学位論文（要約）

The construction of artificial macrocyclic non-standard peptide libraries and discovery of drug leads against proteins related to intracellular proteolysis pathways

(人工特殊ペプチドライブラリーの構築と細胞内タンパク質分解経路関連タンパク質に対する薬剤候補探索)

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Chapters 2, 3, 4, and 5 are not shown due to the reasons involving publication of the paper. All references are included in this text.

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Abstract (abridged)

Peptides exhibiting macrocyclic structures often show properties such as high bioactivity, structural rigidity, membrane permeability, and protease resistance. Peptides containing *N*-alkyl amino acids also show membrane permeability, and protease resistance. Due to these characteristics, peptides exhibiting macrocyclic structures and *N*-alkyl backbone modifications, referred to as macrocyclic non-standard peptides, have attracted recent attention as candidate therapeutic agents. Therefore, it is important to develop a technology for drug discovery of artificial peptides possessing these characteristic structures. This thesis describes the synthetic and screening methodologies of macrocyclic non-standard peptides by means of genetic code reprogramming and peptide selections against the proteins related to intracellular proteolysis pathways.

Chapter 1 describes the background of this thesis, the importance of macrocyclic non-standard peptide drugs, and unique features and contribution for biological activities of peptides derived from natural sources are summarized. Problems of conventional translational synthetic and screening methods and advantages of genetic code reprogramming are also pointed out.

Chapter 2 describes the establishment of the methodology to synthesize peptides containing *N*-alkylated backbone and macrocyclic scaffold by means of genetic code reprogramming. The construction method of the highly diverse libraries, which consist of macrocyclic peptide exclusively, is also described in this chapter.

Chapter 3 describes an *in vitro* selection of macrocyclic non-standard peptides that bind to hAtg3 related to autophagy system by integrated method with mRNA display and genetic code reprogramming.

Chapter 4 describes an *in vitro* selection of macrocyclic non-standard peptide binding to HECT domain of E3 ubiquitin ligase Smurf2 related to breast and esophageal squamous cell carcinomas.

Chapter 5 describes the general conclusion of this thesis and the potential of this study.

Chapter 1

General introduction

1-1. Macrocyclic non-standard peptides

Most of drugs sold on the market are small organic compounds that usually inhibit target proteins by competing against biomolecules. In fact, almost of all small molecule kinase inhibitors approved currently bind at the active sites of target enzymes in competition with ATPs[(1)]. On the other hand, small organic compounds rarely inhibit protein-protein interactions, because these interactions occupy large area (750~1500 $Å^2$)[(2)(3)] that is too wide to block by small compounds. Recently, the important role of protein-protein interactions in major diseases, such as cancers and neurodegenerative diseases have been revealed, and the compounds inhibiting protein-protein interactions can be good treatments for these diseases.

Recently, peptides have attracted a much attention as the new drug candidates to block protein-protein interactions. Peptides bind to target proteins with wide interactions that contribute to specific inhibition of protein-protein interactions due to larger sizes than small organic compounds. In fact, many peptides work as hormones[(4)(5)], antitumors[(6)(7)], antivirotics[(8)(9)] *in vivo*, and these bioactive peptides often possess unique structural features. For example, cyclosporin A[(10)] was isolated from the fungus *Tolypocladium inflatum*, and this peptide possesses a macrocyclic structure and

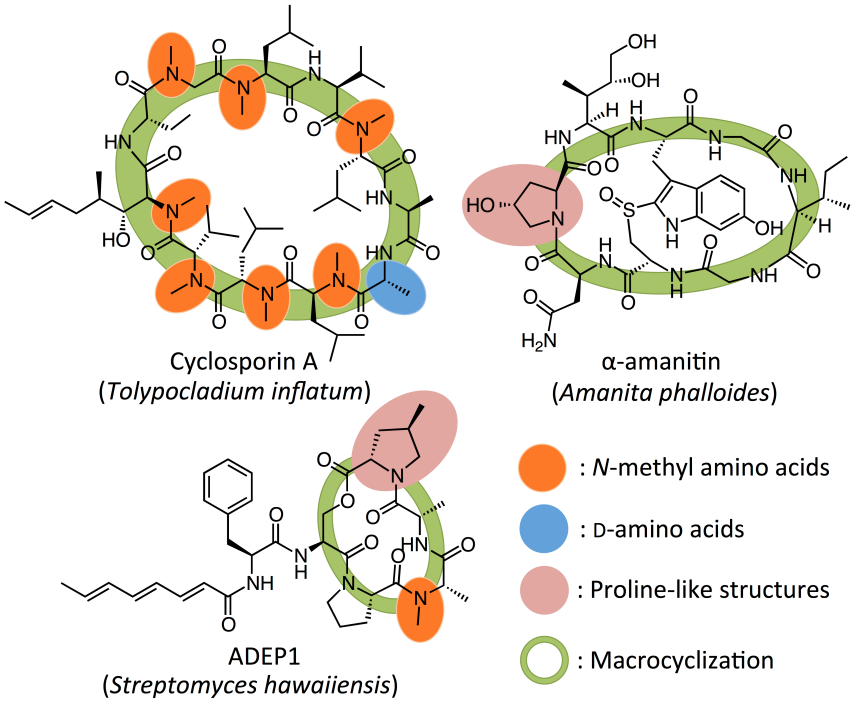

Fig. 1-1 Chemical structures of macrocyclic non-standard peptides derived from natural sources. The compound names and the names of species that generate the compounds are also shown.

several *N*-methyl backbone modifications. α-amanitin[11] (isolated from *Amanita phalloides*), and ADEP1[12] (isolated from *Streptomyces hawaiiensis*) also have the characteristic structures such as D-amino acids, macrocyclic scaffolds, *N*-methyl backbone modifications, and proline-like structures (**Fig. 1-1**). These peptides are usually constructed by non-ribosomal synthesis with non-ribosomal peptide synthetases (NRPSs) or post-translational modification by enzymes in living things. Recent studies have revealed that the peptides containing these characteristic structures (macrocyclic non-standard peptides) often show structural rigidity, proteolytic resistance and membrane permeability due to the characteristic structures. Some macrocyclic non-standard peptides are already in clinical use, for example, cyclosporin A is used as an oral drug against autoimmune diseases and α-amanitin is used as a RNA polymerase II-specific inhibitor in biological studies.

The construction of highly diverse libraries of macrocyclic non-standard peptides derived from natural sources and the screening from these libraries enable us to discover bioactive peptides as new drug candidates effectively and rapidly. However, isolation of a wide variety of natural products is laborious, and *in vitro* reconstitution of synthetic mechanisms of non-standard peptides is not suitable for the preparation of a diversity of compounds because one NRPS can synthesize only one peptide and post-translational modification enzymes modify target peptides in a sequence-dependent manner.

On the other hand, the peptide synthetic method using translation system, referred as a ribosomal synthesis, can construct artificial peptide libraries consisting 10^{13} unique individuals in a microliter scale. However, the peptides synthesized by ribosomal synthesis hardly possess the characteristic structures like natural products because only 20 proteinogenic amino acids can be utilized in translation system.

Thus, the new method to construct the highly diverse libraries of "natural product-like" macrocyclic non-standard peptides can help a breakthrough of the development of peptide drugs, and our laboratory has developed a new methodology to overcome the above barrier based on a genetic code reprogramming.

1-2. Genetic code reprogramming and Flexible In vitro Translation system

Forster *et al.* reported that three non-proteinogenic amino acids were assigned to the three sense codons and incorporated into a peptide in 2003 [13]. To achieve this sense codon suppression, the authors constructed a unique translation system lacking proteinogenic amino acids, aminoacyl tRNA synthetases (ARSs), RFs, and ribosome recycling factor (RRF) for creating "vacant" codons. Then, the non-proteinogenic amino acids were assigned to the vacant codons by adding cognate tRNAs acylated with the non-proteinogenic amino acids (**Fig. 1-2**). This experiment suggested that the vacant codons made by removing amino acids and ARSs can be used for the assignment of non-proteinogenic amino acids, and peptides incorporating non-proteinogenic amino acids (non-standard peptides) can be translated under single turnover translational conditions.

In Forster's study, tRNAs were acylated by the classical chemoenzymatic method[14]-[16] that is laborious and technically demanding to prepare wide array of aminoacyl tRNAs. To prepare the many different aminoacyl tRNA, flexizymes[17]-[22],

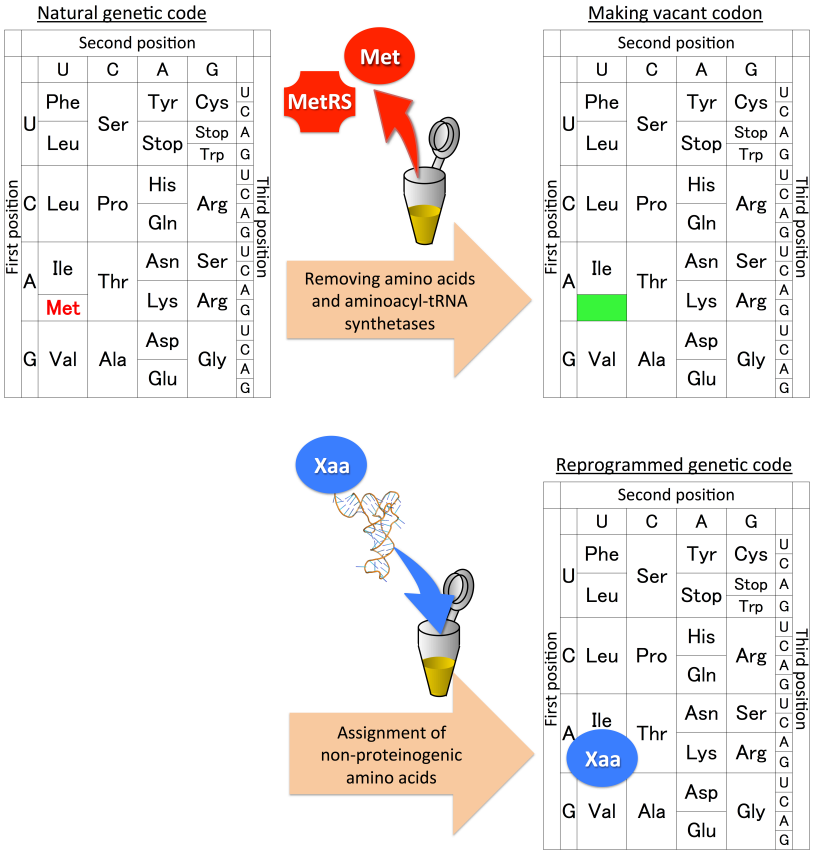

Fig. 1-2 The general concept of genetic code reprogramming. Xaa represents non-proteinogenic amino acids.

de novo artificial tRNA acylation ribozyme developed in our laboratory, are a highly flexible tools. A dinitro-flexizyme (dFx) charges amino acids and hydroxy acids esterified with a 3,5-dinitrobenzyl (DBE) group to tRNAs independent from the type of side chain, while an enhanced-flexizyme (eFx) charges aromatic amino acids activated with cyanomethyl ester (CME)[23][24] (**Fig. 1-3**). Both flexizymes form complementary base pairing with 3'-end sequence of tRNA that is reserved among tRNAs. It means that any amino acid could be charged onto any desired tRNA by flexizymes theoretically[25][26].

On the other hand, several reconstitutive *in vitro* translation systems capable to reconstitute components were reported [27]-[29]. These systems allow us to supply and withdraw the translational components easily. In reconstitutive *in vitro* translation system, any desired vacant codons can be created by withdrawing amino acids and ARSs, and this characteristic is suitable for genetic code reprogramming.

Our laboratory has developed a new technology, composed of the flexizyme technology and *in vitro* translation system, that enables us to reprogram genetic code as we wish and create new engineered translation system to synthesis macrocyclic non-standard peptides easily. This system is called as "FIT (Flexible *In vitro* Translation) system"[30].

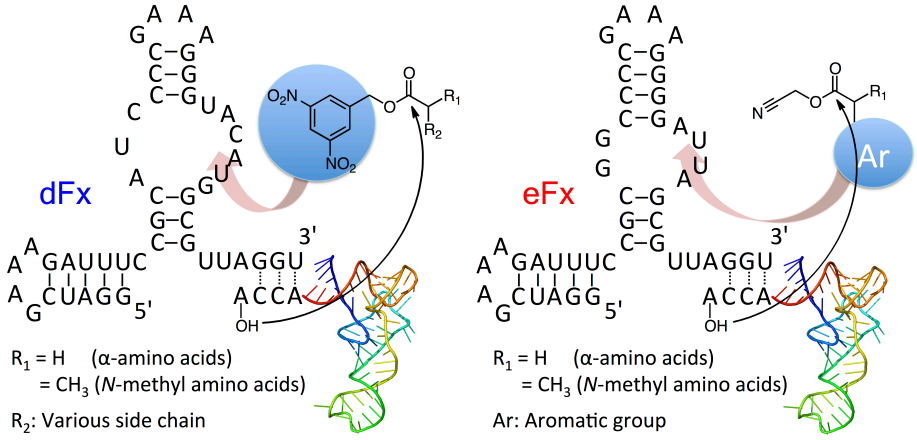

Fig. 1-3 The secondary structures of dFx and eFx. dFx recognizes the 3,5-dinitrobenzylalcohol leaving group, while eFx recognizes the aromatic side chain. Both flexizymes recognize a 5'-DCC-3' (D = G,A,U) sequence at the 3' terminus of tRNA and specifically acylate at the 3'-hydroxy group of tRNA.

1-3. Random non-standard Peptide Integrated Discovery system

FIT system allows us to synthesize macrocyclic non-standard peptides in a template-dependent manner, and we can also construct highly diverse macrocyclic non-standard peptide libraries by translating a wide variety of mRNAs with reprogrammed genetic codes.

To discover potent peptides efficiently from the highly diverse libraries, high-throughput screening methods are needed. *In vitro* selection strategies of bioactive peptides such as phage display[31][32], ribosome display[33][34], and mRNA display[35][36] are powerful screening methodologies for isolation of macrocyclic non-standard peptides binding to target proteins. During *in vitro* selection, macrocyclic non-standard peptides (phenotype) tethered with their genetic information (genotype) are screened from library under an environmental pressure such as temperature, target concentration, and presence of competition. In the case of mRNA display, peptides are tethered with mRNAs via puromycin linker conjugated to 3'-end of mRNAs. After peptide-mRNA complexes are converted into peptide-mRNA-cDNA complexes by reverse-transcriptases, the peptide-mRNA-cDNA complexes are mixed with selection target proteins immobilized on beads. Washing out non-binding compounds, cDNAs of binding peptides are recovered and amplified by PCR. Amplified DNAs are converted into mRNA library, and after mRNAs are conjugated puromycin linkers, peptide-mRNA complexes are generated again. Repeating these operation several times, strong binders would be discovered.

Our laboratory has developed the selection method of macrocyclic non-standard peptides, composed of FIT system coupled with an mRNA display method. This new screening method is called as RaPID (Random non-standard Peptide Integrated Discovery) system (**Fig. 1-4**). In the RaPID system, potent macrocyclic non-standard peptides are selected by the affinity to targets, from non-standard peptide libraries consisting of more than 10^{13} unique compounds in microliter scale. We have discovered bioactive peptides against therapeutic targets using RaPID system[37]-[39].

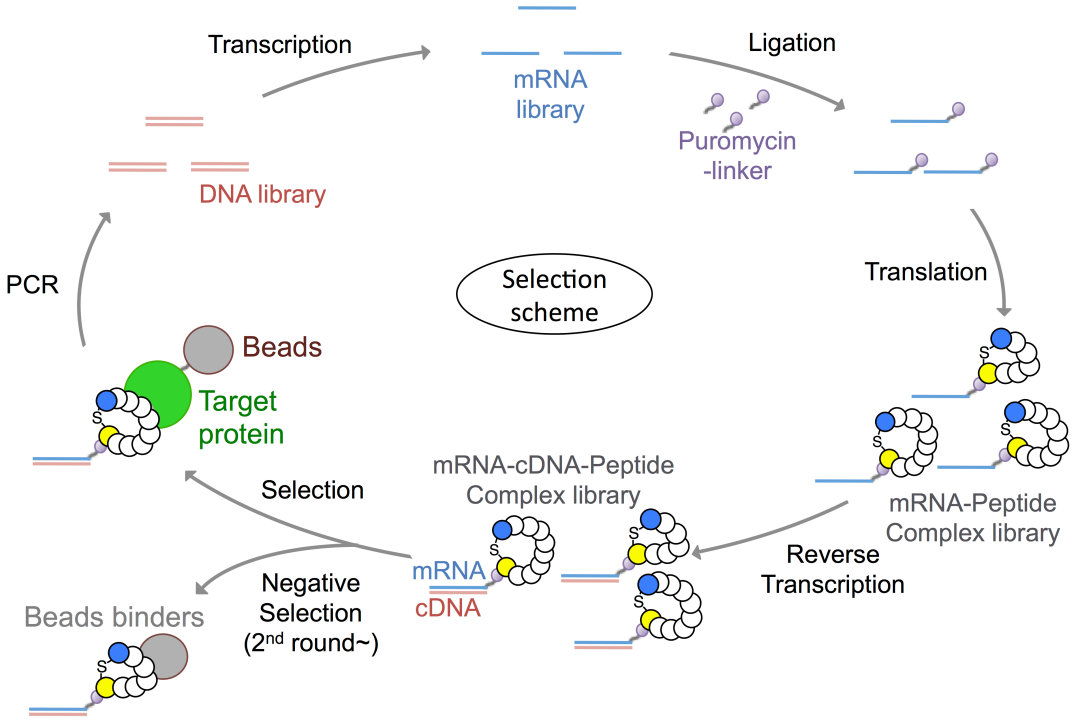

Fig. 1-4 The scheme of RaPID system. Every peptide is evaluated by its binding ability against target proteins immobilized on beads, and strong binding peptides would be discovered after several cycles screening.

1-4. Summary

In chapter 1, the definition and importance of macrocyclic non-standard peptides were described; subsequently unique structures of macrocyclization and *N*-alkyl backbone modifications derived from non-proteinogenic amino acids, and their contribution for biological activities were summarized. The difficulty of the construction of highly diverse natural product-like macrocyclic non-standard peptide library and our approach to overcome the difficulty were also pointed out.

1-5. References

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Chapter 2

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Chapter 3

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Chapter 4

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Chapter 5

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