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20 proteinogenic acids as the building blocks, although this constraint has been partially removed by SICLOPPS with an orthogonal aminoacyl-tRNA synthetase/tRNA$_{CUA}$ pair to add 21st amino acid[69]. Third, the library size is much smaller than that of the in vitro display technologies ($\sim 10^{14}$ at maximum) as described below. For a randomized peptide library, the diversity is of great importance, providing the opportunity for exploration of the sequence space to a greater depth for the search of bioactive compounds.

In Vitro Display Technologies

In vitro display technologies such as phage display[70], ribosome display[71], mRNA display[72,73], etc. allow for the facile construction of polypeptide libraries consisting of hundreds of millions to over trillions mutants and the rapid screening of strong and selective binders to target proteins. These significant features of in vitro display technologies are largely achieved by linking the phenotype polypeptide to the corresponding genotype nucleic acid. In in vitro display, "hit" molecules are typically selected in vitro on an affinity basis from a library consisting of genotype-phenotype fusions, and the recovered genotypes are amplified. Thus the iterative rounds of these library construction, selection and enrichment allow molecular evolution in vitro, followed by easy identification of the selected phenotypes by sequencing of the encoded genotypes (**Figure 6**).

In vitro display technologies can be separated into cellular approaches using cell to express and clone the polypeptide libraries and acellular approaches in which the libraries are expressed truly in vitro and display is achieved without the need for transformation. For example, phage display is a representative technique of the cellular approaches, where the phenotype peptides or proteins are expressed and displayed on bacteriophage particles that have their corresponding DNAs inside. The cellular approaches also include yeast cell display[74] and bacterial display[75]. Despite the versatility of phage display, however, it has the limitations, particularly in terms of speed and library

diversity. This is mainly because transformation, the process which is indispensable for the cellular approaches, is inefficient and sets a practical threshold of ~10^9 clones[76], leading to a poor probability of affinity hits. This is the reason why the development of the acellular approaches has been prompted. The first cell-free in vitro display is ribosome display, and in ribosome display the genotype mRNA templates are connected with the nascent peptides in a complex with ribosome. mRNA display is a related technique and differentiates itself from ribosome display by the formation of covalent link between the mRNA templates and the expressed peptides via puromycin linker. In addition, DNA-based displays have also been developed where phenotype peptides and genotype DNA (not mRNA) are linked via DNA binding proteins[77,78]. Consequently, diverse libraries with as many as 10^{12} to 10^{14} unique sequences can be readily generated with these cell-free displays, which are several orders of magnitude higher than phage display[79].

On the other hand, the classic in vitro display technologies have a limitation that the displayed libraries are principally restricted to the 20 proteinogenic amino acid building blocks since the library generation depends on ribosomal synthesis, limiting the functions and structural motifs that can be explored. To overcome this issue, our group has recently developed a novel nonstandard peptide screening technology, referred to as RaPID (Rapid non-standard Peptide Integrated Discovery) display, by integrating mRNA display with the FIT system, a convenient genetic code reprogramming technique as mentioned above. We have already reported various nonstandard peptide libraries such as thioether macrocyclic peptide libraries[80,81], including *N*-methylated peptide libraries[82] and those with mechanism-based chemical warheads to the target proteins[83] expressed by the RaPID display. Thus the RaPID display has greatly expanded not only the sequence space of peptide libraries but also the diversity of structures and chemical entities in the peptides. However, even the RaPID display, as well as other in vitro displays, still has a limitation in terms of peptide structures to be expressed. For example, backbone-cyclized

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peptides have not been compatible with the RaPID display. In mRNA display including the RaPID display, genotype mRNA is covalently linked to the *C*-terminus of downstream peptide via puromycin linker. This linkage allows for iterative rounds of selection and amplification shown in Figure 6 and thereby is indispensable for mRNA display. Backbone-cyclized peptides can be ribosomally synthesized by expressing peptides possessing *N*-terminal cysteine and *C*-terminal thioester followed by intramolecular NCL. In this backbone macrocyclization, however, the peptide downstream of thioester connected with mRNA always works as a leaving group and genotype-phenotype linkage is disrupted. Thus backbone-cyclized peptides have not been compatible with mRNA display (**Figure 7**).
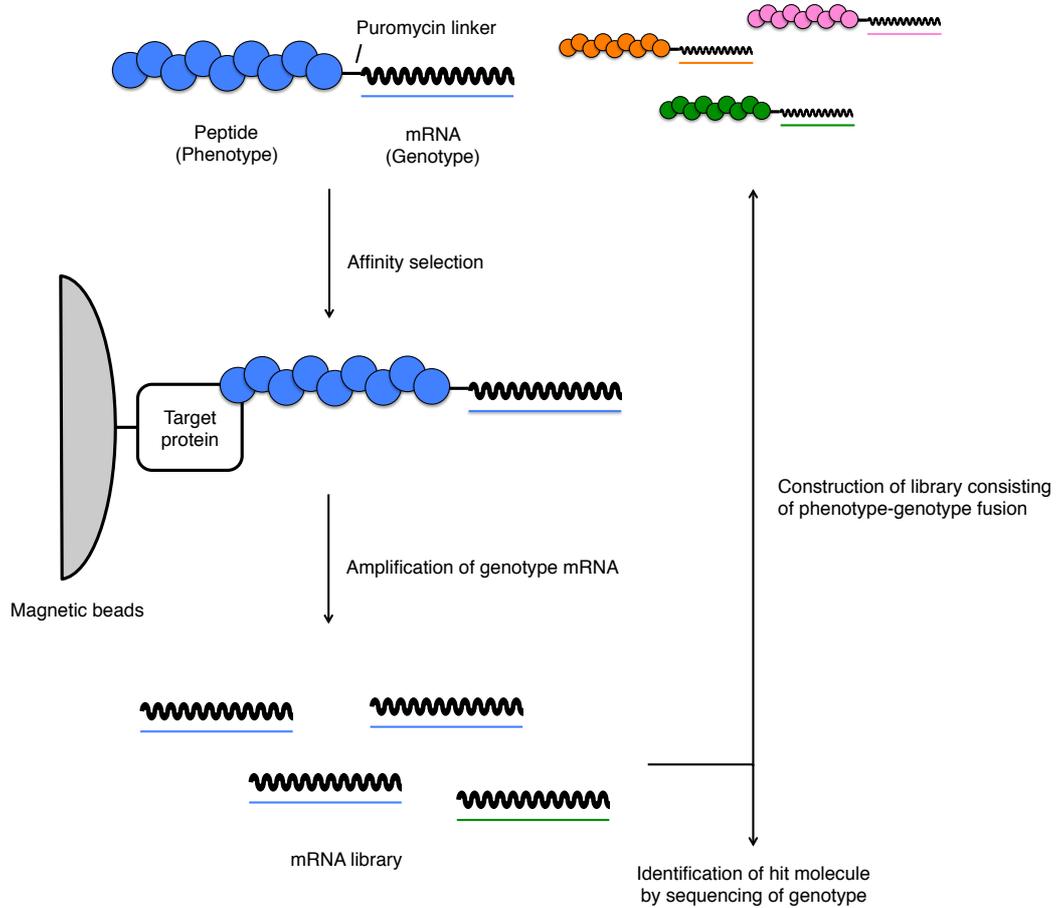
Figure 6 | Scheme of in vitro display using mRNA display as an example. In mRNA display, phenotype-genotype fusion is prepared in which phenotype and genotype are covalently connected via puromycin. Then target-binding peptide-mRNA fusions are selected from the library on an affinity basis. Selected mRNA sequences are amplified by reverse transcription, PCR, and transcription to afford an mRNA library. Thus obtained mRNAs are used for construction of next-generation peptide library or for identification of hit peptides by sequencing.
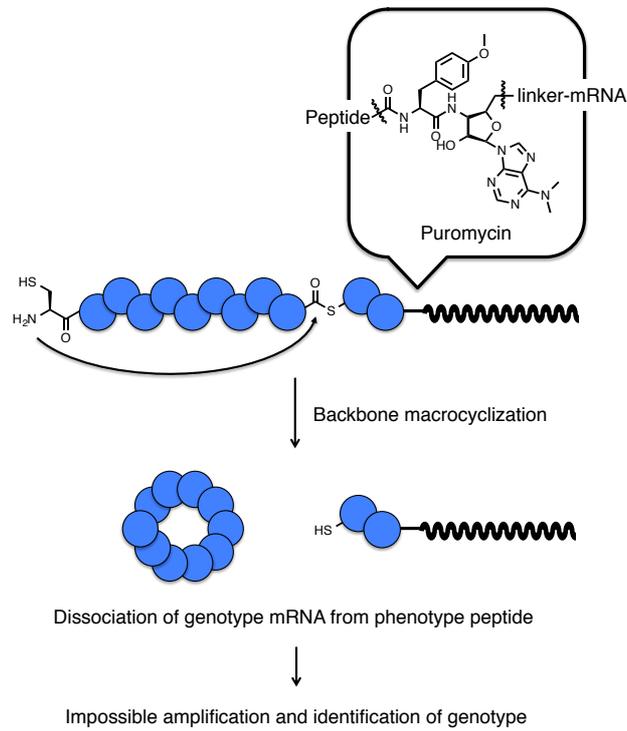
Figure 7 ǀ Backbone macrocyclization disrupting the linkage between genotype and phenotype. In this figure, backbone macrocyclization using intramolecular NCL is illustrated, but other backbone macrocyclization methods would also disrupt the linkage and not be compatible with mRNA display nor other in vitro display techniques.

Ribosome-Mediated Thioester Bond Formation (a Brief Introduction of Chapter 2)

As described above, ribosome-mediated thioester bond formation has not been shown nor used even though thioesters in polypeptides are turned out to be of great use in protein chemistry and chemical biology. Thus, the alternative recombinant protein thioester preparation methods have been developed and applied. However, if thioester bond formation by ribosome were turned out to be possible and compatible with applications, it would be an alternative technique for preparation of site-selectively thioester-incorporated proteins and peptides.

Therefore, in chapter 2, we investigated ribosome-mediated thioester bond formation by using the FIT system. Especially, the flexizymes were used for the easy preparation of thioacyl tRNAs, which were tRNAs charged with thio acid and indispensable for ribosomal incorporation of α-thio acids. Furthermore, a proof-of-concept application of ribosomal thioester bond formation toward NCL was studied. Here the NCL of VENUS, a yellow fluorescent protein, was demonstrated.

Backbone-Cyclized Peptides Compatible with In Vitro Display (a Brief Introduction of Chapter 3)

Since the backbone-cyclized peptides are attractive lead compounds for drugs as mentioned above, a high-throughput screening method from a highly diverse backbone-cyclized peptide library is expected to be developed. For that purpose, mRNA display including the RaPID display has notable advantages. First, diverse libraries with as many as 10^{12} to 10^{14} unique sequences can be readily generated and screened, which is several orders of magnitude higher than the genetic selection with SICLOPPS. Second, the covalent linkage of the peptide to its mRNA makes it possible to set arbitrary conditions in a selection in vitro. However, mRNA display, as well as other in vitro displays, has not been compatible with backbone-cyclized peptides since genotype leaves off from phenotype backbone-cyclized peptide in macrocyclization as mentioned above.

To overcome this issue, I speculated that an additional covalent bond linkage formation between genotype and phenotype would enable backbone-cyclized peptides to be used for in vitro display.

In chapter 3, the backbone macrocyclization compatible with mRNA display via a complex rearrangement strategy was investigated to realize this idea. The strategy was made possible with ribosome-mediated thioester bond formation, demonstrating the utility of this straightforward polypeptide thioester preparation method.

Chapter 2

Ribosome-Mediated Thioester Bond Formation

本章に関しては、特許申請のため特許申請のため、非公開。

Chapter 3

Backbone-Cyclized Peptides Compatible with

In Vitro Display

本章に関しては、特許申請のため特許申請のため、非公開。

Chapter 4

General Conclusion

To date, ribosome-mediated thioester bond formation has not been demonstrated nor used for applications despite the fact that not only peptide bond (amide bond) but also other analogues of peptide bond such as *N*-alkyl amide bond and ester bond can be formed by ribosome. Although the alternative recombinant protein thioester preparation methods such as intein-based approaches have been developed due to the utility of thioester bond, I envisioned that direct ribosome-mediated thioester bond formation would provide a at least supplementary or possibly better technique depending on the kinds of applications. Thus ribosome-mediated thioester bond formation was clearly demonstrated for the first time to our best knowledge in chapter 2. In addition, its utility was also demonstrated with NCL of a yellow fluorescent protein, VENUS. Although only such a proof-of-concept experiment was performed in this thesis for application to NCL, this original thioester bond formation method has several features and advantages that differentiate itself from other alternative methods. For example, in intein-based approaches, the size of intein (>150 aa) could be problematic for NCL of protein because it could disrupt the forming secondary or tertiary structure of protein-intein fusion depending on the insertion site. This drawback could be solved by my method because incorporation of one residue of α-thio acid, $^{HS}F^{p-Cl}$ would be less likely to influence the forming structure. In addition, thioester bond is prepared by conversion from thioester equivalent via *N* to *S* acyl shift from cysteine thiol in a lot of alternative methods, whereas thioester bond is directly formed by ribosome in my method. Thus possibly slow and inefficient thiolysis step can be omitted in my method. Besides, downstream peptide of thioester can be used for application, and no cysteine is required to form thioester bond in my method. These features would possibly make novel applications that are not accessible by alternative methods.

In fact, the features are largely utilized for backbone macrocyclization of peptides performed in chapter 3. In this chapter, backbone macrocyclization of peptides compatible with mRNA display was achieved via a complex rearrangement strategy where ribosome-mediated thioester bond formation, spontaneous thioether bond formation after translation, *N*-terminal ^{Me}Cys liberation, and intramolecular NCL occur. Backbone-cyclized peptides have not been compatible with in vitro displays because backbone macrocyclization would disrupt the indispensable linkage between the

genotype and the phenotype peptide. I speculated that this was circumvented by an additional side chain-to-side chain covalent bond linkage formation between genotype and phenotype before the macrocyclization. I used thioether bond formation reaction between chloroacetyl group and thiol for the linkage because this reaction has desirable characteristics for mRNA display. First, genetic incorporation of only one non-canonical residue, Cab, is needed for the bond formation and thereby the limited resource of building blocks to be used in ribosomal synthesis can be saved. Second, since this reaction occurs fast and spontaneously, reducing the possibility of thioester hydrolysis and any extra steps such as addition of reagents and purification. Importantly, this reaction could be used in the strategy by ribosome-mediated thioester bond formation. In the alternative methods, not downstream cysteine but cysteine in thioester equivalent would react with Cab, resulting in no thioester bond formation nor backbone macrocyclization. Intein-based approaches are not favorable especially for the RaPID display because expression of intein requires 20 proteinogenic amino acids, making ribosomal incorporation of other non-canonical substrates and expression of libraries with diverse structures and chemical entities impossible. In the strategy using ribosome-mediated thioester bond formation, backbone cyclized peptides maintaining downstream peptide were successfully obtained even though thiol-thioester exchange was observed with downstream cysteine. mRNA display can readily generate and screen diverse libraries with as many as 10^{12} to 10^{14} unique sequences, which is several orders of magnitude higher than the genetic selection using SICLOPPS with up to 10^9 sequences. For a randomized peptide library, the diversity is of great importance, providing the opportunity for exploration of the sequence space to a greater depth for the search of bioactive compounds. Thus this result would be an important milestone toward the goal of the screening of drug candidates with a backbone-cyclized peptide scaffold.

The studies carried out in this thesis have validated the utility of ribosome-mediated thioester bond formation for not only classic native chemical ligation but also a backbone-peptide cyclic scaffold of peptides via a complex rearrangement strategy compatible with in vitro display. Particularly, not other alternative peptide-thioester preparation medthods but only ribosome-mediated thioester bond formation could be used for the strategy for the synthesis of the backbone-cyclized

peptides. Thus, this novel and original thioester bond formation method would extend the range of accessible applications and contribute to the development of protein chemistry and chemical biology.

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List of accomplishments

Paper

1. "Preparation of Mercaptoacyl-tRNAs for Ribosomal Thioester Bond Formation"

R. Takatsuji, T. Katoh, H. Suga, *Peptide Science 2014*, **2015**, 139-140

Oral presentations

1. "リボソームによるチオエステル結合とそのネイティブケミカルライゲーションへの応用"

高辻諒、加藤敬行、菅裕明、第 9 回バイオ関連化学シンポジウム、熊本、2015 年 9 月

2. "Thioester bond formation in ribosome"

R. Takatsuji, T. Katoh, H. Suga, 日本化学会第 95 春季年会、千葉、2015 年 3 月

Poster presentations

1. "Thioester bond formation by ribosome and its application"

R. Takatsuji, T. Katoh, H. Suga, Pacifichem 2015 Congress, Hawaii, December 2015

2. "リボソームによるチオエステル結合の形成"

高辻諒、加藤敬行、菅裕明、第 8 回バイオ関連化学シンポジウム、岡山、2014 年 9 月

3. "RIBOSOMAL THIOESTER BOND FORMATION"

高辻諒、加藤敬行、菅裕明、第 51 回ペプチド討論会、徳島、2014 年 11 月

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