Doctoral Dissertation

博士論文

Synthesis of γ/δ-peptide linkages in ribosomally synthesized peptides by means of chemical modifications

（化学的修飾反応による翻訳ペプチドにおける
γ/δ-ペプチド結合の形成）

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Abstract

Represented by statine in natural bioactive peptides, γ-peptides represent a promising building block for the peptide drugs. Contrary to their attractive structures, the synthesis of peptides containing γ-peptides is highly challenging in the translation reaction. To lead the discovery of novel peptidic drugs, a platform to synthesize various γ-peptides is essential. In this thesis, I describe a facile methodology to produce γ- and δ-peptides in ribosomally synthesized peptides by means of posttranslational chemical modification reactions. The reactions also allow me to achieve the total synthesis of a peptide inhibitor containing a statine residue, suggesting that the established reactions are applicable for the design and synthesis of novel bioactive peptides.

Chapter 1 mentions the general introduction of this thesis. In this chapter, natural bioactive peptides are quickly reviewed, and the importance and synthetic difficulties of γ-peptides are also discussed. Furthermore, translation system, which is mainly utilized in this thesis, and related various tools are described to show advantages and limitations.

Chapter 2 describes the concept of this study: posttranslational chemical modification reactions. In this chapter, I also demonstrated that the designed reactions are able to form the γ-peptide linkages in ribosomally synthesized peptides through several optimizations for maximizing the conversion yield of the reactions.

Chapter 3 describes the investigation of substrate scope for the posttranslational chemical modification reactions. It was elucidated that not only the statine and its analogue but also various γ- and δ-peptides are applicable, indicating the generality of the developed reactions. The studies also provided me the mechanistic knowledge of modification reactions.

Chapter 4 describes the total synthesis of a peptide inhibitor named P10-P4'statV containing a statine residue. For this purpose, an additional posttranslational deprotection reaction step was combined to the developed reactions. As the results, total synthesis of P10-P4'statV by utilizing translation system and chemical modifications was successfully demonstrated.

Chapter 5 is the general conclusion of my thesis. In this topic, the achievements of this thesis and perspectives are described.

Contents

Abbreviation list

ARS	Aminoacyl-tRNA synthetase
AcOH	Acetic acid
aq.	Aqueous
BPB	Bromophenol blue
Boc	*Tert*-butoxycarbonyl
Calc.	Calculated mass value
ClAc	Chloroacetyl
CBT	4-chlorobenzyl thioether
CME	Cyanomethyl ester
DBE	2,4-dinitrobenzyl ester
DCM	Dichloromethane
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine-*N,N,N',N'*-tetraacetic acid
ESI	Electrospray ionization
EtOAc	Ethyl acetate
EtOH	Ethanol
GMP	Guanosine monophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LC-MS	Liquid Chromatography-Mass spectrometry
MALDI	Matrix Assisted Laser Desorption/Ionization
MeCN	Acetonitrile
MeOH	Methanol
mRNA	Messenger RNA
MS	Mass spectrum
NaOAc	Sodium acetate
N.C.	Negative control
NMR	Nuclear magnetic resonance
NTP	Nucleoside triphosphate
Obs.	Observed mass value

PAGE	Poly-Acrylamide Gel Electrophoresis
P.C.	Positive control
PCR	Polymerase chain reaction
sat.	Saturated
TBS	Tris-Buffered Saline
Temp.	Temperature
TFA	Trifluoroacetic acid
THF	Tetrahydrofran
Tris	Tris(hydroxymethyl)aminomethane
tRNA	TransferRNA
TOF	Time of flight
XIC	Extracted ion chromatogram
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil
Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
Cys (C)	Cysteine
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Gly (G)	Glycine
His (H)	Histidine
Ile (I)	Isoleucine
Leu (L)	Leucine
Lys (K)	Lysine
Met (M)	Methionine
Phe (F)	Phenylalanine
Pro (P)	Proline
Ser (S)	Serine
Thr (T)	Threonine
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine

Val (V)	Valine
Sta	Statine
δ	Chemical shift
s	singlet
t	triplelet
m	multiplet
br	broad
h	hour(s)

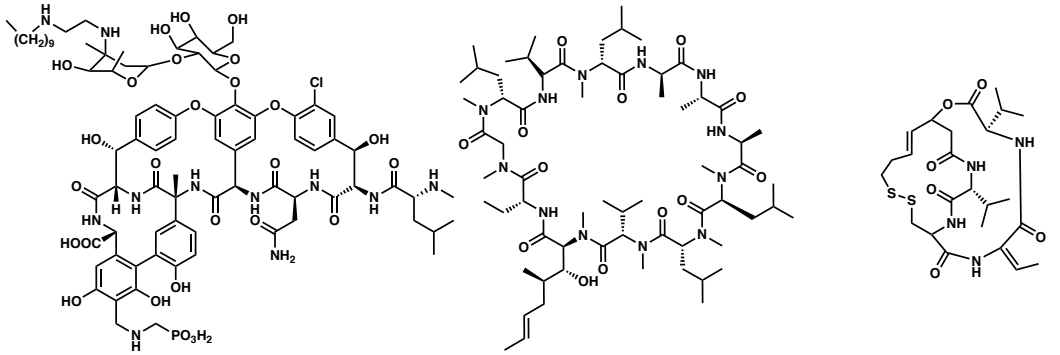
Chapter 1 General introduction

1.1. Natural bioactive peptides as drug candidates

Nature has been proven as the fertile source of bioactive peptide drugs: today, several peptides isolated from nature are available as drugs (**Figure 1.1**)[1–4]. The most characteristic point of natural bioactive peptides is that they often bear macrocyclic structures via various types of connections such as head-to-tail, disulfide bond, and ester linkage. Compared to the linear feature, macrocyclic peptides are able to bind to target proteins more tightly by reducing their binding entropy[5,6]. Furthermore, the large surface of macrocycles also contributes to their selective inhibitory activities[7]. In addition to the macrocyclic structure, backbone modification is another important factor for peptide drugs. Compared to normal peptides composed of α-proteinogenic amino acids, backbone modified peptides, for instance peptides containing N-methylated amino acids or D-amino acid, resist to proteolytic degradation[8,9]. Backbone modification with a macrocycle also adjusts the pattern of internal hydrogen bonding to increase cell-permeability[10,11]. These characters enable the artificial macrocyclic peptides to inhibit protein-protein interaction, which is still difficult drug target in today's drug development[12,13]. Although the drug discovery today is mainly focusing on small molecules or antibodies, nowadays macrocyclic peptides are regarded as next generation drugs because of their potentials[7,14].

Toward the discovery of novel bioactive peptides, a number of efforts have been made to discover novel peptides, or to develop the platform for accelerate the drug discovery. Traditionally, bioactive peptides have beem reported through isolation of compounds from natural organisms, elucidation of their structures, and investigation of their bioactivity by screening to targets. Although such method is still a powerful today, the limitations of this approach were also emerged: the shortage of resources led the re-discovery of bioactive compounds, or the spread of resistant pathogens were much faster than the discovery of novel drugs[15–17]. Recent advances, such as improvement of the screening system[18] and genome mining methodology[19,20], has been able to improve the situations to discover "overlooked" peptides. Indeed, recent studies discovered numerous peptides containing

highly unique structures, for instance, C-C bond crosslinked structures between Lys and Trp[21,22]. These surprising examples remind us that we can learn the structural diversity of natural bioactive peptides, and can rationally design novel bioactive peptides based on these knowledges.



Telavancin
antibacterial

Cyclosporin A
immunosuppressive property

Romidepsin
anticancer activity

Figure 1.1 | Examples of natural bioactive peptides bearing macrocyclic structures

1.2. γ-Peptides in natural bioactive peptides

Natural bioactive peptides often contain unique non-proteinogenic structures. Among all, γ-peptides are quite interesting because of their inhibitory mechanism of bioactive peptide. For example, pepstatin A, a well-studied potent inhibitor to aspartic protease family, is a linear peptide bearing two γ-peptide structures named as statines[23] (**Figure 1.2a**). Generally, aspartic protease recognizes the sequence of substrate peptides and catalyzes hydrolytic cleavage of a peptide linkage by utilizing two aspartic acid residues. To this protease, pepstatin A is able to show sub-micromolar order inhibitory activity by stacking into the catalytic pocket[24–28] (**Figure 1.2b**). The mechanistic study suggested that such inhibitory activity of pepstatin A is derived from the its analogous structure of a tetrahedral intermediate[29,30] (**Figure 1.2c**). Although most of bioactive peptides possess the cyclic feature, such a unique mechanism allows pepstatin A to work as a peptide inhibitor even in the linear peptide structure.

Pepstatin A was discovered from actinomycetes in 1970[23]. Initiated from this study, a number of researches have revealed the existence of bioactive peptides containing γ-peptide(s) (**Figure 1.3**). Six kinds of grassystatins A-F were isolated from marine cyanobacteria as linear peptides, in which all of them contain one statine[31,32]. A series of statine analogues are also found in nature; tasiamide B and F contain unique phenylstatine structures, in which an isobutyl group of the statine side chain to a benzyl group[33–35]. All of the peptides described above have linear structures and inhibit aspartic proteases: it is considered that the mode of action of them would be the same as that described in pepstatin A. Didemnins and tamandarins are bioactive peptides which contain different statine analogues[36–39]. Both of them are composed of small cyclic structures, nonproteinogenic amino acids like *O*-methylated tyrosine, and alanine/leucine-like statine analogues. The study for inhibitory activity of didemnins demonstrated that they can bind to the catalytic pocket of eukaryotic elongation factor eEF1A to inhibit translation, and the residue of statine analogue contributes to stabilize the binding state by forming hydrogen bond network[40]. Microsclerodermin[41], theonellamide[42], and nagahamide[43] are other examples of bioactive peptides bearing γ-peptides or even the δ-peptide structures.

These examples strongly suggest that γ-peptides possess an attractive feature for discovery of novel peptide drugs. Indeed, some of the structural analogues based on peptides

shown in **Figure 1.3** were applied to the drug usage; Aplidin, also known as Plitidepsin, is the analogue of didemnin. It has been recently approved as a drug for multiple myeloma[44]. The example suggests us that the establishment of synthetic methodology to produce γ-peptides in drug discovery systems would accelerate the discovery of novel peptide drugs.

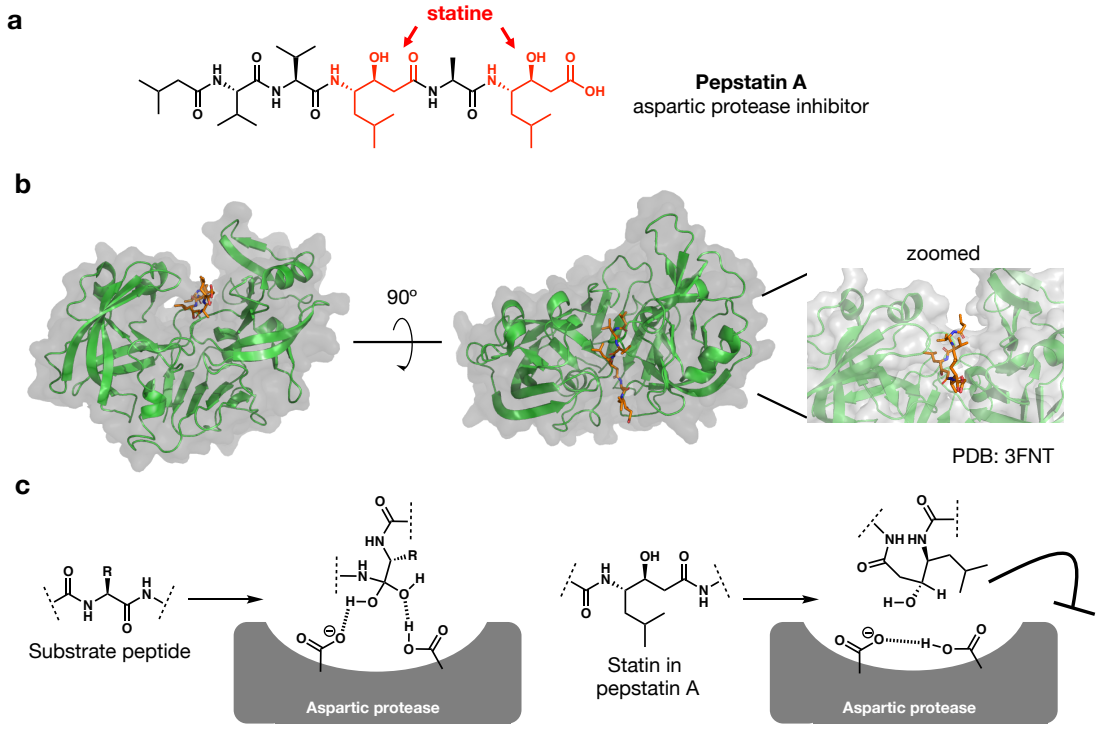


Figure 1.2 | Pepstatin A and its inhibitory mechanism

(a) Structure of pepstatin A. Statine is highlighted in red.

(b) Crystal structures of pepstatin A (orange stick) bound to the aspartic protease (green cartoon).

(c) Schematic illustration of a tetrahedral intermediate of substrate peptides and inhibitory mechanism of peptstatin A.

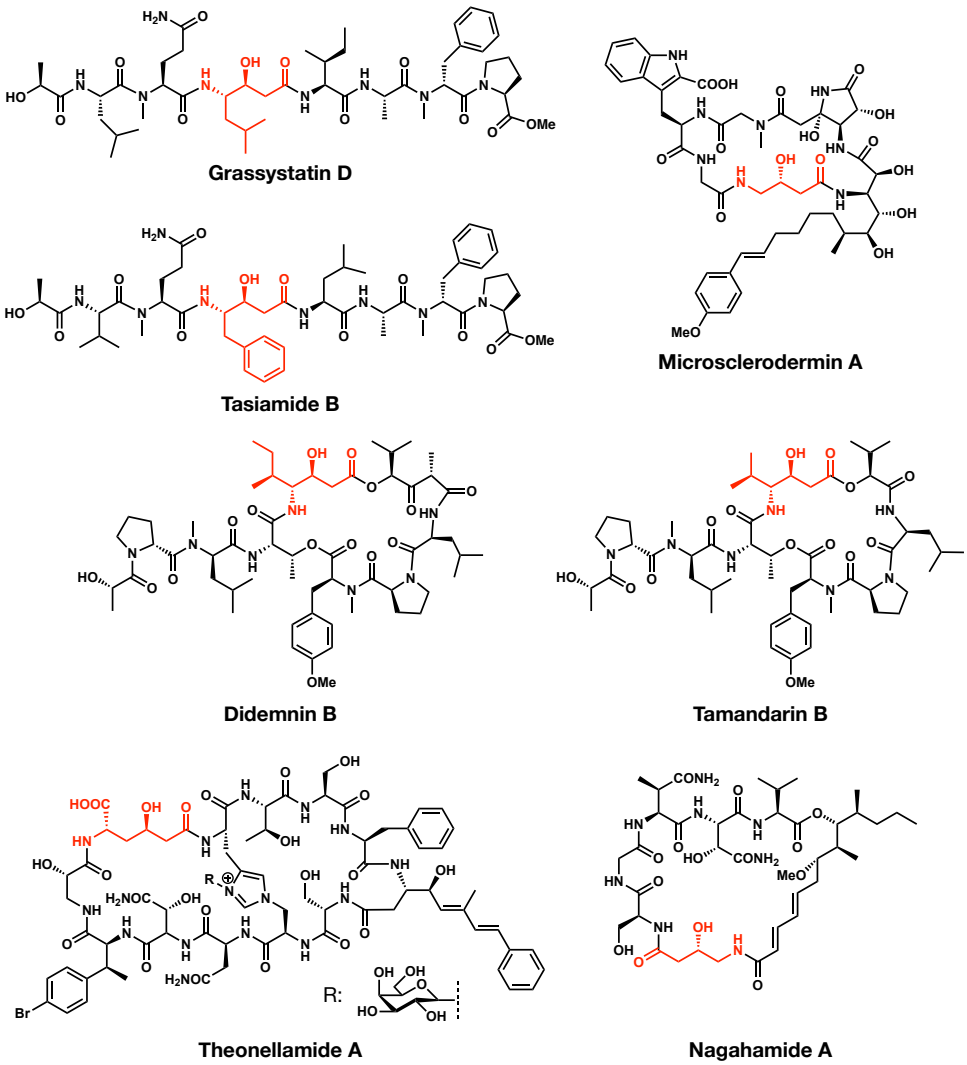


Figure 1.3 | Structural examples of natural bioactive peptides containing γ- or δ-peptides

1.3. Synthesis of γ-peptides by NRPS-mediated pathway

In nature, bioactive peptides containing γ-peptides are synthesized by nonribosomal peptide synthetase (NRPS). The structure of NRPS can be divided into domain units called modules. The module unit is composed of at least three domains: adenylation domain (A) to activate substrate amino acid, thiolation domain (T) as a substrate carrier, and condensation domain (C) for formation of a peptide linkage (**Figure 1.4a**). Each module is responsible for recognition of substrate and elongation of peptide chain, and finally thioesterase (TE) domain facilitates hydrolysis or cyclization to produce mature peptides. The analysis of discovered natural peptides suggests that NRPS can incorporate not only β-, or γ-amino acids, but also α-hydroxy acids into peptides[45,46]. Furthermore, NRPS is capable of complex formation with polyketide synthetase (PKS), an enzymatic family with the module system, and provide peptides modified with fatty acid moieties or highly modified carbon chains[47,48]. Indeed, statine is synthesized from PKS-NRPS enzyme, where a leucine and a malonyl-CoA are condensed to elongate a carbon chain by ketosynthesaze (KS), followed by an enantioselective reduction of a carbonyl group by keto reductase (KR) domain (**Figure 1.4b**)[46,49–51].

Although NRPS is able to synthesize complicated and sophisticated peptide structures, the poor substrate acceptability is always accompanied due to the strict selectivity toward substrates by each modules. To expand the substrate scope, a number of studies have been energetically conducted to obtain *de novo* bioactive peptides. To date, swapping[52], deletion[53], and insertion of the modules[54] are acceptable to generate novel nonribosomal peptides[55–57]. In addition, recent progress of the directed evolution methodology enables facile engineering of NRPS[58–61]. However, these engineering often suffered from the problems, such as dropping the yield, lack of generality, and limited diversity of products up to 10^2 order[57]. Owing to the poor diversity, a general procedure to discover bioactive peptides by engineering NRPS has not been established yet, although their structures are attractive for their inhibitory activity.

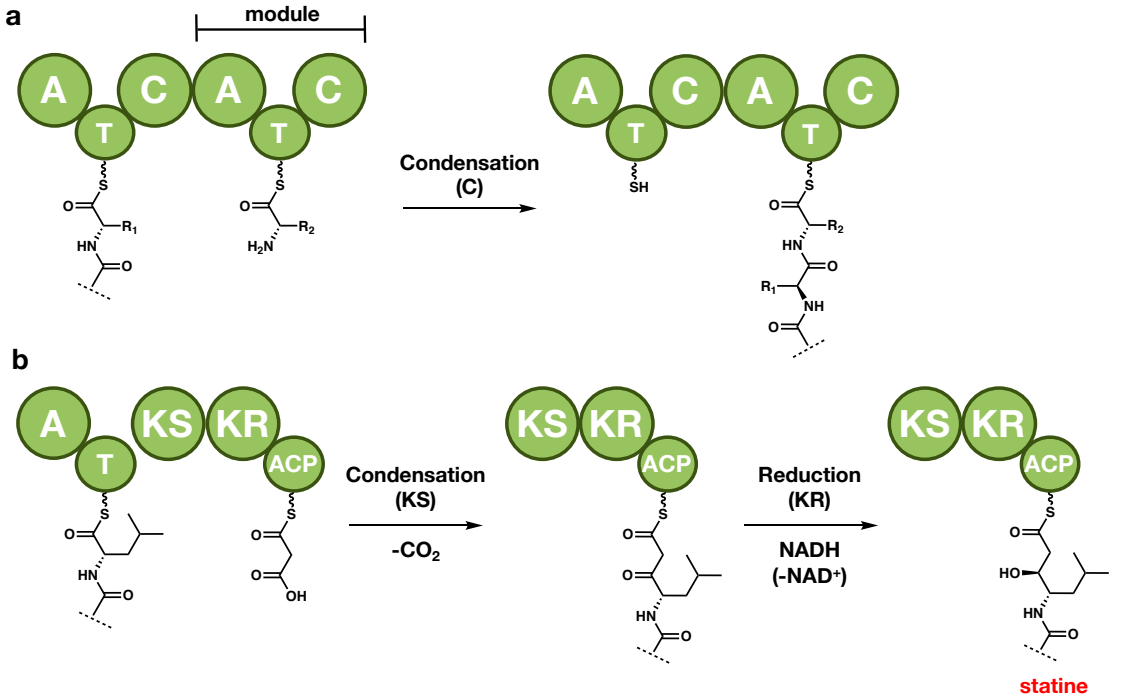


Figure 1.4 | Schematic illustration of nonribosomal peptide synthetase

(a) Formation of peptide linkages in NRPS system. Abbreviation of each domains is mentioned in the main text.

(b) Synthetic scheme of statine residue in PKS-NRPS system. ACP: acyl career protein.

1.4. Translation reaction and its engineering tools

1.4.1. Translation reaction in nature

Compared to NRPS system, translation is another pathway to synthesize polypeptides, in which ribosome catalyzes to form nascent peptidyl bonds. In the translation reaction, each proteinogenic amino acids are charged onto correspond tRNAs catalyzed by ARSs, then aminoacyl-tRNAs are accommodated into ribosome based on their anticodon and mRNA sequence. The sequence of synthesized peptides depends on mRNA template according to the genetic codes. Owing to its mRNA templated dependency, translation reactions are widely used for the synthesis of diverse peptides and proteins in nature. Indeed, in addition to nonribosomal peptides, a large number of natural peptides are classified into the large family called RiPPs (Ribosomally synthesized and Post-translationally modified Peptides), in which peptides are initially synthesized by translation and then are highly modified by a series of post-translational enzymatic reactions[62,63].

The limitation of natural translation system is that it typically utilizes only 20 kinds of α-proteinogenic amino acids, i.e. backbone of synthesized peptides is limited to α-peptides. However, contrary to this limitation, ribosome is able to accept wide variety of substrates; not only α-amino acids with various artificial side chains but also D-amino acids[64], α-hydroxy acids[65], β-amino acids[66], hydrazino acids[67], and β-hydroxy acids[68] are reported to be incorporated into peptides (**Figure 1.5**). These results imply that the facile preparation of artificial acyl-tRNA would lead the synthesis of diverse artificial peptides.

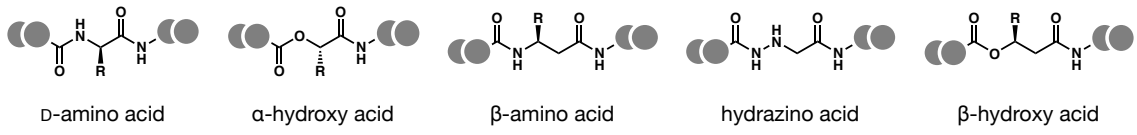


Figure 1.5 | Ribosomally synthesized peptides containing artificial substrates

1.4.2. Flexizymes as aminoacylation ribozymes

The studies mentioned above prepared artificial acyl-tRNAs by chemical synthesis of acylated adenosine and ligation to tRNA with it, which accompanies with laborious works and low yields[69]. For the ribosomal synthesis of artificial peptides, facile tRNA acylation method had been highly demanded. Based on this background, our laboratory previously developed artificial ribozyme called flexizyme which is able to catalyze tRNA acylation reaction with various artificial amino acids (**Figure 1.6a**)[70–73]. In this reaction, flexizyme recognize both of the CCA bases at 3'-end of tRNA and the activated ester of amino acids, and charge them onto tRNA[74]. To date, several combinations of flexizymes and the types of ester activation are practically available (**Figure 1.6b**). Cyanomethyl ester (CME) is used for amino acids bearing aromatic side chain with "eFx" flexizyme. In the case of amino acids which do not have aromatic side chains, the usage of "dFx" flexizyme and 2,4-dinitrobenzyl ester (DBE) allow us to charge onto tRNA. The eFx is also able to utilize 4-chlorobenzyl thioester (CBT), which has lower steric hindrance, and thus structurally constrained substrates such as β-branched amino acids are suitable. Sometimes flexizyme reactions suffer from the hydrophobicity of substrate. In this case, the combination of 4-[2-aminoethyl]carbomyl] benzyl thioester (ABT) and "aFx" flexizyme offers a good solution to enhance solubility of substrates. The significance of flexizyme system is that only the activated ester is essential for tRNA acylation reactions, in other words, other structures including side chains are not involved in the reactions.

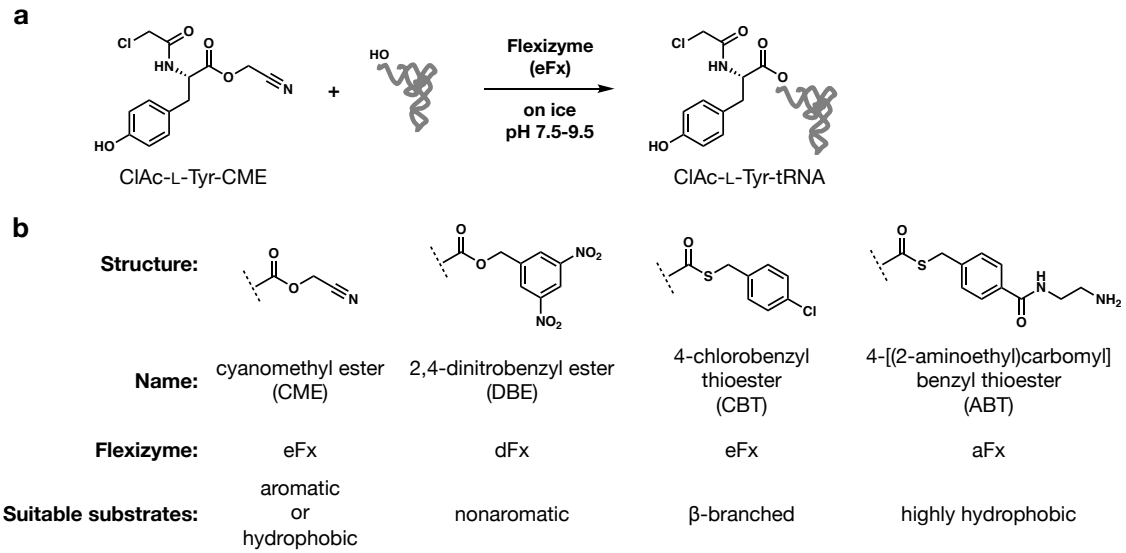


Figure 1.6 | Flexizyme as tRNA acylation ribozyme

(a) Catalytic synthesis of acyl-tRNAs assisted by flexizymes.

(b) List of activated esters, flexizymes, and appropriate substrates for acylation reaction.

1.4.3. Flexible in vitro translation (FIT) system assisted by flexizymes

The development of flexizyme enabled the facile preparation of artificial acyl-tRNAs. By combining flexizyme technology, genetic code reprogramming methodology, and reconstitution of translation system, novel translation system, referred as flexible in vitro translation (FIT) system, enabled us to express *de novo* artificial peptides (**Figure 1.7**). In this system, arbitral amino acids are removed from reconstituted translation system to make vacant codons in the codon box. Instead of them, artificial acyl tRNAs bearing corresponding anticodons are added to the mixture to reprogram the genetic codes. In this environment, translation system can utilize artificial amino acids charged on tRNAs as its substrate, and produce peptides containing the designed amino acids. Because of the generality of flexizyme and mRNA template dependency of translation reaction, the FIT system enables us to design and synthesize diverse artificial peptides. Indeed, FIT system could reproduce the incorporation of α-hydroxy acids[75,76], β-amino acids[77,78], and D-amino acids[79,80] as mentioned above, in the elongation steps. Furthermore, initiation of translation step seems to be much flexible for incorporating various structures, such as fluorescent-labeled amino acids[81,82], di-peptides to hepta-peptides[83], and even the polyketide-mimicked backbones[84,85].

One of the most beneficial strategy for FIT system is the synthesis of macrocyclic peptides by incorporating a chloroacetyl group (ClAc) into peptide N-terminus[86]. After translation by the FIT system where a natural initiator amino acid Met is omitted, the nucleophilic thiol group on the downstream cysteine attacks to a ClAc group spontaneously, resulting in the formation of a thioether macrocycle structure. In addition, other methodologies have been developed for the synthesis of different types of macrocyclization such as head-to-tail macrocyclic peptides[87,88], bicyclic peptides by click chemistry[89], and tricyclic peptides[90]. These techniques enable the FIT system to synthesize “pseudo-natural” peptides, which have not only the macrocyclic structures but also a numerous nonproteinogenic amino acids.

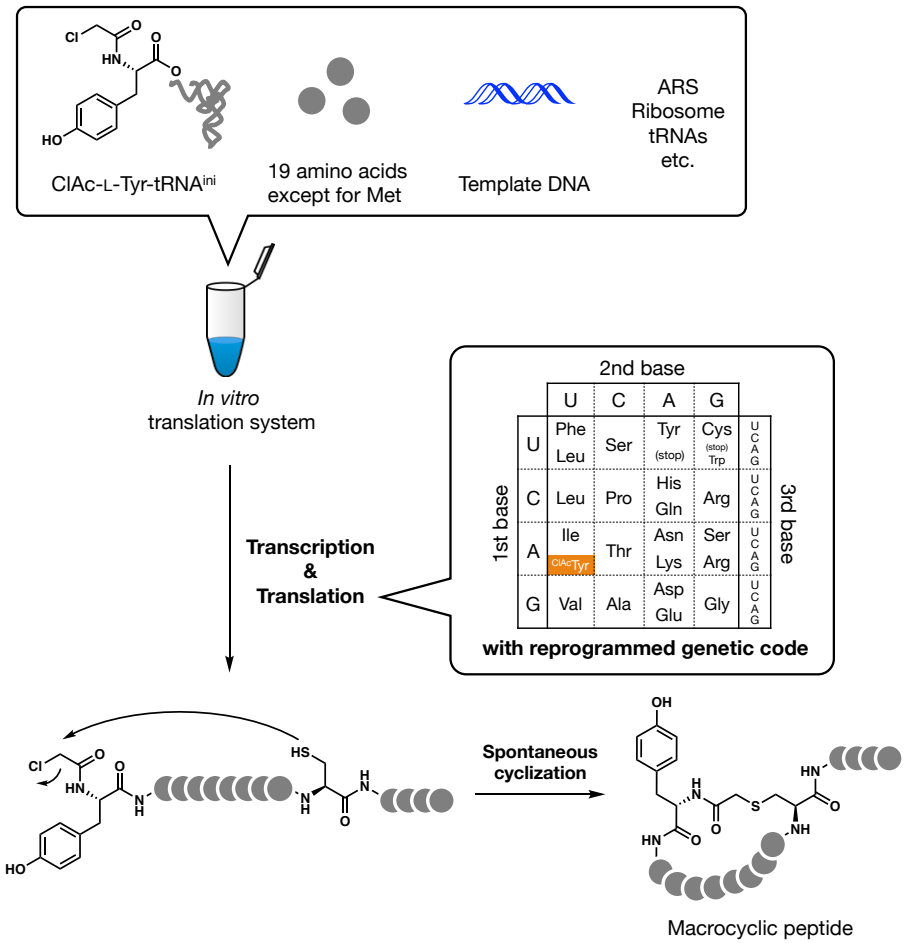


Figure 1.7 | FIT system as the synthetic tool of artificial macrocyclic peptides

1.5. RaPID system for the discovery of bioactive macrocyclic peptides

In the past two decades, display techniques, represented by phage display[91–95] and mRNA display[96,97] have emerged to obtain *de novo* bioactive peptides. In the display technologies, diverse peptides are screened toward targeted proteins based on affinity and obtain peptide ligands. Especially, mRNA display technology can display highly diverse peptides (10^{12} to 10^{13}) owing to the mRNA template dependency of translation reactions. Basically, association of phenotype and genotype is essential for the display techniques. mRNA display achieves the connection of peptides (phenotype) and mRNA (genotype) by attaching puromycin into mRNA terminus to form a covalent bond after translation[98,99]. Although mRNA display could afford various peptides binding to nM order activity, conventional systems could utilize only limited α-amino acids.

To such obstacles, the combination of mRNA display technology and the FIT system enabled the construction of nonstandard peptide libraries, named as RaPID (Random nonstandard Peptide Integrated Discovery) system (**Figure 1.8**)[100–102]. Here, introducing ClAc cyclization method mentioned above is applicable to mRNA display technique, allowing for the construction of macrocyclic peptide library. After screening against proteins of interest immobilized on beads, DNA is recovered and used for the next selection round. By repeating the cycles, RaPID system provide the information of peptide sequences which can strongly bind to the targets. In the RaPID system, the building blocks of peptide libraries are easily modified by means of genetic code reprogramming. Therefore, libraries of natural peptide mimetics, for instance highly N-methylated macrocyclic peptides, are prepared and screened toward draggable target proteins[100].

The RaPID system have been demonstrated as the useful methodology to discover novel bioactive peptides with high affinity and high selectivity. For example, a deacylase human SIRT2 was targeted of the RaPID screening with the macrocyclic peptide library containing an acetylated lysine, which is able to work as "warhead" to inhibit the target[103,104]. Although several homologues are known for SIRTs, the obtained peptide showed not only the strong affinity but also selectivity; it bound to SIRT2 with IC_{50} ~ 3.2 nM whereas IC_{50} ~47 nM against SIRT1 and 480 nM against SIRT3. Furthermore, the peptide discovered by the RaPID system could be the activator of proteins to control the signaling pathway. The dimerization of the peptide obtained by the RaPID screening against the Met protein was found to work as a novel agonist as the same manner to natural ligand, and activate the downstream signaling pathway[105].

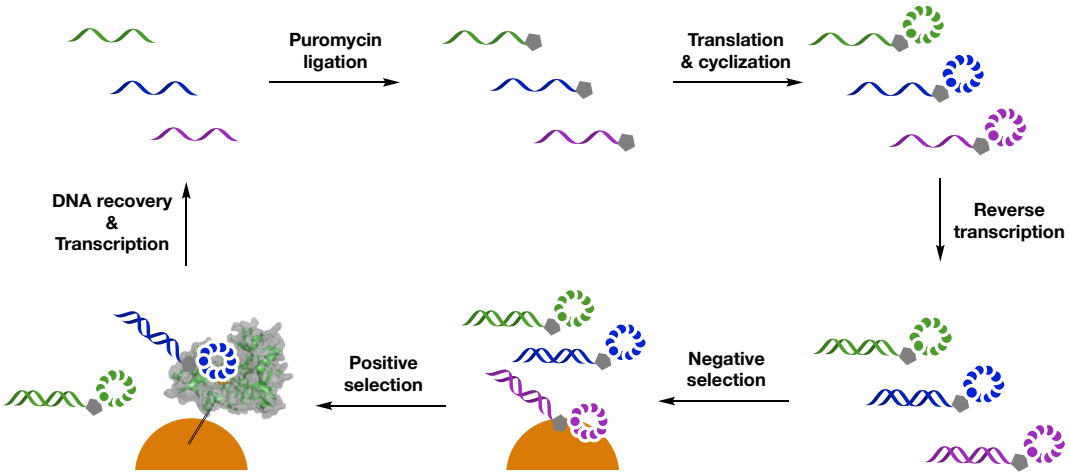


Figure 1.8 | Overview of RaPID system

1.6. Modification reactions for generating various peptide backbones

1.6.1. Enzymatic modifications of peptide backbones

As described above, translation reactions assisted by the FIT system are able to synthesize peptides with various peptide backbones. Looking back to natural bioactive peptides, however, diverse structures including γ-peptides are still difficult to synthesize. One of the contributions of the diversity of natural peptides is enzymatic modification reactions of RiPPs. Typically, RiPPs are biosynthesized from precursor peptides, which composed of leader peptide region for enzymatic recognition and core region to be enzymatically modified. To date, various enzymes and their structures are elucidated (**Figure 1.9**)[106–108]. For instance, azole and azoline structures are widely seen in natural bioactive peptides. It is also reported that the prenylation of tryptophan produced backbone-constrained structures. Since such unique structures are difficult for the selective chemical synthesis, a number of studies conducted the reconstitution of enzymatic reactions to reproduce natural biosynthesis. The enzymatic reaction is also applicable to *in vitro* translation system to produce highly modified peptides. FIT-PatD system is an example, where Ser/Thr cyclodehydrase PatD is introduced to FIT system, enabling the one pot synthesis of azoline/thiazoline-containing peptides[109,110].

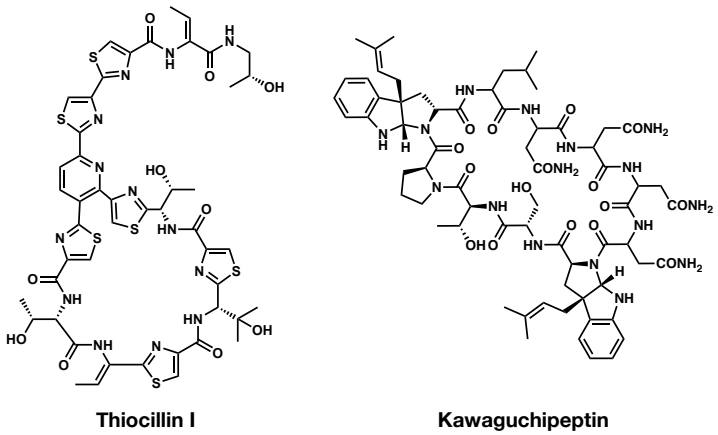


Figure 1.9 | Structural examples of backbone modified peptides synthesized by RiPPs

1.6.2. Chemical modifications of peptide backbones

In the modifications of peptide backbones, natural enzymatic reactions are able to modify peptides selectively with high conversion yield. However, because enzymatic reactions that are applicable for the library construction are often derived from RiPPs, the existence of leader peptide sequences is typically essential for the recognition of enzymes. From this viewpoint, chemoselective reactions offers a promising method for the synthesis of various peptides without any limitations of peptide sequences. Indeed, a number site specific chemical modification reactions have been developed for peptide/protein labeling[111–113].

Several reactions were developed for the modification of peptide backbones by means of chemical reagent(s). The site specific arylation/alkenylation of peptide bonds was reported with the use of boronate reagents and copper (II) reagents (**Figure 1.10a**)[114,115]. This reaction is able to modify the residue adjacent to His selectively in spite of the existence of several amide bonds in a peptide. The formation of dehydroamino acids, seen in RiPPs peptides, was achieved by Cys-selective bis-alkylation and elimination reactions in the basic conditions (**Figure 1.10b**)[116]. Since dehydroalanine is reactive to thiol groups, this reaction is compatible for further modification of proteins and macrocyclic peptides[117]. Succinimide or isoaspartic acid are well-known side products in the solid-phase peptide synthesis (**Figure 1.10c**)[118,119]. In this side reaction, side chain protected aspartic acid undergos intramolecular cyclization to form succinimide. The hydrolysis of succinimide have the possibility of producing both of aspartic acid or isoaspartic acid. The reaction occurs *in vivo* to cause harmful effects, but it is also reported that it is utilized for controlling protein functions[120].

The idea of rearrangement of peptide backbones is a more facile strategy to adjust the peptide properties. Native chemical ligation is a well-established method to chemically synthesize large peptides/proteins (**Figure 1.10d**)[121,122]. A peptide fragment containing a N-terminal free Cys undergoes thioester exchange with a C-thioesterified peptide fragment, and then free amine group spontaneously attack to the thioester to yield a novel peptide bond. The resulted product is a full-length peptide ligated at the Cys residue. Since desulfization reaction to convert Cys to Ala are applicable to the resulted peptides[123], ligation point is not limited to Cys: ligation at almost arbitral amino acids such as Val[124], Phe[125], and Lys[126] are available for this method. *O*-acyl isopeptide method is another strategy to proceed backbone

rearrangement, in which synthesized peptides containing isoserine or its analogue residue proceed *O*-to-*N* acyl migration in physiological conditions (**Figure 1.10e**)[127–131]. The resulted residue is native serine. This method is utilized for the chemical synthesis of highly hydrophobic peptides to increase their solubility.

The reactions shown above were studied with chemically synthesized peptides. Theoretically, they are also applicable to ribosomally expressed peptides: application of native chemical ligation enabled the library construction of backbone-macrocyclic peptides[88]. By utilizing the FIT system, production of C-terminal lactams, thiolactones, and alkylamides was also achieved by introducing an ester linkage into peptide backbones[132]. These examples demonstrated that chemical modifications of peptide backbones are also compatible with ribosomally synthesized peptides, and imply that the combination of translation system and modification reactions would generate diverse peptides with interesting structures.

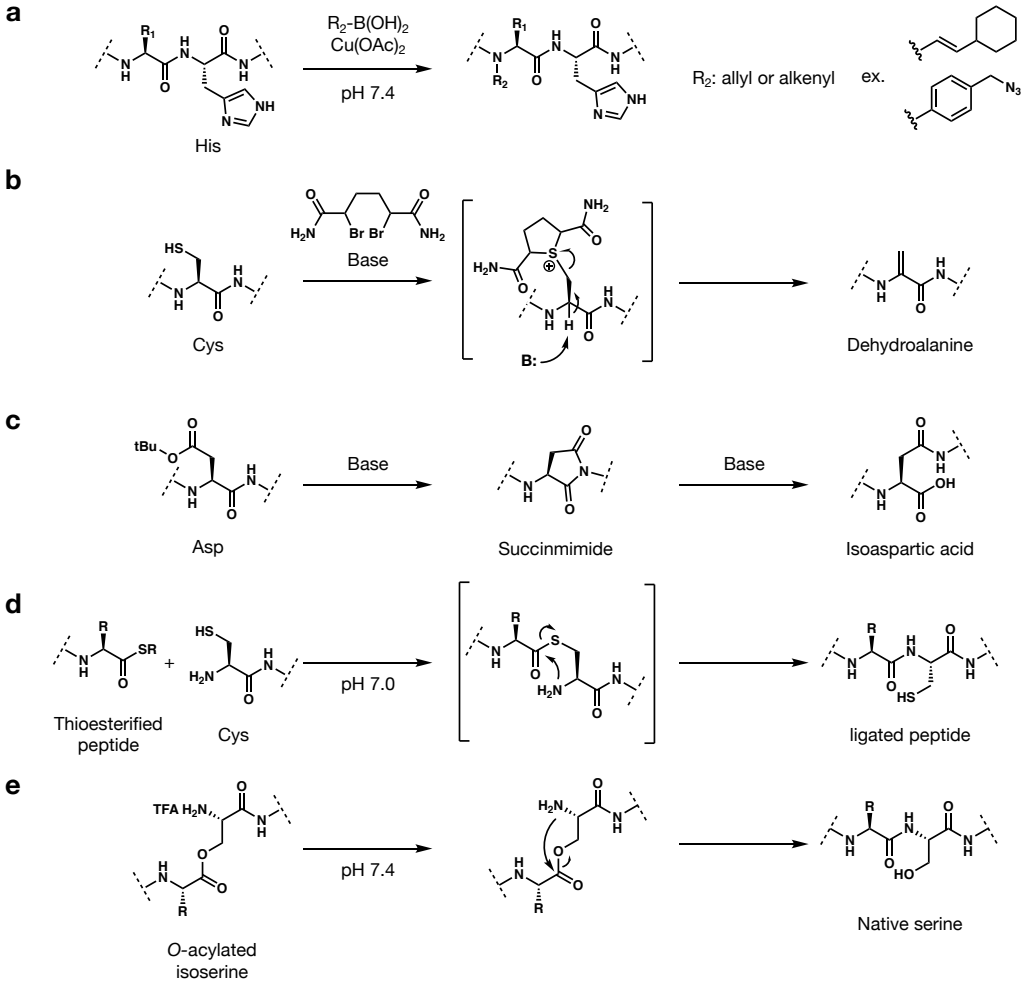


Figure 1.10 | Examples of chemical reactions to modify peptide backbones

(a) Selective arylation/alkenylation of peptide bond by boronate reagents and copper (II).

(b) Synthesis of dehydroalanine from Cys.

(c) Formation of succinimide and isoaspartic acid from protected Asp.

(d) Native chemical ligation to synthesize full-length peptides/proteins.

(e) *O*-acylated isopeptide method.

1.7. Purpose of this study

In this study, I focused on γ-peptides in ribosomally expressed peptides because they offer attractive structures for peptide drug discovery, and ultimately applicable to the RaPID system. Although numerous methodologies are available today to modify peptide backbones, generation of γ-peptides is not reported with the use of translation system. To enable the synthesis of diverse peptides containing γ-peptide structures, this research aimed to establish a novel methodology to synthesize γ-peptide linkages in ribosomally synthesized peptides. Because chemical modification reactions for modifying peptide backbones are reliable methodology independent of peptide sequences, I conceived to combine translation system and posttranslational chemical reactions.

In Chapter 2, I achieved the formation of γ-peptide linkages by developing novel posttranslational chemical modification reactions. In Chapter 3, the substrate scope of the established methodology was investigated. As the results, natural γ-peptides like statine were incorporated into expressed peptides. In Chapter 4, I synthesized a peptide inhibitor containing a γ-peptide linkage to demonstrate its applicability.

Chapter 2 Development of posttranslational chemical modification reactions to produce γ-peptide linkages in ribosomally synthesized peptides

2.1. Introduction

Ribosomal synthesis of peptides bearing γ-peptide structures is promising to construct natural peptide mimetic library and discover novel bioactive peptides. However, contrary to the diversity of γ-peptide structures in bioactive peptides, ribosomal synthesis of γ-peptides is highly restricted. To utilize γ-aminoacyl-tRNAs for translation reaction, two problems should be taken into consideration: self-cyclization reactions and substrate tolerance of ribosome. Since γ-amino groups could be a nucleophile, intramolecular attack to the ester linkage proceed spontaneously, resulting in the decomposition of γ-aminoacyl-tRNA (**Figure 2.1**). In addition, substrates should be compatible with all of the translation steps (i.e., form a complex with EF-Tu, accommodate into ribosomal peptidyl transfer center, and catalyze the formation of a novel peptide linkage by ribosome). Although γ-amino acids with the specific structures are demonstrated to be ribosomally incorporated into peptides, the structural limitation cannot allow statine analogues to be incorporated into peptides. Thus, a general methodology is required for the ribosomal synthesis of peptides containing γ-peptides.

In this chapter, I aimed to develop a novel methodology for generating γ-peptide structures in ribosomally expressed peptides. To achieve this, I designed substrates that can undergo posttranslational chemical modification reactions to generate γ-peptide linkages in the backbone of ribosomally expressed peptides. The ribosomal incorporation of the designed substrate and the formation of the γ-peptide linkage by chemical modifications were confirmed by utilizing both of MALDI-TOF MS and LC-MS. Although the first trial of chemical modifications caused the formation of the undesired byproducts, a series of optimizations improved the conversion yield of the γ-peptide linkage formation.

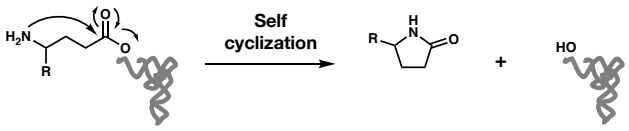


Figure 2.1 | Self-deacylation reactions to decompose γ-aminoacyl-tRNA.

2.2. Results and discussion

2.2.1. Substrate design for formation of γ-peptide linkages

To overcome the above-mentioned issues, γ-azide-β-hydroxy acid was designed in this study (**Figure 2.2**). A γ-azide group was adopted to prevent the self-cyclization reactions because an azide group do not have nucleophilicity. In addition, Staudinger reactions can easily reduce an azide group to an amine group in water. Another important design of this substrate is that it contains a β-hydroxy group to be recognized as the substrate of ribosome and used for translation reactions[68].

After incorporating this substrate into peptides by translation reactions, I further conceived to conduct posttranslational chemical modification reactions to produce new γ-peptide linkages. By adding the phosphine reagent, Staudinger reactions proceed to reduce the azide group to the amine group. Subsequently, the reduced amine group would cause *O*-to-*N* acyl transfer reactions with the β-ester linkage, which lead to the rearrangement of peptide backbone. As a consequence, a γ-peptide linkage is newly formed in the peptide backbone. Theoretically, the substrate design requires only β-hydroxy group and γ-azide group for the substrate, therefore substrates with various side chains are applicable for this strategy. Although β-hydroxy group can be a structural limitation of the substrate, natural γ-peptides like statines widely contain β-hydroxy group. In addition, α-hydroxy acids are also available for substrates of translation reactions, and thus the usage of α-hydroxy acids containing azide group can expand the substrate scope of this concept.

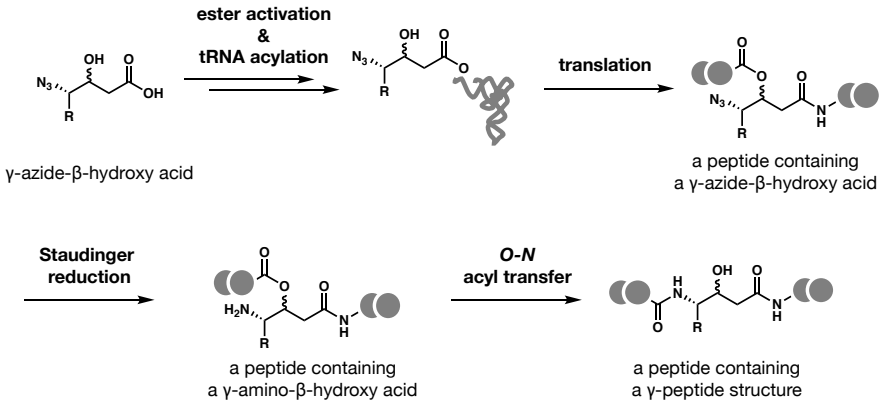


Figure 2.2 | Concept of posttranslational chemical modification reactions

2.2.2. Ribosomal synthesis of the peptide containing γ-azide-β-hydroxy acid

To demonstrate this concept, I firstly designed the simple γ-azide-β-hydroxy acid (**1**) to test whether the substrate structure could be accepted for translation reaction, and the posttranslational modifications might convert the correspond γ-peptide structure (**Figure 2.3a**). The activated hydroxy acid **1-CBT** was charged onto tRNA with the optimized conditions and used for the translational experiments (supplemental results 2.4.1).

For the sake of (i) detecting the mass shift easily for further analysis, and (ii) mimicking the structure of natural bioactive peptides, the expressed peptide was treated as a cyclic peptide by introducing a chloroacetylated tyrosine (^{ClAc}Y), a cysteine residue, and substrate **1** by means of genetic code reprogramming (**Figure 2.3b**). This peptide would spontaneously cyclize to form a thioether bond after the translation reaction (**Figure 2.3c**). To reprogram the genetic code, Met and Gln were omitted from the genetic code and instead of them ^{ClAc}Y-tRNA^{ini} and tRNA$^{Pro1E2\#3}_{cug}$ charged with **1** were reprogrammed (**Figure 2.3d**). The mixture was incubated at 37ºC for 30 min for translation reactions, and then the mixture was subjected to MALDI-TOF MS. As expected, the spectrum clearly showed a peak corresponding to the desired cyclic peptide bearing an β-ester linkage (named **1-pep-N_3**). The result demonstrated that the designed γ-azide-β-hydroxy acid (**1**) was successfully incorporated into the peptide via the translation reaction.

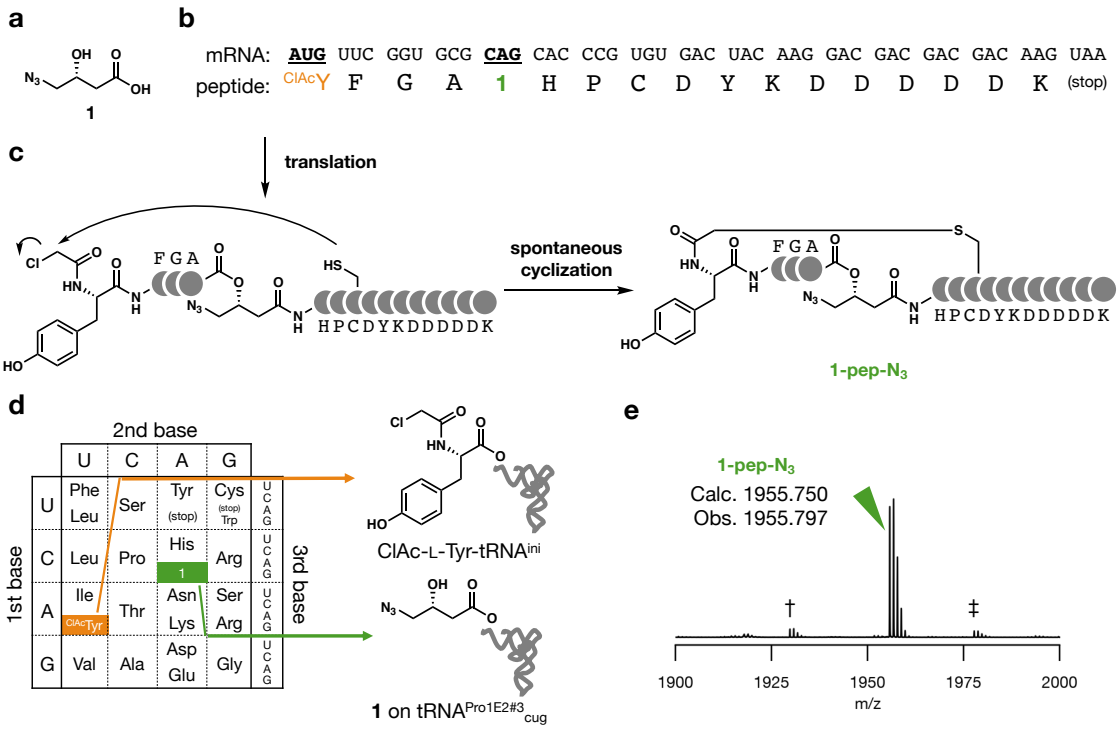


Figure 2.3 | Incorporation of γ-azide-β-hydroxy acid (1) into the peptide

(a) Structure of γ-azide-β-hydroxy acid **1** used in this study.

(b) mRNA sequence and corresponding peptide sequence after reprogramming of genetic code.

(c) Scheme of spontaneous macrocyclization and resulted peptide structure.

(d) Reprogrammed genetic code. Met and Gln codons are assigned to ^{ClAc}Y and **1**, respectively.

(e) MALDI-TOF mass spectrum of ribosomally synthesized peptide. †: [M-N_2+3H] reduced peptide probably due to the laser energy of MALDI, ‡: $[M+Na]^+$ sodium adduct.

2.2.3. Initial trial of posttranslational chemical modification reactions

After confirming the translation reaction, the peptide was further subjected to posttranslational modification reactions. As an initial trial, this peptide was incubated with 50 mM tris(2-carboxyethyl)phosphine (TCEP) at 25 ℃ in pH 8.0. TCEP is a water-soluble phosphine reagent to be utilized for Staudinger reactions in water. After 4 hours, the reaction mixture was quenched by the addition of TFA aq. and subjected to LC-MS. The mixture was analyzed by extracted ion chromatograms (XICs) for the mass of expected structures (namely, **1-pep-N_3** as a starting material, and **1-pep-NH_2**/**1-pep-γ** as a product) (**Figure 2.4a, b**). Since the acyl transfer reaction does not generate the mass difference, **1-pep-NH_2** and **1-pep-γ** are not distinguishable. Thus, the results were not enough to conclude whether whole modification reactions proceeded, but at least, I confirmed that the Staudinger reduction consumed all of the starting material **1-pep-N_3**.

Unfortunately, I also found that the undesired byproducts were generated in the reactions. In the XIC of **1-pep-NH_2**/**1-pep-γ** +1Da, two peaks were newly detected. After analyzing the products carefully, I elucidated the byproducts as **1-pep-OH**, where the azide group was substituted to the hydroxy group. The reason of emerging the two peaks is considered that **1-pep-OH** could have two possible structures by ester exchange. The other byproducts were not observed here.

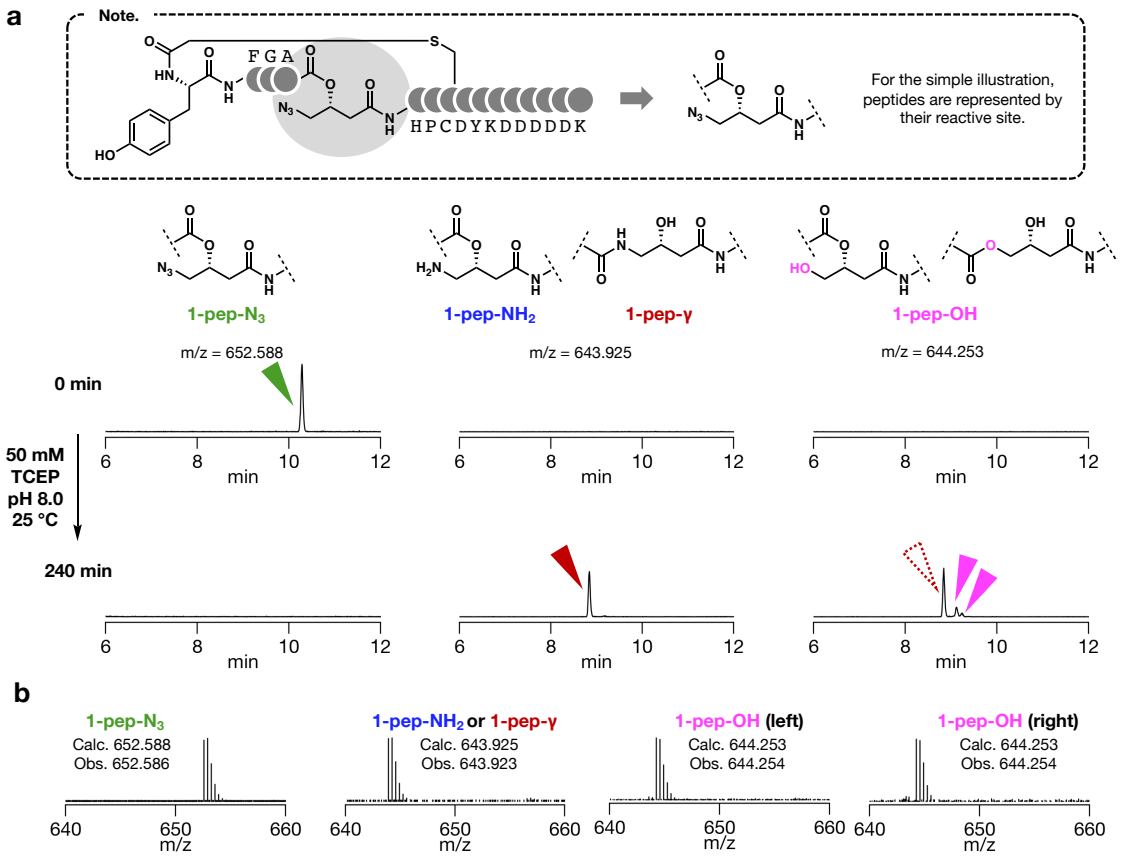


Figure 2.4 | XIC spectra of the peptides after posttranslational chemical modification reactions conducted in pH 8.0

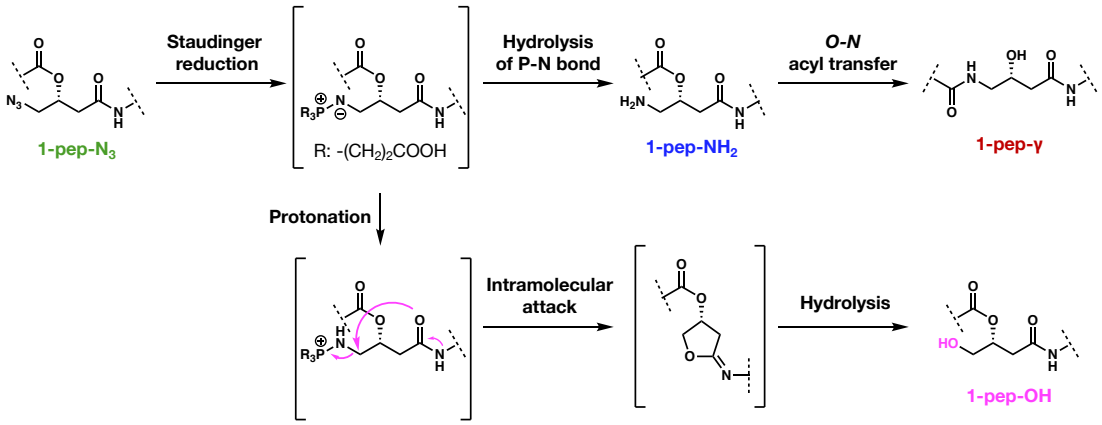
(a) XIC spectra of the each peptides after reactions. The dashed arrow shows the detection of isotopic mass of **1-pep-NH$_2$**/**1-pep-γ**.

(b) Mass spectra of each peaks.

2.2.4. Reaction mechanism for the byproduct formation

In the Staudinger reaction, an azide and a phosphine react to form an aza-ylide structure as an intermediate. Normally, Staudinger reactions in water cleaves the P-N bond of the aza-ylide by hydrolysis and generate a free amine group (**Scheme 2.1**). However, it was also reported that (i) a protonated aza-ylide can work as a leaving group, and (ii) an amide bond can work as a nucleophile[133]. The former study implies that the peptide bond in **1-pep-N_3** is able to attack to the protonated γ-aza-ylide. Consequently, the 5-membered cyclic structure is formed as an intermediate. Quenching the reaction in acidic conditions would open the ring to produce the undesired byproduct, **1-pep-OH**.

To suppress the byproduct pathway, I hypothesized that protonation of aza-ylide would promote the byproduct pathway, and higher pH would suppress it. On the other hand, because the peptides contain a β-ester linkage in their backbone, high pH may cause the ester hydrolysis. Therefore, the appropriate conditions were investigated to suppress both of the byproduct pathway and hydrolysis.

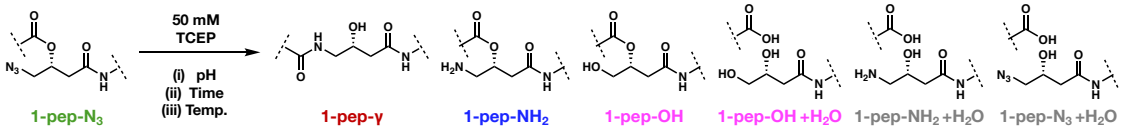


Scheme 2.1 | Plausible mechanism to generate 1-pep-OH during Staudinger reaction

2.2.5. Optimization of reaction conditions

To maximize the yield of the γ-peptide, optimizations of the Staudinger reaction were conducted. In the experiments, (i) pH, (ii) reaction time, and (iii) reaction temperature were investigated. After the reactions, six expected peptides were analyzed by extracting the ion chromatograms. The conversion rates of each peptides were calculated from the peak areas of each XIC spectra by (the peak area of peptide of interest) / (the sum of the peak areas of expected six peptides). I chose the LC-MS as an analysis method here because (i) it can analyze the several products of translation reactions and following reactions simultaneously, and (ii) the sensitivity of LC-MS is tolerable to the scale of the reactions, which provided the peptides with μM to nM order. In addition, the LC-MS analysis is applicable to the semi-quantitative yield calculation, since it is reported that the ion intensity of the peptides bearing the same sequence except for one residue showed the correlation to the yields[134].

The summary of the results is shown in **Table 2.1** (the discussion for the distinction of **1-pep-NH_2** and **1-pep-γ** is described in the next topic). As I hypothesized, higher pH was effective to suppress the formation of byproducts (**Table 2.1**, entries 1 to 7). To further minimize the byproduct formation, the reactions were conducted at 0 ℃, which resulted that the entire reactions became much slower (**Table 2.1**, entries 8 to 21). Simultaneously, the slow reaction promoted the formation of byproducts. To investigate more appropriate reaction conditions, reactions were conducted at 0 ℃ for 2 hours and then further incubated at 42 ℃ for 2 hours, because the azide reduction completed in 2 hours at 0 ℃ (**Table 2.1**, entries 22 to 28). In these conditions, the sample incubated in pH 9.9 showed the best conversion yield (99%) (**Figure 2.5**). Here, I succeeded to minimize both of the byproduct pathway and the hydrolysis, and decided to use these conditions for the following experiments.



Entry	(i) pH	(ii) time & (iii) temp.	1-pep-N_3 (%)	1-pep-γ (%)	1-pep-NH_2 (%)	1-pep-OH (%)	1-pep-OH +H_2O (%)	1-pep-NH_2 +H_2O (%)	1-pep-N_3 +H_2O (%)
1	7.5	25 °C, 2 h	0	0	68	29	2	1	0
2	8.0		0	0	75	22	2	1	0
3	8.5		0	0	77	20	2	1	0
4	9.0		0	1	66	25	6	2	0
5	9.5		0	0	95	2	1	2	0
6	9.9		0	0	94	4	1	1	0
7	10.5		0	0	96	0	0	4	0
8	7.5	0 °C, 2 h	18	23	32	24	1	0	2
9	8.0		6	28	41	24	0	1	0
10	8.5		4	40	27	27	1	1	0
11	9.0		8	47	19	23	1	1	1
12	9.5		6	38	42	12	0	1	1
13	9.9		8	37	37	16	1	0	1
14	10.5		10	18	59	11	0	1	1
15	7.5	0 °C, 4 h	1	4	63	30	1	1	0
16	8.0		0	5	67	26	1	1	0
17	8.5		0	21	56	22	1	0	0
18	9.0		0	37	42	19	1	1	0
19	9.5		0	13	78	7	0	2	0
20	9.9		0	7	84	8	0	1	0
21	10.5		1	3	88	6	0	2	0
22	7.5	0 °C, 2 h + 42 °C, 2 h	0	0	68	27	4	1	0
23	8.0		0	0	73	22	4	1	0
24	8.5		0	0	79	14	6	1	0
25	9.0		0	0	86	8	5	1	0
26	9.5		0	0	96	0	2	2	0
27	9.9		0	0	99	0	1	0	0
28	10.5		0	0	97	0	0	3	0

Table 2.1 | Summary of the conversion yields with various conditions

All data were acquired for three times, and the average values are described.

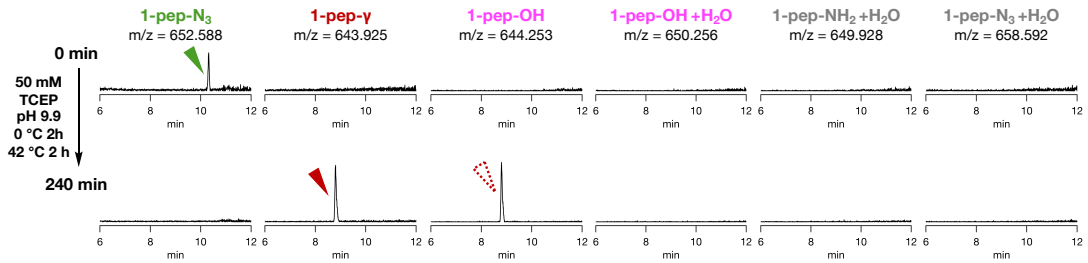


Figure 2.5 | XIC spectra of expected products before/after reactions with the optimized conditions

2.2.6. Analysis of posttranslational chemical modification reactions with the optimized conditions

Using the optimized conditions, the posttranslational chemical modification reactions were conducted again to demonstrate the formation of a γ-peptide linkage in the ribosomally expressed peptide. The peptide **1-pep-N_3** was exposed to the conditions optimized above, and then the mixture was analyzed by both of MALDI-TOF MS and LC-MS.

In the study of MALDI-TOF MS, not only the purposed reactions but also the control experiment were conducted. The sample incubated with TCEP showed the -26 Da mass shift, which corresponded to the azide reduction (**Figure 2.6a**). The result also informed us that the hydrolysis of ester did not occur. On the other hand, the sample incubated in pH 9.9 without TCEP resulted in the observation of β-ester hydrolysis, indicating that the condition of pH 9.9 was enough to cleave the β-ester linkage of peptide backbone (**Figure 2.6b**). Compared to the control experiment, these results revealed that the product obtained with TCEP did not contain the ester linkage in its backbone, indicating that the *O-N* acyl transfer reaction proceeded to form the γ-peptide linkage. The MALDI-TOF mass spectra also showed that the posttranslational chemical modifications underwent without producing any byproducts, since XIC of LC-MS was not able to discuss the whole mass spectrum.

In the LC-MS study, the time course analysis was carried out. The XIC spectra of **1-pep-N_3** mass showed that the posttranslational modification reactions consumed the starting material in 2 hours (**Figure 2.7a**). On the other hand, the XIC spectra of reduced peptide mass showed two peaks in the reactions, and finally converged to one peak (**Figure 2.7b**). From the results of MALDI-TOF MS, the peak seen in the XIC of after 4 hours reactions was assigned to the **1-pep-γ**. Considering the reaction mechanism, the other peak seen in after 2 hours should be **1-pep-NH_2** as the intermediate of the reactions. The results clearly showed us that LC-MS study could distinguish **1-pep-NH_2** and **1-pep-γ**.

Considering the results of both of the LC-MS and MALDI-TOF MS, here I concluded that the posttranslational chemical modifications converted γ-azide-β-hydroxy acid **1** into the γ-peptide structure.

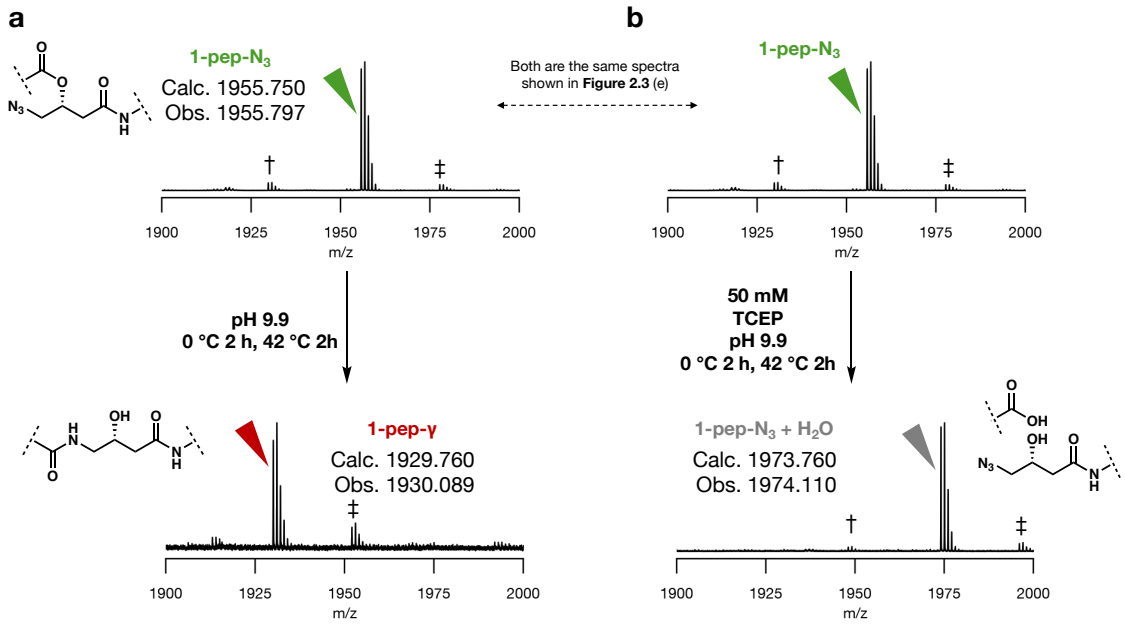


Figure 2.6 | MALDI-TOF mass spectra of peptides before/after reactions with the optimized conditions

(a) Posttranslational chemical modification reactions to produce the γ-peptide. †: [M-N_2+3H] reduced peptide probably due to the laser energy of MALDI, ‡: $[M+Na]^+$ sodium adduct.

(b) Hydrolytic cleavage of the β-ester linkage by incubating without TCEP.

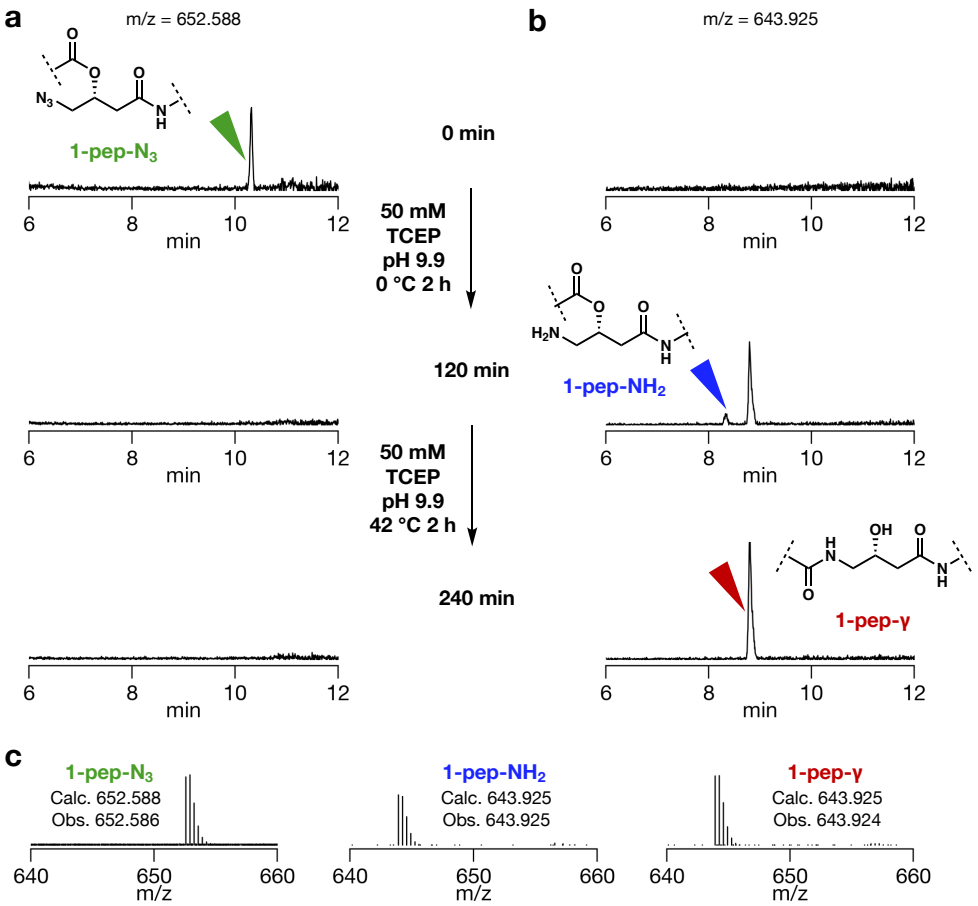


Figure 2.7 | Time course study of posttranslational chemical modification reactions by means of LC-MS

(a) XIC spectra corresponding to the mass of **1-pep-N_3**.

(b) XIC spectra corresponding to the mass of **1-pep-NH_2** and **1-pep-γ**.

(c) Mass spectra of each peaks.

2.3. Conclusion

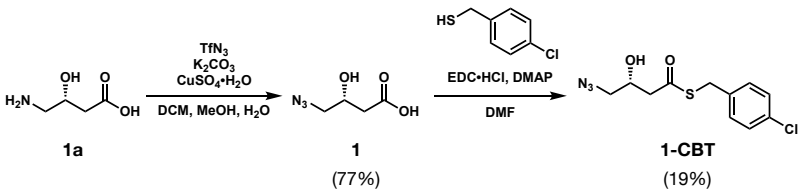
In this chapter, I established the novel methodology to form a γ-peptide linkage in ribosomally synthesized peptides. For the demonstration, γ-azide-β-hydroxy acid (**1**) was designed to overcome the problems by incorporating two essential structural motives: γ-azide for preventing the decomposition of acyl-tRNA and β-hydroxy acid form as the substrate of translation. Although the incorporation of this substrate by the FIT system was succeeded, the byproduct formation was unexpectedly observed due to the intramolecular cyclization reaction. Such byproducts were suppressed by elevating pH and controlling the reaction time and temperature. By applying the optimized conditions, the γ-peptide was obtained with the almost quantitative conversion yield.

2.4. Supplemental results

2.4.1. Synthesis of γ-azide-β-hydroxy acid (1) and tRNA acylation reaction

The γ-azide-β-hydroxy acid (**1**) used in this chapter was synthesized according to **scheme 2.2**. The purchased 4-azide-(3*R*)-hydroxy butanoic acid **1a** was converted to **1** by diazo transfer reaction with TfN_3 reagent[135–137]. Next, activation of the ester was conducted by the condensation reaction with 4-chlorobenzyl mercaptan. I chose the activation method as CBT ester for **1**, because of its β-branched structure.

The synthesized **1-CBT** was then mixed with flexizyme (eFx) and microhelix (small tRNA-mimic RNA) to determine the efficiency of acylation reaction. The mixture was incubated in pH 8.0 on ice for 6 hours to 48 hours. The resulted mixtures were applied to the gel electrophoresis to separate the acylated microhelix (upper) and non-reacted microhelix (lower). Although these experimental schemes are enough to separate them in the case of typical amino acids (lane 1, and 2 as control samples, **Figure 2.8**), I found that they were hardly separated in the case of **1-CBT**. Therefore, $NaIO_4$ treatment followed by deacylation reaction were conducted before applying to the electrophoresis[138]. $NaIO_4$ cleaves the diol structure of 3'-terminal free microhelix, and deacylation reaction eliminates one base. Whereas acylated microhelix is intact to $NaIO_4$ and deacylation allows us to detect it as microhelix. The $NaIO_4$ treatment resulted in showing the gel shift (lane 3, and 4, **Figure 2.8**). By quantifying the band intensity of the reactant (lane 5 to 8, **Figure 2.8**), the sample reacted for 40 hours showed the best acylation efficiency. Therefore, **1-CBT** was mixed with eFx and tRNA and incubated with the optimized conditions, and this acylated tRNA was further utilized for the translation.



Scheme 2.2 | Synthesis of 1-CBT

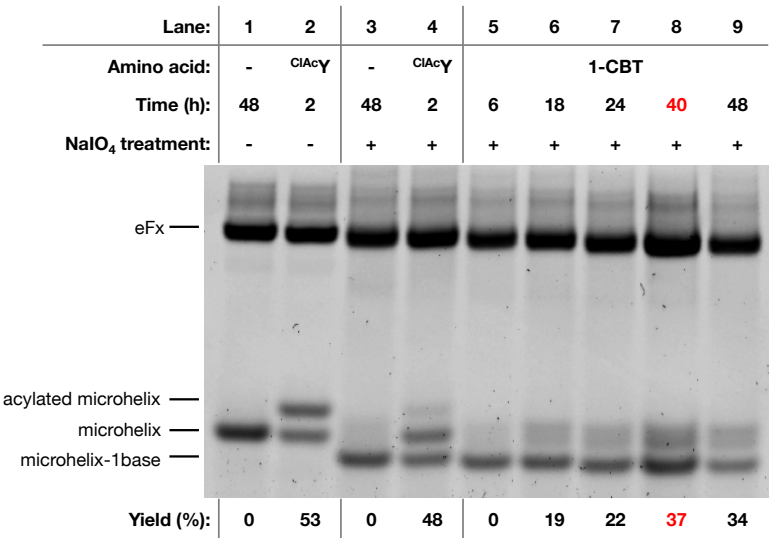


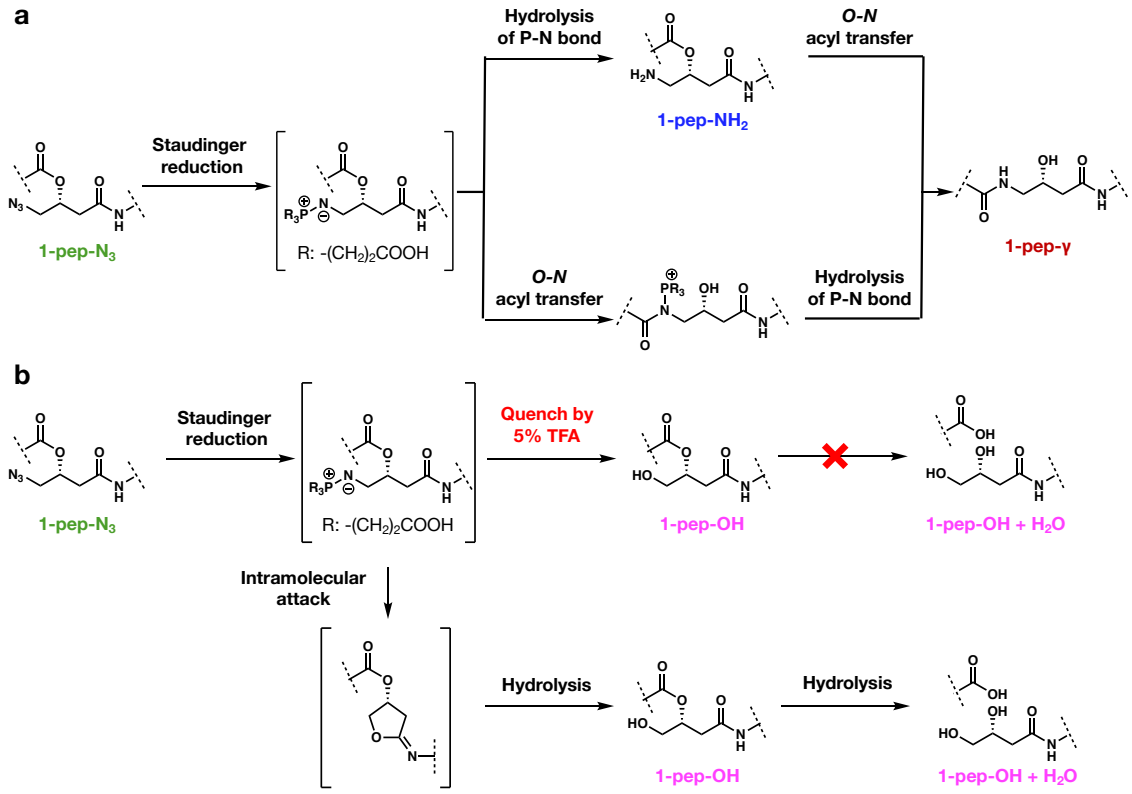
Figure 2.8 | Confirmation of acylation reaction with 1-CBT by acid PAGE analysis

2.4.2. Additional discussion for the reaction scheme

Although posttranslational modification reactions are discussed in this chapter, there are still rooms to discuss the entire reaction mechanism. In the **Figure 2.2**, I described the reaction mechanism as that acyl transfer reaction would proceed after the formation of the amine group. On the other hand, it is noteworthy that the aza-ylide could be the nucleophile[139]. Thus, the other pathway is also possible in the developed reactions, where the aza-ylide directly attack to the β-ester, and spontaneously the aza-ylide is hydrolyzed to produce the desired γ-peptide (**Scheme 2.3a**).

Related to this argument, **1-pep-OH** could be generated by the other reaction route. In fact, the XIC spectra of **1-pep-OH** in the time course study showed the peaks even in the optimized conditions (**Figure 2.9a**). However, the **1-pep-OH** and **1-pep-OH + H_2O** were completely diminished after the reaction (**Figure 2.9b**). This phenomenon is probably because that the quenching the reactions by TFA aq. would decompose aza-ylide intermediate to **1-pep-OH** (**Scheme 2.3b**). Therefore, two pathways should be taken into consideration for the observation of **1-pep-OH**.

It seems that the usage of LC-MS made the entire reactions much complicated because it is necessary to quench the reaction by adding TFA aq. for detecting samples. I emphasize here, however, that the LC-MS analysis was essential because it provided me a lot of information, such as the distinction of peptides bearing the same mass and semiquantitative conversion yields.



Scheme 2.3 | Other conceivable reaction schemes

(a) Reaction scheme of acyl transfer reaction and P-N bond hydrolysis.

(b) Two pathways for the generation of **1-pep-OH** by the quench of intermediate aza-ylide.

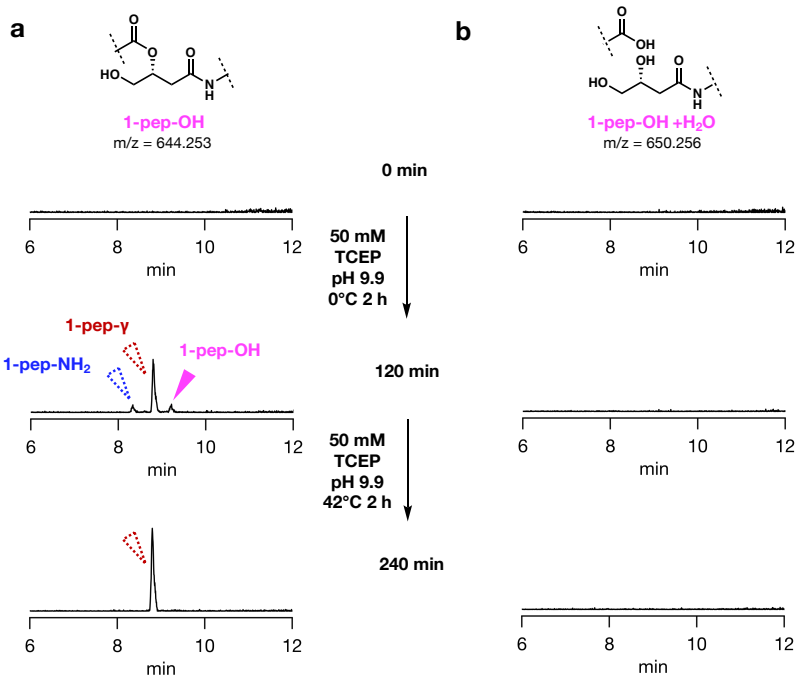


Figure 2.9 | Time course XIC spectra of byproducts

(a) XIC spectra corresponding to the mass of **1-pep-OH**

(b) XIC spectra corresponding to the mass of **1-pep-OH + H_2O**

2.5. Material and methods

・Materials

All chemicals and reagents were purchased from Tokyo Chemical Industry, Kanto Chemical, Nacalai Tesque, Sigma-Aldrich Japan, Watanabe chemical Industry, or Fujifilm Wako Pure Chemical Industries. All DNA oligomers were purchased from Eurofin Genomics or GeneDesign.

・List of DNA oligomers

Primer	Sequence
Fx-5'.F36	GTAATACGACTCACTATAGGATCGAAAGATTTCCGC
eFx.R45	ACCTAACGCTAATCCCCTTTCGGGGCCGCGGAAATCTTTCG ATCC
T7ex5.F22	GGCGTAATACGACTCACTATAG
eFx.R18	ACCTAACGCTAATCCCCT
Pro1E.F50	GTAATACGACTCACTATAGGGTGATTGGCGCAGCCTGGTAG CGCACTTCG
Pro1E2#3CUG_Gln. R62	TGGCGGGTGATAGGGGATTCGAACCCCTGACCCCTTCGTTC AGAGCGAAGTGCGCT
Pro1E2#3-OMe.R20	TGGCGGGTGATAGGGGATTC
Ini-3'.R20	TGGTTGCGGGGGCCGGATTT
Ini-3'.R38	TGGTTGCGGGGGCCGGATTTGAACCGACGATCTTCGGG
Ini1-1G-5'.F49	GTAATACGACTCACTATAGGCGGGGTGGAGCAGCCTGGTAG CTCGTCGG
Ini cat.R44	GAACCGACGATCTTCGGGTTATGAGCCCGACGAGCTACCAG GCT
eSD_MFG_Q(7G)_f lag.R51	GTCGTCGTCCTTGTAGTCACACGGGTGCTGCGCACCGAACA TGTTTTTCTC
Flaguaa.R33	CGAAGCTTACTTGTCGTCGTCGTCCTTGTAGTC
T7eSD6M.F46	TAATACGACTCACTATAGGGTTAACTTTAAGAAGGA GAAAAACATGTACAAGAAGTACAAAAAG

・**Combination of DNA primers**

Name	Extension	1st PCR	2nd PCR
DNA template	T7eSD6M.F46 eSD_MFG_Q(7G)_flag.R51	-	T7ex5.F22 Flaguaa.R33
eFx	Fx-5'.F36 eFx.R45	-	T7ex5.F22 eFx.R18
$tRNA^{ini}$	Ini1-1G-5'.F49 Ini cat.R44	T7ex5.F22 Ini-3'.R38	T7ex5.F22 Ini-3'.R20
$tRNA^{Pro1E2\#3}_{cug}$	Pro1E.F50 Pro1E2#3CUG_Gln.R62	-	T7ex5.F22 Pro1E2#3-OMe.R20

・**Preparation of DNA templates**

1 μL of 100 μM appropriate primers for extension were mixed in PCR mixture (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% (v/v) Triton X-100, 2.5 mM $MgCl_2$, 250 μM each dNTPs, and 1.5% (v/v) Taq DNA polymerase). Extension reaction [95℃, 1 min → (50℃, 1 min → 72℃, 1 min)×5] was carried out.

1st PCR (if necessary)

190 μL of PCR master mix, 10 μL of extension reaction product, and 1 μL of 100 μM appropriate primers for 1st PCR were mixed and PCR [95℃, 40 sec → 50℃, 40 sec → 72℃, 40 sec×5] was carried out.

2nd PCR

1000 μL of PCR master mix, 5 μL of 1st PCR product or extension product, and 5 μL of 100 μM appropriate primers for 2nd PCR were mixed and PCR [95℃, 40 sec → 50℃, 40 sec → 72℃, 40 sec×15] was carried out. The amplification was checked by 3% agarose gel electrophoresis.

This DNA template was purified by phenol/chloroform extraction and ethanol precipitation.

・Preparation of flexizymes (eFx)

The DNA template of eFx was prepared as described above from appropriate combination of primers.

The obtained DNA was dissolved in 100 μL of water and added to the transcription mixture (345 μL of water, 100 μL of T7 buffer, 100 μL of 100 mM DTT, 120 μL of 250 mM $MgCl_2$, 15 μL of 2M KOH, 200 μL of 25 mM each NTPs, and 20 μL of T7 RNA polymerase). After incubating the mixture at 37°C for overnight, 115 μL of DNase buffer (40 mM Tris-HCl pH8.0, 10 mM $MgSO_4$, and 1 mM $CaCl_2$) and 30 μL of RQ1 RNase-free DNase were added, and incubated for further 1 h to degrade the DNA template. The obtained RNA was precipitated with isopropanol and purified by 8% denaturing PAGE containing 6M urea. The concentration was adjusted to 250 μM by determining the concentration with NanoDrop.

・Preparation of tRNAs

The DNA templates of $tRNA^{ini}$ and $tRNA^{Pro1E2\#3}_{cug}$ were prepared as described above from appropriate combination of primers.

The obtained DNA was dissolved in 100 μL of water and added to the transcription mixture (345 μL of water, 100 μL of T7 buffer, 100 μL of 100 mM DTT, 90 μL of 250 mM $MgCl_2$, 11.25 μL of 2M KOH, 50 μL of 100 mM GMP, 150 μL of 25 mM each NTPs, and 20 μL of T7 RNA polymerase). After incubating the mixture at 37°C for overnight, 115 μL of DNase buffer (40 mM Tris-HCl pH8.0, 10 mM $MgSO_4$, and 1 mM $CaCl_2$) and 30 μL of RQ1 RNase-free DNase were added, and incubated for further 1 h to degrade the DNA template. The obtained RNA was precipitated with isopropanol and purified by 8% denaturing PAGE containing 6M urea. The concentration was adjusted to 250 μM by determining the concentration with NanoDrop.

・Optimization of acylation conditions

Acylation reaction was conducted with microhelix, flexizyme, and substrate **1**. For one sample, 1.5 μL of water, 0.5 μL of 500 mM HEPES•KOH pH 8.0, 0.5 μL of 250 μM microhelix, and 0.5 μL of 250 μM eFx were mixed and incubated 95°C for 2 min. The

mixture was cooled to room temperature, then 1 μL of 3 M $MgCl_2$ was added and incubated on ice for 5 min. 1 μL of 25 mM substrate **1** in DMSO was added and incubated for 6, 18, 24, 40, and 48 hours. In addition, positive control was prepared by adding 1 μL of 25 mM ^{ClAc}Y in DMSO and incubated for 2 h, and negative control was added 1 μL of DMSO and for 48 h. The acylation reaction was quenched by addition of 20 μL of 0.3 M NaOAc (pH 5.2), 50 μL of EtOH, and RNA was recovered by ethanol precipitation.

$NaIO_4$ oxidization was conducted as follows. The pellet was dissolved in 11.55 μL of the mixture containing 662 mM of NaOAc and 19.5 mM of $NaIO_4$. The mixture was incubated on ice for 1 h in the dark. 100 μL of 80% EtOH was added to the mixture, and RNA was recovered by ethanol precipitation. The pellet was dissolved in 50 mM Bicine (pH 9.0) and incubated at 42˚C for 1 h. 80 μL of 75% EtOH containing 1.125 M NaCl was added to the mixture, and RNA was recovered by ethanol precipitation.

The pellet was dissolved in 0.75 μL of 10 mM NaOAc (pH 5.2), 7.25 μL of acid PAGE loading buffer was added into this solution and mixed well. 2 μL of this mixture was loaded to acid PAGE and electrophoresis was carried out at 120 V for 2.5 h. The gel was dyed with ethidium bromide and analyzed the efficiency of acylation by FLA 7000.

・Preparation of acylated-tRNA

tRNA acylation was conducted with prepared tRNA, flexizyme, and substrate **1**. 1.5 μL of water, 0.5 μL of buffer, 0.5 μL of 250 μM $tRNA^{Pro1E2\#3}_{cug}$, and 0.5 μL of 250 μM eFx were mixed and incubated 95°C for 2 min. The mixture was cooled to room temperature, then 1 μL of 3M $MgCl_2$ was added and incubated on ice for 5 min. 1 μL of 25 mM substrate **1** in DMSO was added and incubated for 40 h. The acylation reaction was quenched by addition of 20 μL of 0.3 M NaOAc (pH 5.2) and 50 μL of EtOH, and acylated tRNA was recovered by ethanol precipitation.

・Translation

sol A contains 2 mM ATP, 2 mM GTP, 1 mM CTP, 1 mM UTP, 50 mM creatine phosphate, 50 mM HEPES-KOH pH 7.6, 100 mM potassium acetate, 12 mM Mg acetate, 2

mM Spermidine, 1 mM DTT.

sol B contains 0.3 mM magnesium acetate, 1.2 μM *E.coli* ribosome, 0.6 μM MTF, 2.7 μM IF1, 0.4 μM IF2, 1.5 μM IF3, 0.26 μM EF-G, 10 μM EF-Tu, 0.66 μM EF-Ts, 0.25 μM RF2, 0.17 μM RF3, 0.5 μM RRF, 4 μg/mL crearine kinase, 3 μg/mL myokinase, 0.1 μM inorganic pyrophosphatase, 0.1 μM T7 RNA polymerase, 0.73 μM AlaRS, 0.03 μM ArgRS, 0.38 μM AsnRS, 0.13 μM AspRS, 0.02 μM CysRS, 0.06 μM GlnRS, 0.23 μM GluRS, 0.09 μM GlyRS, 0.02 μM His RS, 0.4 μM IleRS, 0.04 μM LeuRS, 0.11 μM LysRS, 0.03 μ MetRS, 0.68 μM PheRS, 0.16 μM ProRS, 0.04 μM SerRS, 0.09 μM ThrRS, 0.03 μM TrpRS, 0.02 μM TyrRS, and 0.02 μM ValRS

The solution containing 10% (v/v) DNA template, 0.5 mM amino acid mixture (F, G, A, H, P, C, D, Y, and K), 10.9% (v/v) solA, 12% (v/v) solB, 25 μM prepared acyl-tRNAs, and 200 μM NaOAc was prepared. This mixture was incubated at 37°C for 30 min.

For LC-MS study, 24.5 μL of 5% TFA was added to 0.5 μL of translation mixture, then centrifuged at 4℃, 13000 rpm for 10 min. The supernatant was recovered and subjected to LC-MS.

・Posttranslational chemical modification reactions

The translation mixture was incubated on ice for 1 min. To the mixture, the cooled solution containing 2 vol. of 250 mM TCEP in pH 9.9 and 7 vol. of 500 mM Na_2HPO_4-NaOH buffer at pH 9.9 was added. The reaction mixture was incubated at 0℃ for 2 h, and 42℃ for 2 h.

For LC-MS study, 20 μL of 5% TFA was added to 5.0 μL of reaction mixture, then centrifuged at 4℃, 13000 rpm for 10 min. The supernatant was recovered and subjected to LC-MS.

・MALDI-TOF MS study

The peptides after posttranslational chemical modification reactions were added to 20 μL of 5% TFA and 300 μL of acetone. The mixture was incubated at -20℃ for at least

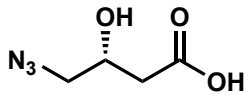
30 min, then centrifuged at 4°C, 13000 rpm for 10 min. The pellet was dried for 3 min and dissolved in 5.0 μL of 50 mM Tris-HCl pH 7.6. For peptides without chemical modification reactions, 1 vol. of 100 mM Tris-HCl pH 7.6 was directly added to the mixture.

1.5 vol. (to translation mixture) of Anti-Flag M2 affinity gel (Sigma) was washed with 3 vol. of 100 mM of Gly-HCl pH 3.5 buffer once, followed by 5 vol. of 50 mM Tris-HCl pH 7.6 twice. To this gel, the solution of Tris-HCl buffer containing peptides were added and rotated at room temperature for 1 h. The gel was recovered and washed with 5 vol. of 50 mM Tris-HCl buffer twice. 1 vol. of 0.2 % TFA was added and rotated for 5 min at room temperature. The peptides were further purified by solid phase extraction (C-Tip), and subjected to MALDI-TOF-MS (ultrafleXtreme, Bruker Daltionics), calibrated based on peptide calibration standard II (Bruker Daltonics).

・LC-MS study

3 μL of the prepared samples were injected to Hclass/Xevo G2-XS (Waters) equipped with ACQUITY UPLC Peptide BEH C18 column, 300Å, 1.7 μm, 2.1 mm diameter, 150 mm (Waters). The loaded samples were separated with the linear gradient (0.01% formic acid in water : 0.1% formic acid in acetonitrile) = 99:1 to 70:30 within 10 min. The resulted LC-MS spectra were calibrated by the mass of GFB peptide (Sigma) as an internal standard and analyzed by the software of MassLynx (Waters) or UNIFI (Waters).

・Chemical synthesis



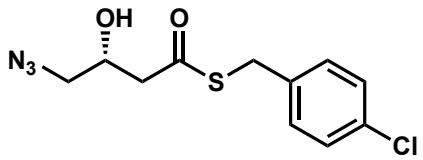
4-azide-(3*R*)-hydroxy butanoic acid (1)

Sodium azide (218.4 mg, 3.36 mmol) was dissolved in 1.5 mL of water and 2.5mL of DCM. This solution was cooled to 0 °C, then trifluoromethanesulfonic anhydride (353 μL, 2.10 mmol) was added dropwise within 5 minutes. This solution was kept vigorous stirring for 3

hours. The organic layer of this solution was washed with water followed by sat. $NaHCO_3$ aq. This TfN_3 solution was used without further purification.

4-amino-(3*R*)-hydroxy butanoic acid **1a** (100.0 mg, 0.839 mmol), K_2CO_3 (174.1 mg, 1.26 mmol), and $CuSO_4$•H_2O (2.0 mg, 7.98 μmol) were dissolved in 1 mL of water and 2 mL of MeOH. To this solution, all of TfN_3 solution was added and stirred vigorously for overnight. The solution was evaporated until MeOH was vaporized. DCM was added and the organic layer was washed with 1M HCl twice. The water layer was back extracted again with DCM twice. The combined organic layer was dried over Na_2SO_4, filtrated, and evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 2), afforded compound **1** (76.9 mg, 63%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 4.23 (m, 1H), 3.40 (t, *J*=5.40 Hz, 2H), 2.63 (t, *J*=3.77 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.91, 67.27, 55.67, 38.32. HR-ESI-MS: Calculated for $C_4H_7N_3O_3$ $[M-H]^-$ 144.0415, Observed: 144.0406.



4-azide-(3*R*)-hydroxy butanoic acid CBT ester (1-CBT)

4-azide-(3*R*)-hydroxy butanoic acid (**1**) (26.0 mg, 0.179 mmol) and 4-chlorobenzyl mercaptan (26.4 μL, 0.179 mmol) were dissolved in 4 mL of dry THF. EDC•HCl (41.2 mg, 0.215 mmol) was added to this solution, then stirred for 24 h. 1M HCl was added, and the water layer was extracted with diethyl ether three times. The combined organic layer was washed with 1M HCl followed by brine. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 1), afforded compound **1-CBT** (10.9 mg, 19%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29-7.21 (m, 4H), 4.26 (m, 1H), 4.11 (s, 2H), 3.34 (m, 2H), 2.82 (t, *J*=6.15 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 197.64, 135.71, 133.48, 130.32,

129.00, 55.67, 47.42, 32.84. HR-ESI-MS: Calculated for $C_{11}H_{12}N_3O_2SCl$ $[M-H]^-$ 284.0266, Observed: 284.0272.

Chapter 3 Expansion of substrate scope for posttranslational chemical modification reactions

3.1. Introduction

In the chapter 2, I demonstrated that post-translational chemical modification methodology is able to produce γ-peptide structures in ribosomally synthesized peptides. Generally speaking, one of the advantages of chemical reactions is their selectivity to substrates. However, because structures of peptides are much complicated than small molecules, reactions highly depend on their structures or peptide sequences. Therefore, although the model reaction with **1** resulted in the good conversion yield, the generality of the developed reactions should be investigated.

In this chapter, the applicability of posttranslational chemical modification reactions was investigated by using a series of substrates (**Table 3.1**). The studies in this chapter aimed at two proposes: the first topic aimed to produce natural γ-peptides seen in bioactive peptides to enable the synthesis of pseudo-natural peptides by utilizing the translation system. Because of the unique activities of statine and its analogue, I focused on the incorporation of these moieties into ribosomally synthesized peptides. Second, the analysis of an acyl transfer reaction step was carried out by preparing various types of α-hydroxy acids. The improvement of conversion yield was also carried out by designing α,β-dihydroxylated acids.

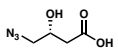
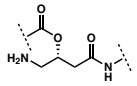
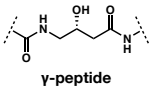
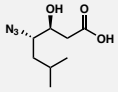
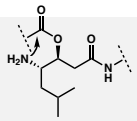
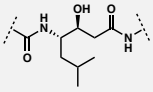
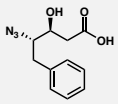
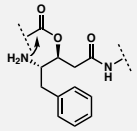
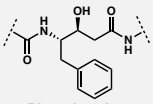
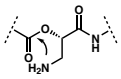
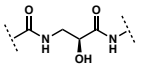
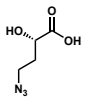
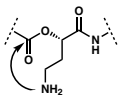
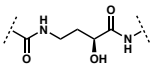
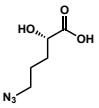
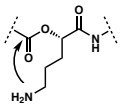
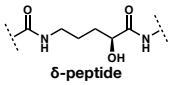
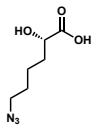
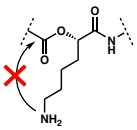
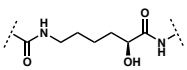
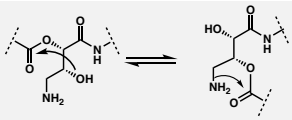
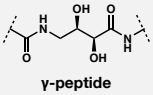
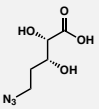
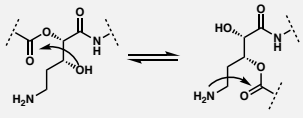
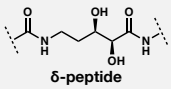
Entry	Hydroxy acid	Reduced structure in peptide	Structure after reactions	Conversion yields (%)		
				γ-peptide	OH-peptide	NH_2-peptide $+H_2O$
1		 5-member ring transition state	 γ-peptide	99	1	0
2		 5-member ring transition state	 Statine	89	0	11
3		 5-member ring transition state	 Phenylstatine	89	0	11
4		 5-member ring transition state	 β-peptide	95	2	3
5		 6-member ring transition state	 γ-peptide	76	14	10
6		 7-member ring transition state	 δ-peptide	41	48	11
7		 8-member ring transition state	 ε-peptide	0	50	50
8		 5-member ring transition state for ester exchange / 5-member ring transition state for acyl transfer	 γ-peptide	Quant.	0	0
9		 5-member ring transition state for ester exchange / 6-member ring transition state for acyl transfer	 δ-peptide	70	24	6

Table 3.1 | Summary of the substrates used in this chapter

Entry number and compound number of hydroxy acids are identical.

3.2. Results and discussion

3.2.1. Incorporation of natural γ-peptides into ribosomally expressed peptides

I applied the established reactions for generating statine or its analogous structures to ribosomally expressed peptides. Based on the structure of statine and phenylstatine, γ-azide-β-hydroxy acids **2** and **3** were prepared and subjected to translation and posttranslational reactions in the same manner as **1**. Although I found the translation efficiency became lower than **1**, a cyclic peptide **2-pep-N_3** containing γ-azide-statine **2** was expressed by the FIT system (**Figure 3.1a, b**). The low translation efficiency may be derived from flipped stereochemistry of β-hydroxy group compared to **1**. The product obtained with TCEP reactions showed the hydrolysis tolerance, whereas the peptide was fragmented by the hydrolysis to produce **2-pep-N_3 + H_2O**, indicating that the incorporation of statine into peptide **2-pep-γ** succeeded (**Figure 3.1b, c**). The conversion yield of **2-pep-γ** from **2-pep-N_3** was calculated as 89% from the peak areas of XIC spectra (**Table 1, entry 2**). Instead of OH-peptides, the hydrolyzed peptides **2-pep-NH_2 + H_2O** was obtained as a byproduct, probably because the acyl transfer step became slower by the steric hindrance of the side chain.

Furthermore, we also succeeded in the incorporation of phenylstatine, a phenylalanine-type statine analogue, from the correspond hydroxy acid **3** with 89% conversion yield (**Table 1, entry 3**). The results here showed that the developed reactions were applicable to not only various γ-azide-β-hydroxy acids but also γ-peptides seen in natural bioactive peptides. These results also implied the possibility to synthesize bioactive peptides which possess the inhibitory function derived from statine structures.

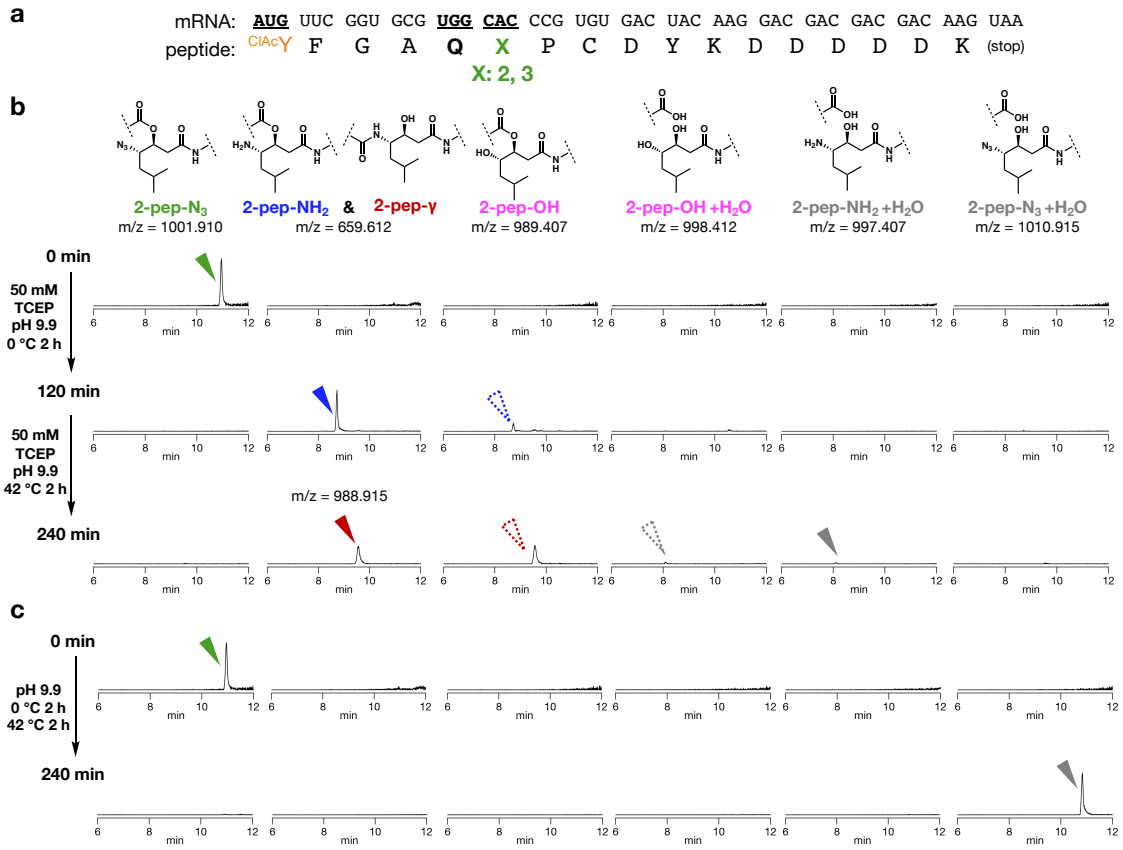


Figure 3.1 | LC-MS XIC spectra of peptides bearing a statine residue

(a) mRNA sequence and correspond peptide sequence used in this study.

(b) XIC spectra of the expected peptides before/after reactions.

(c) The control experiment incubated without TCEP.

3.2.2. Investigation for the effect of transition state in acyl transfer step with a series of α-hydroxy acids

I next applied the modification reactions to the α-hydroxy acids **4–7** which possess the different length of side chains. The purpose of this study is (i) to form various peptide bonds, e.g., β-, γ-, and δ- peptides, and (ii) to obtain the information about the acyl transfer reaction step. Each acyl-tRNAs is prepared and used for translation to synthesize peptides containing the same peptide sequence except for the prepared substrates (**Figure 3.2a**). The peptides before/after reactions were analyzed by LC-MS. From the XIC spectra of LC-MS, β-, γ-, and δ-peptides are obtained with 95%, 70%, and 41% conversion yield from **4, 5,** and **6** respectively, whereas the synthesis of a ε-peptide linkage from **7** completely failed (**Table 3.1, entries 4-7**). In detail, conversion of β-peptide from **4** only showed the tiny peaks of byproducts (**Figure 3.2b**). In the case of **5-7**, the conversion yield dropped because of the formation of hydroxylated byproduct and reduced but hydrolyzed peptides (**Figure 3.2c-e**). Especially in the case of **7**, all of the starting peptide **7-pep-N_3** was converted to the byproducts **7-pep-OH**, **7-pep-OH + H_2O**, and **7-pep-NH_2 + H_2O**.

These results informed me of the importance of transition state at acyl transfer reactions: 5-membered ring transition state of **4** showed the best conversion yield, on the other hand, 8-membered ring of **8** couldn't proceed the acyl transfer reaction. Surprisingly, δ-peptide formation via the 7-membered ring transition state was also confirmed, which is considered relatively slow reaction. The tendency observed in this study also explained the good conversion yields of **1-3**, which underwent acyl transfer via the 5-membered ring transition state. This also explains the higher yield of OH-peptides, because α-hydroxy acids are able to undergo the byproduct pathway (shown in **Scheme 2.1**) via "minus one" transition state, (e.g., the acyl transfer of substrate **7** undergoes via 8-member ring transition state, whereas byproduct pathway undergoes via 7-membered ring transition state).

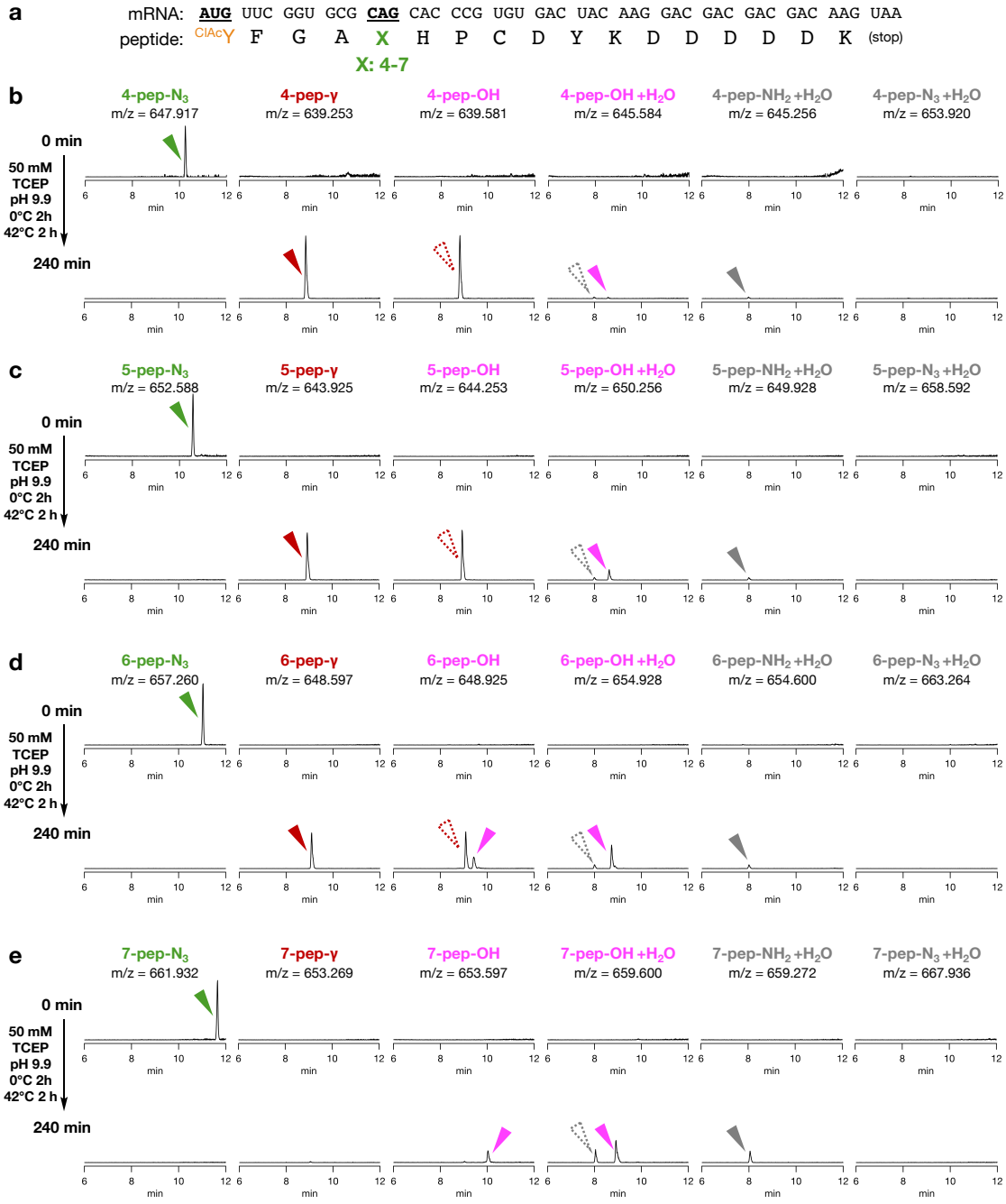


Figure 3.2 | LC-MS XIC spectra of peptides after reactions.

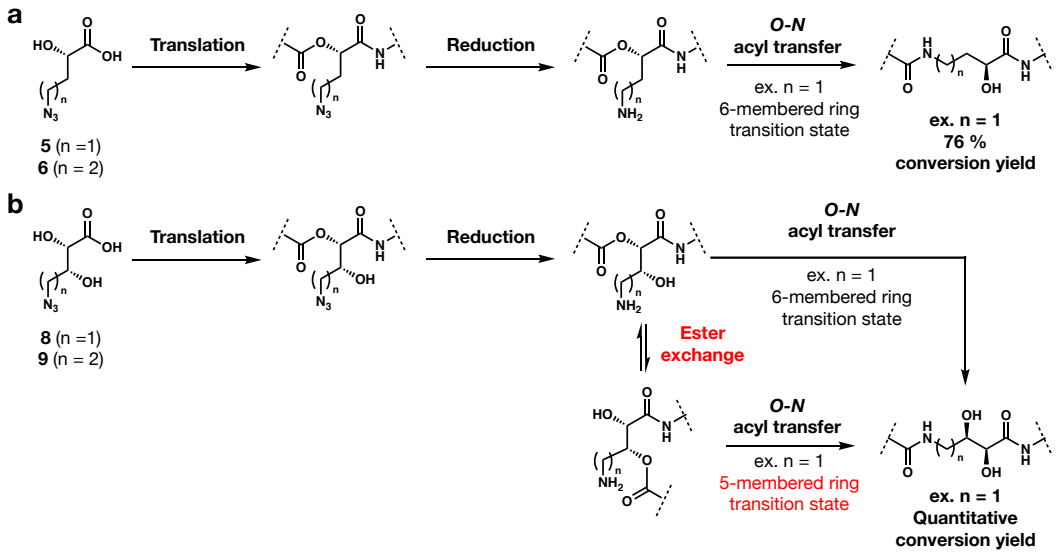
(a) mRNA sequence and correspond peptide sequence used in this study.

(b)-(e) XIC spectra of expected products using **4-7**. The dashed arrow shows the detection of isotopic mass of other product.

3.2.3. Strategy to improve the conversion yield of acyl transfer reaction

The series of studies with α-hydroxy acids suggested that 5-membered ring transition state is the most suitable for the acyl transfer step. Based on this hypothesis, α,β-dihydroxy acids **8** and **9** were newly designed. These substrates were expected to be incorporated into peptides by forming an α-ester linkage, followed by ester exchange reactions between α- and β-hydroxy groups. As a result, these substrates are able to undergo acyl transfer reaction with more closer reaction points (**Scheme 3.1a, b**). For instance, **8** is able to undergo acyl transfer with 5-membered ring transition state, and α-hydroxy acid **5** proceeds with 6-member ring transition state.

The conversion yields of **8** and **9** were calculated by LC-MS to record the quantitative yield and 70% yield, respectively (**Table 3.1, entries 8 and 9**). Both of them could improve the conversion yields from α-hydroxy acid **5** and **6**, and thus the design to append hydroxy group(s) onto the side chains is proven to be a good way for improving the conversion yields. The resulted γ- or δ-peptides are regarded as hydrophilic carbon chains. Although, to the best of my knowledge, exactly the same structures are not seen in natural peptides, the resulted structures have the similarity to hydrophillic polyketide carbon chains, which sometimes enhance the solubility of hydrophilic natural molecules[140].



Scheme 3.1 | Ester exchange strategy to improve conversion yield.

(a) acyl transfer reaction of α-hydroxy acids **5** and **6** with 6/7-membered ring transition state.

(b) acyl transfer reaction of α,β-dihydroxy acids **8** and **9** with 5/6-membered ring transition state.

3.3. Conclusion

In this chapter, I demonstrated that the developed posttranslational chemical modification reactions were applicable to various substrates, such as statine and phenylstatine as a building block of natural γ-peptide and δ-peptide linkages. In addition, the investigation for the acyl transfer reaction revealed that the conversion yield of the acyl transfer reaction was highly dependent on their transition states. In the investigation, I found that even the 7-membered ring transition state is acceptable to synthesize δ-peptides albeit with relatively low conversion yield. The conversion yield was recovered by designing α,β-dihydroxy acids.

The achievement of incorporation of statine residues is quite important for the rational design of peptide drugs, because of the flexibility of translation system. Hereby the method allows us to synthesize various statine-containing peptides just by changing the sequence of template mRNA, and it would provide candidates of bioactive peptides.

The ester exchange strategy was proven as a good way to improve the efficiency of acyl transfer reactions. Although the addition of hydroxy groups to the substrates may restrict the substrate scope, natural NRPS-PKS products often have the similar structure, where carbon chains of their backbone are highly hydrophilic. Moreover, the addition of hydroxy group can theoretically incorporate any side chain length of peptides. Therefore, the chemical modification reactions are probably applicable for the synthesis of the mimetics of carbon backbones seen polyketide chains.

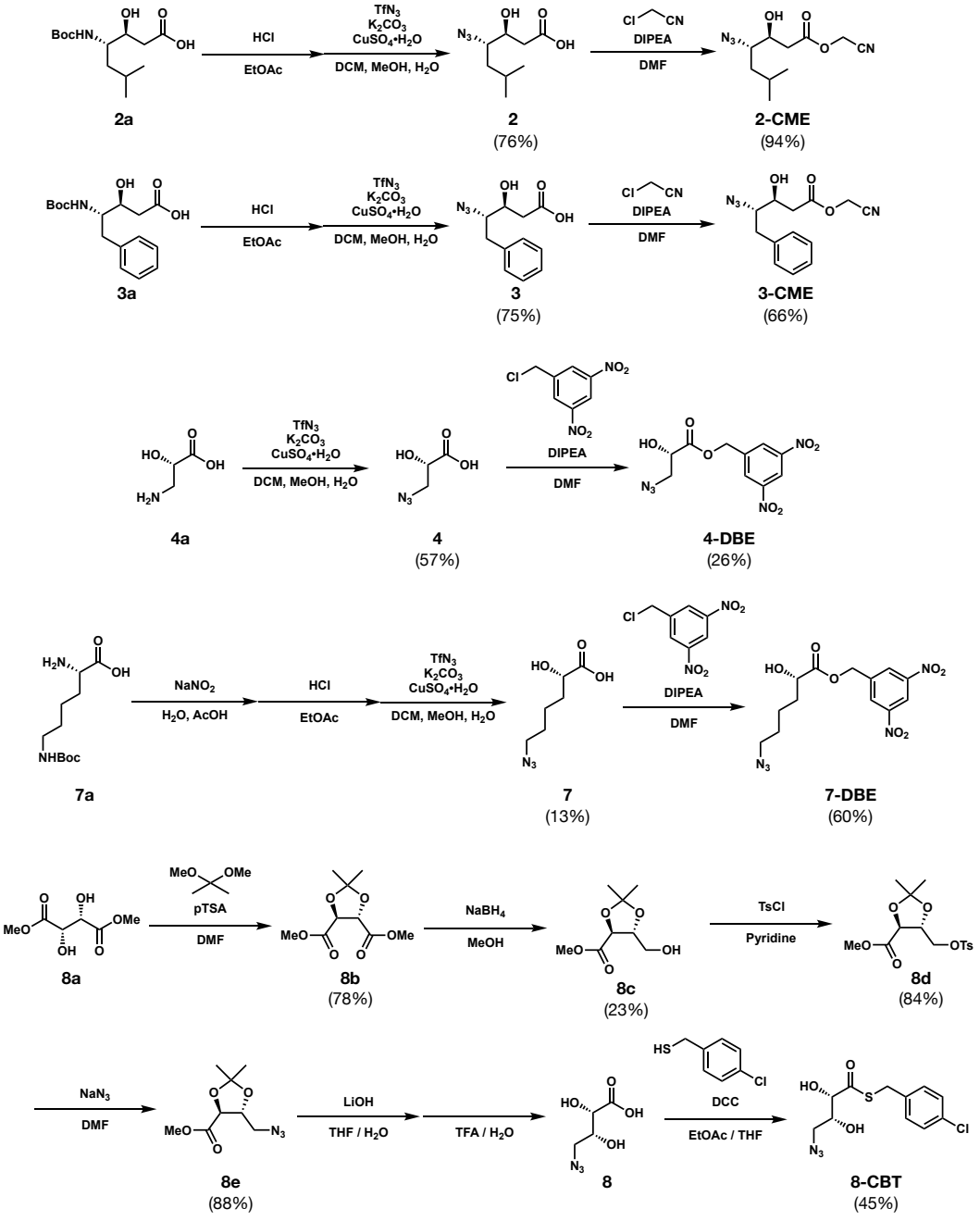
3.4. Supplemental results

3.4.1. Synthesis of substrates (2-9) and tRNA acylation reaction with them

All of the substrates used in this chapter were synthesized as followed. For **2-CME** and **3-CME**, Boc-protected statine/phenylstatine (**2a** and **3a**) are deprotected, followed by azide conversion provide **2** and **3** respectively. Both of the compounds adopted CME ester based on their side chain characters. **4-DBE** was synthesized by azide conversion and DBE esterification. **7-DBE** was prepared form side chain-protected lysine **7a**: α-amine group was converted to α-hydroxy group by selective hydroxylation in water. After deprotecting Boc group, azide conversion provided **7**, and DBE esterification provided **7-DBE**. Synthesis of α,β-dihydroxy acid **8-CBT** was composed of 7 steps. After protecting the diol group of **8a** by acetal, reported mono-reduction reaction provided compound **8c**[141,142]. Although **8b** contains the two reactive esters, the resulted stereochemistry is identical. The hydroxy group was then tosylated and substituted to azide. Deprotection of acetal and ester followed by CBT esterification produced **8-CBT.**

The synthesized compounds were then confirmed the acylation reaction catalyzed by flexizymes. All of the experiments were conducted with the use of $NaIO_4$ treatment. The reacted or unreacted microhelix were analyzed by acid PAGE analysis (**Figure 3.3**). As a result, all of the substrates were confirmed to be acylated by appropriate flexizymes.

5-DBE, **6-DBE**, and **9-DBE** were already synthesized and checked acylation reaction in my master's thesis.



Scheme 3.1 | Synthesis of substrates used in this chapter.

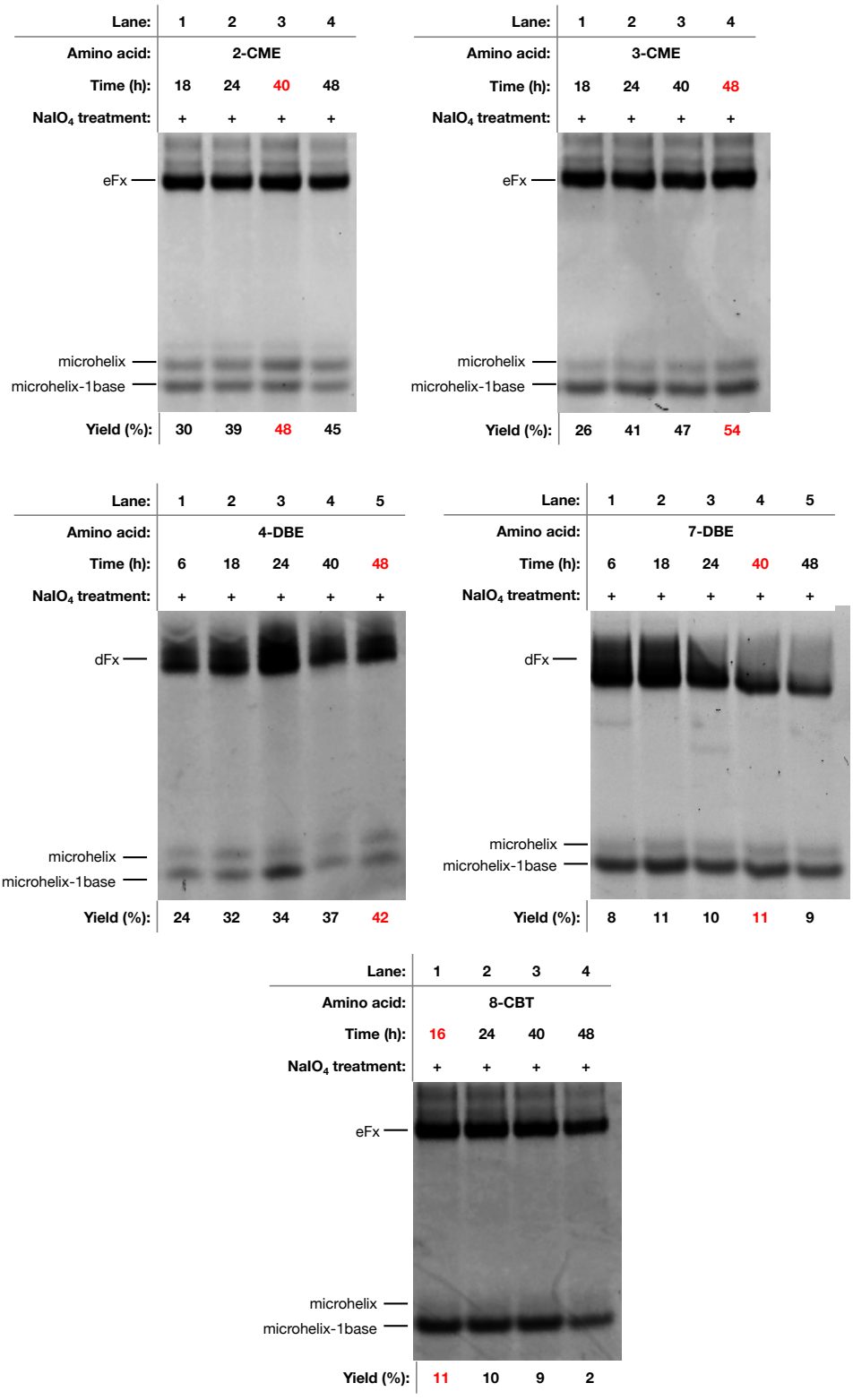
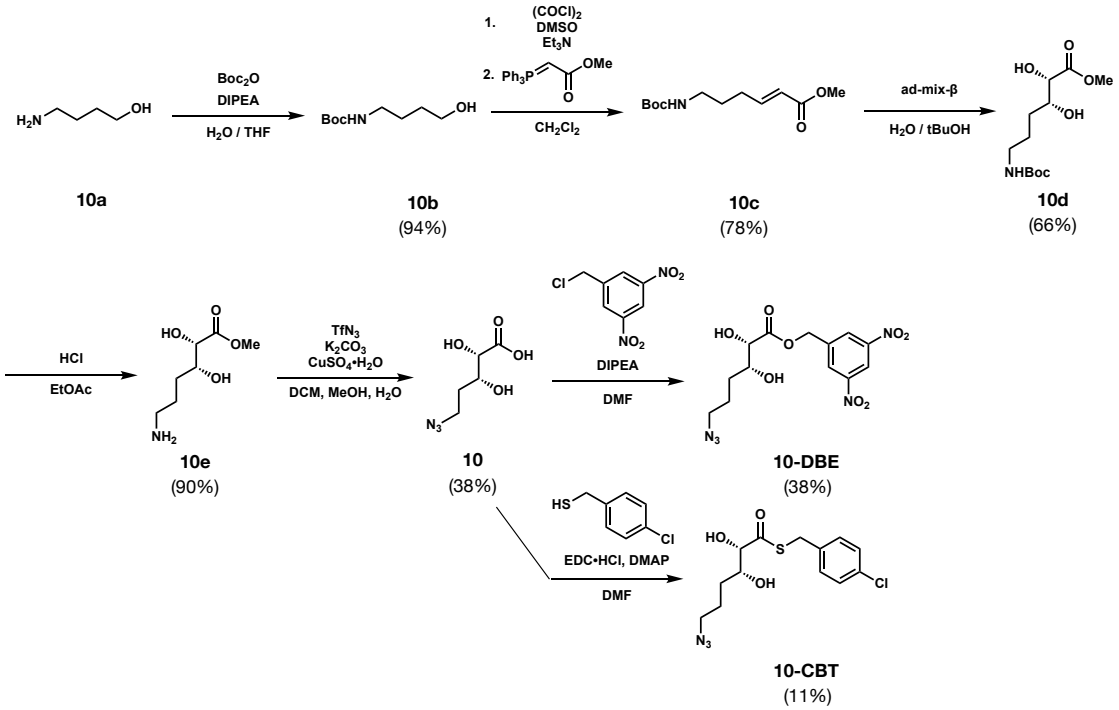


Figure 3.3 | Confirmation of acylation reaction with synthesized compounds by acid PAGE analysis

3.4.2. Attempt to produce ε-peptide linkages by means of the ester exchange strategy

Since the formation of a ε-peptide linkage from the α-hydroxy acid **7** failed, I designed an α,β-dihydroxy acid **10** to undergo the acyl transfer via 7-member ring transition state. **10-CBT** was synthesized according to **scheme3.2**. The starting material **10a** was firstly protected by the Boc group. To this compound **10b**, Swern oxidation and Wittig reaction were conducted to provide **10c**. The stereoselective osmium oxidation yielded **10d**. After deprotecting the Boc group, the amine group was converted to the azide group, resulting in the formation of **10**. In this step, the methyl ester was hydrolyzed spontaneously in the basic conditions. **10-CBT** and **10-DBE** were prepared by the esterification of **10** based on the knowledge that both of the α,β-dihydroxy acids **8-CBT** and **9-DBE** could be the substrates of the flexizymes.

Next, the acylation reaction of microhelix was tested with **10-CBT** and **10-DBE** in the condition of pH 8.0. Both of them showed the failure of the acylation reactions in the acid-PAGE analysis (**Figure 3.4**). Furthermore, the efforts to optimize pH did not show the improvements. I estimated that α,β-dihydroxy acids would be acylated with the CBT/DBE ester, since **9-DBE** was acylated with 67% yield**,** and **8-CBT** was acylated with the relatively low acylation yield (11%). However, the flexizymes would not accept the structure of **10** as their substrates. From these results, the attempt to form ε-peptide linkages was gave up.



Scheme 3.2 | Synthesis of 10-DBE and 10-CBT.

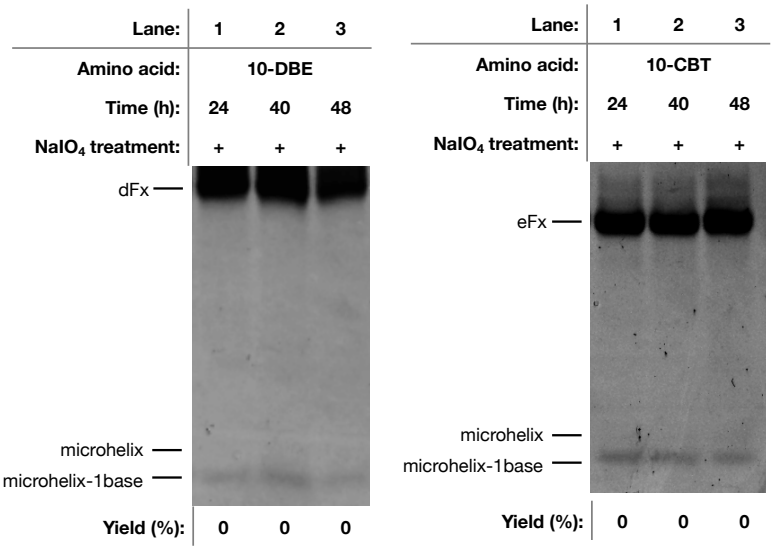


Figure 3.4 | Confirmation of acylation reaction with 10-DBE and 10-CBT by acid PAGE analysis

3.5. Material and methods

・Materials

All chemicals and reagents were purchased from Tokyo Chemical Industry, Kanto Chemical, Nacalai Tesque, Sigma-Aldrich Japan, Watanabe chemical Industry, or Fujifilm Wako Pure Chemical Industries. All DNA oligomers were purchased from Eurofin Genomics or GeneDesign.

・List of DNA oligomers

Primer	Sequence
dFx.R46	ACCTAACGCCATGTACCCTTTCGGGGATGCGGAAATCTTTC GATCC
dFx.R19	ACCTAACGCCATGTACCCT
SD6MK3_W(4G)H(7C)flag.R45	GTCGTCGTCCTTGTAGTCGTGCCACTTCTTCTTCATGTTTT TCTC
Pro1E2#3CCA_Trp.R62	TGGCGGGTGATAGGGGATTCGAACCCCTGACCCCTTCGTTT GGAGCGAAGTGCGCTACCAGG
Pro1E2#3GUG_His.R62	TGGCGGGTGATAGGGGATTCGAACCCCTGACCCCTTCGTTC ACAACGAAGTGCGCTACCAGG

・Combination of DNA primers

Name	Extension	1st PCR	2nd PCR
DNA template	T7eSD6M.F46 SD6MK3_W(4G)H(7C)flag.R45	-	T7ex5.F22 Flaguaa.R33
dFx	Fx-5'.F36 dFx.R46	-	T7ex5.F22 dFx.R19

$tRNA^{Pro1E2\#3}_{cca}$	Pro1E.F50 Pro1E2#3CCA_Trp.R62	-	T7ex5.F22 Pro1E2#3-OMe.R20
$tRNA^{Pro1E2\#3}_{gug}$	Pro1E.F50 Pro1E2#3GUG_His.R62	-	T7ex5.F22 Pro1E2#3-OMe.R20

・Preparation of DNA template, flexizyme (dFx), $tRNA^{Pro1E2\#3}_{cca}$, and $tRNA^{Pro1E2\#3}_{gug}$

The DNA template used in this chapter and eFx were prepared as described in chapter 2 from appropriate primers. $tRNA^{Pro1E2\#3}_{cca}$ and $tRNA^{Pro1E2\#3}_{gug}$ were prepared in the same manner as $tRNA^{Pro1E2\#3}_{cug}$.

・Summary of acylation conditions for the substrates

Substrates	tRNA	Flexizyme	pH	Reaction time (h)
2-CME	$tRNA^{Pro1E2\#3}_{gug}$	eFx	8.0	40
3-CME	$tRNA^{Pro1E2\#3}_{gug}$	eFx	8.0	48
4-DBE	$tRNA^{Pro1E2\#3}_{cug}$	dFx	8.5	48
5-DBE	$tRNA^{Pro1E2\#3}_{cug}$	dFx	7.5	40
6-DBE	$tRNA^{Pro1E2\#3}_{cug}$	dFx	8.5	24
7-DBE	$tRNA^{Pro1E2\#3}_{cug}$	dFx	8.0	40
8-CBT	$tRNA^{Pro1E2\#3}_{cug}$	eFx	8.5	16
9-DBE	$tRNA^{Pro1E2\#3}_{cug}$	dFx	7.5	40
Gln-DBE	$tRNA^{Pro1E2\#3}_{cca}$	dFx	7.5	2

The procedure of tRNA acylation is described in chapter 2. To adjust pH of acylation reaction, HEPES•KOH was used for pH 7.5 and 8.0, Bicine•KOH was used for pH 8.5.

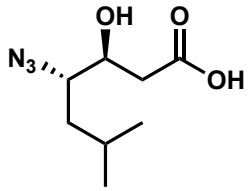
・**Translation**

The ribosomal synthesis of peptides containing **2-9** were performed as the same manner described in chapter 2.

For the ribosomal synthesis of peptides containing **2** or **3**, in addition to translation mixture (10% (v/v) DNA template, 0.5 mM amino acid mixture (M, K, D, Y), 10.9% (v/v) solA, 12% (v/v) solB, 25 μM **2**-$tRNA^{Pro1E2\#3}_{gug}$ or 25 μM **3**-$tRNA^{Pro1E2\#3}_{gug}$, Gln-$tRNA^{Pro1E2\#3}_{cca}$ and 200 μM NaOAc), 5 μM of EF-P was added to this mixture. The mixture was incubated at 37°C for 30 min. The mixtures were further analyzed by both of MALDI-TOF MS and LC-MS.

・**Chemical synthesis**

*Synthesis of **5-DBE**, **6-DBE**, and **9-DBE** are described in my master's thesis.*



(3*S*,4*S*)-4-azide-3-hydroxy-6-methyl-heptanoic acid (2)

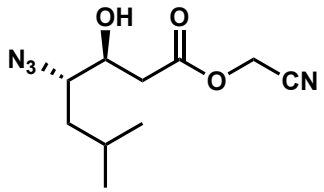
TfN_3 solution in DCM (0.625 mmol) was prepared as previously described.

Boc-statine (**2a**) (68.8 mg, 0.250 mmol) was dissolved in 2 mL of 4N HCl in EtOAc. The solution was stirred for 1 h, the solvent was evaporated under reduced pressure. The crude product was used for the next step without further purification.

The crude product, K_2CO_3 (51.9 mg, 0.375 mmol), and $CuSO_4•H_2O$ (2.0 mg, 7.98 μmol) were dissolved in 1 mL of water and 2 mL of MeOH. To this solution, freshly prepared TfN_3 solution (0.625 mmol) was added and stirred vigorously overnight. The solution was evaporated until MeOH was vaporized. DCM was added and the organic layer was washed with 1M HCl twice. The water layer was back extracted with DCM twice. The combined organic layer was dried over Na_2SO_4, filtrated, and evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 2), afforded

compound **2** (38.1 mg, 76%).

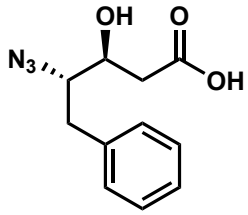
^{1}H NMR (300 MHz, $CDCl_3$): δ 5.46 (br, 1H), 4.07 (m, 1H), 3.28 (m, 1H), 2.62 (ddd, J=6.31, 16.29, 35.08 Hz, 2H)1.87-1.39 (m, 3H), 0.97 (dd, J=3.92, 6.56 Hz, 6H).^{13}C NMR (75 MHz, $CDCl_3$): δ 176.95, 70.41, 63.53, 39.23, 38.57, 25.13, 23.21, 22.00. HR-ESI-MS: Calculated for $C_8H_{15}N_3O_3$ $[M-H]^-$ 200.1041, Observed: 200.1040.



(3*S*,4*S*)-4-azide-3-hydroxy-6-methyl-heptanoic acid CME ester (2-CME)

Compound **2** (4.9 mg, 24.4 μmol) was dissolved in 800 μL of dry DMF. *N*,*N*-diisopropyl ethyl amine (6.36 μL, 36.5 μmol) and chloroacetonitrile (9.73 μL, 153.4 μmol) were added to this solution and stirred for 12 h. 1 M HCl and diethyl ether were added and the water layer was extracted with diethyl ether twice. The combined organic layer was washed with 1 M HCl followed by brine. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 2 : 1), afforded compound **2-CBT** (3.4 mg, 66%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 4.77 (d, *J*=3.00 Hz, 2H), 4.09 (m, 1H), 3.27 (m, 1H), 2.70 (ddd, *J*=6.33, 16.52, 40.17 Hz, 2H), 2.61 (br, 1H), 1.90-1.39 (m, 3H), 0.98 (dd, *J*=3.18, 6.57 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.70, 114.12, 70.24, 63.40, 48.68, 39.21, 38.43, 25.12, 23.17, 22.05. HR-ESI-MS: Calculated for $C_{10}H_{16}N_4O_3$ $[M+Cl]^-$ 275.0916, Observed: 275.0914.



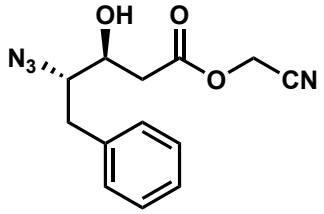
4-azide-2,4,5-trideoxy-5-phenyl-*L-threo*-pentonic acid (3)

TfN_3 solution in DCM (0.625 mmol) was prepared as previously described.

Boc-phenylstatine (**3a**) (77.4 mg, 0.250 mmol) was dissolved in 2 mL of 4N HCl in EtOAc. The solution was stirred for 1 h, the solvent was evaporated under reduced pressure. The crude product was used for the next step without further purification.

The crude product, K_2CO_3 (51.9 mg, 0.375 mmol), and $CuSO_4 \cdot H_2O$ (2.0 mg, 7.98 μmol)were dissolved in 1 mL of water and 2 mL of MeOH. To this solution, freshly prepared TfN_3 solution (0.625 mmol) was added and stirred vigorously overnight. The solution was evaporated until MeOH was vaporized. DCM was added and the organic layer was washed with 1M HCl twice. The water layer was back extracted with DCM twice. The combined organic layer was dried over Na_2SO_4, filtrated, and evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 2), afforded compound **3** (44.3 mg, 75%).

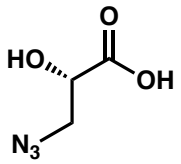
^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36-7.24 (m, 5H), 5.91 (br, 1H), 4.05 (m, 1H), 3.44 (m, 1H), 3.05 (ddd, *J*=7.31, 13.69, 31.62 Hz, 2H), 2.66 (ddd, *J*=4.65, 8.25, 66.79 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.46, 137.09, 129.43, 128.95, 127.17, 68.74, 66.49, 38.65, 36.76. HR-ESI-MS: Calculated for $C_{11}H_{13}N_3O_3$ $[M-H]^-$ 234.0884, Observed: 234.0889.



4-azide-2,4,5-trideoxy-5-phenyl-*L-threo*-pentonic acid CME ester (3-CME)

Compound **3** (4.2 mg, 17.9 μmol) was dissolved in 800 μL of dry DMF. *N*,*N*-diisopropyl ethyl amine (4.66 μL, 26.8 μmol) and chloroacetonitrile (7.14 μL, 112.5 μmol) were added to this solution and stirred for 12 h. 1 M HCl and diethyl ether were added and the water layer was extracted with diethyl ether twice. The combined organic layer was washed with 1 M HCl followed by brine. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 2 : 1), afforded compound **3-CME** (4.6 mg, 94%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.37-7.26 (m,5H), 4.74 (d, J=1.77 Hz, 2H), 4.07 (m, 1H), 3.45 (dt, *J*=2.73, 3.65 Hz, 1H), 3.06 (ddd, *J*=7.37, 13.64, 27.96 Hz, 2H), 2.70 (ddd, *J*=6.32, 16.58, 64.90 Hz, 2H), 2.68 (br, 1H).^{13}C NMR (75 MHz, $CDCl_3$): δ 170.71, 136.90, 129.42, 129.02, 127.27, 114.08, 68.57, 66.37, 48.68, 38.47, 36.72. HR-ESI-MS: Calculated for $C_{13}H_{14}N_4O_3$ $[M-H]^-$ 273.0993, Observed: 273.1000.



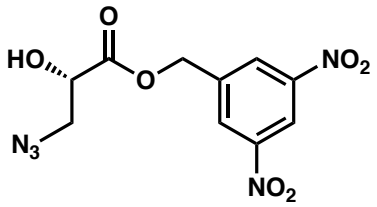
3-azide-(2*S*)-hydroxy propanoic acid (4)

TfN_3 solution in DCM (4.76 mmol) was prepared as previously described.

L-isoserine (**4a**) (200.0 mg, 1.90 mmol), K_2CO_3 (394.5 mg, 2.86 mmol), and $CuSO_4$•H_2O (4.75 mg, 19.0 μmol) were dissolved in 5 mL of water and 5 mL of MeOH. To this solution, freshly prepared TfN_3 solution (4.76 mmol) was added and stirred vigorously overnight. The solution was evaporated until MeOH was vaporized. DCM was added and the organic layer

was washed with 1M HCl twice. The water layer was back extracted with DCM twice and EtOAc five times. The combined organic layer was dried over Na_2SO_4, filtrated, and evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 2), afforded compound **4** (140.9 mg, 57%).

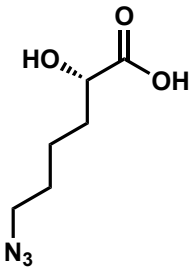
^{1}H NMR (300 MHz, DMSO-d_6): δ 8.92 (br, 2H), 4.21 (dd, *J*=3.75, 5.82 Hz, 1H), 3.44 (ddd, *J*=4.79, 12.80, 22.97 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 173.03, 69.75, 53.47. HR-ESI-MS: Calculated for $C_3H_5N_3O_3$ $[M-H]^-$ 130.0258, Observed: 130.0253.



3-azide-(2*S*)-hydroxy propanoic acid DBE ester (4-DBE)

Compound **4** (42.0 mg, 0.318 mmol) was dissolved in 2 mL of dry DMF. *N*,*N*-diisopropyl ethyl amine (83.2 μL, 0.478 mmol) and 2,4-dinitrobenzyl chloride (82.8 mg, 0.382 mmol) were added to this solution and stirred for 12 h. 1 M HCl and diethyl ether were added and the water layer was extracted with diethyl ether twice. The combined organic layer was washed with 1 M HCl followed by brine. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 2 : 1), afforded compound **4-DBE** (25.3 mg, 26%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 9.03 (t, *J*=1.94 Hz, 1H), 8.59 (d, *J*=1.95 Hz, 2H), 5.47 (q, *J*=13.69 Hz, 2H), 4.52 (dd, *J*=3.54, 8.73 Hz, 1H), 3.69 (ddd, *J*=1.65, 7.72, 23.55 Hz, 2H), 3.14 (d, *J*=5.37 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.03, 69.75, 53.47. HR-ESI-MS: Calculated for $C_{10}H_9N_5O_7$ $[M-H]^-$ 310.0429, Observed: 310.0440.



6-azide-(2*S*)-hydroxy hexanoic acid (7)[140]

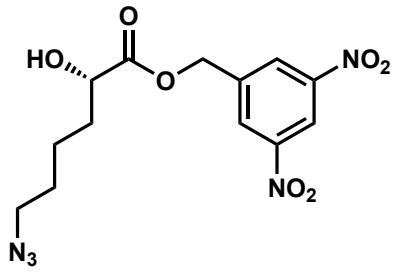
H-Lys(Boc)-OH **7a** (246.3 mg, 1.00 mmol) was dissolved in the mixture of 8 mL of water and 2 mL of AcOH. The mixture was cooled to 0℃, then the solution of 2 M $NaNO_2$ (2.5 mL, 5.00 mmol) were added to this solution dropwise over 15 min. The mixture was stirred for 3 h. 1 M HCl and EtOAc were added and the water layer was extracted with EtOAc twice. The combined organic layer was washed with 1 M HCl followed by brine. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was used for the next step without further purification.

The crude product was dissolved in 2 mL of 4N HCl in EtOAc. The solution was stirred for overnight, then the solvent was evaporated under reduced pressure. Diethyl ether was added to the crude and the precipitant was recovered. The crude product was used for the next step.

TfN_3 solution in DCM (0.786 mmol) was prepared as previously described.

The crude product, K_2CO_3 (65.1 mg, 0.471 mmol), and $CuSO_4•H_2O$ (0.8 mg, 3.14 μmol) were dissolved in 5 mL of water and 5 mL of MeOH. To this solution, freshly prepared TfN_3 solution (0.786 mmol) was added and stirred vigorously overnight. The solution was evaporated until MeOH was vaporized. DCM was added and the organic layer was washed with 1M HCl twice. The water layer was back extracted with DCM twice and EtOAc five times. The combined organic layer was dried over Na_2SO_4, filtrated, and evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 2), afforded compound **7** (23.1 mg, 13% in 3 steps).

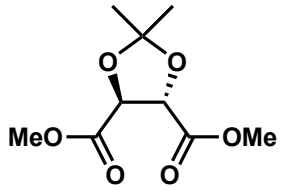
^{1}H NMR (300 MHz, DMSO-d_6): δ 4.18 (m, 1H), 3.29 (t, J=6.9 Hz, 2H), 1.85-1.46 (m, 6H).
^{1}HR-ESI-MS: Calculated for $C_6H_{11}N_3O_3$ $[M-H]^-$ 172.0728, Observed: 172.0726.



6-azide-(2*S*)-hydroxy hexanoic acid DBE ester (7-DBE)

Compound **7** (5.5 mg, 31.8 μmol) was dissolved in 500 μL of dry DMF. *N*,*N*-diisopropyl ethyl amine (6.1 μL, 34.9 mmol) and 2,4-dinitrobenzyl chloride (7.6 mg, 34.9 μmol) were added to this solution and stirred for 12 h. 1 M HCl and diethyl ether were added and the water layer was extracted with diethyl ether twice. The combined organic layer was washed with 1 M HCl followed by brine. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 1), afforded compound **7-DBE** (6.7 mg, 60%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 9.05 (s, 1H), 8.57 (d, *J*=1.95 Hz, 2H), 5.42 (s, 2H), 4.34 (q, *J*=3.74 Hz, 2H), 3.30 (t, J=6.45 Hz, 2H), 2.66 (s, 1H), 1.69-2.65 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.50, 148.80, 139.59, 127.94, 118.87, 64.76, 51.16, 33.78, 28.51, 22.11. HR-ESI-MS: Calculated for $C_{13}H_{15}N_5O_7$ $[M+Na]^+$ 376.0864, Observed: 376.0852.

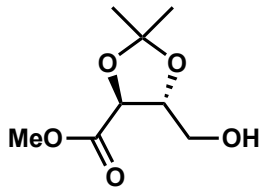


Dimethyl 2,3-O-isopropylidene-D-tartrate (8b)[143]

Dimethyl-D-(-)-tartrate (**8a**) (1.00 g, 5.61 mmol) was dissolved in 10 mL of dry DCF. *p*-toluene sulfonic acid monohydride (483.3 μL, 2.81 mmol) and acetone dimethyl acetal (4.0 mL, 33.49 mmol) were added to this solution and stirred for 8 h. Water was added and the water layer was extracted with EtOAc three times and chloroform once. The combined organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced

pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 2 : 1), afforded compound **8b** (952.4 mg, 78%).

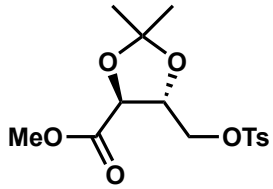
^{1}H NMR (300 MHz, $CDCl_3$): δ 4.81 (s, 1H), 3.83 (s, 3H), 1.50 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.09, 113.90, 77.23, 52.82, 26.32. HR-ESI-MS: Calculated for $C_9H_{14}O_6$ $[M+Na]^+$ 241.0683, Observed: 241.0684.



(4*S*, 5*R*)-Methyl 5-hydroxymethyl-2,2dimethyl-1,3-dioxolane-4-carboxylate (8c)[141]

Compound **8b** (735.1 mg, 3.37 mmol) was dissolved in 2 mL of dry MeOH. $NaBH_4$ (76.5 mg, 2.02 mmol) was added portionwise to this solution over 1 hand stirred for 30 min. The solvent was evaporated under reduced pressure. Water was added and the water layer was extracted with EtOAc three times. The combined organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 2 : 1), afforded compound **8c** (149.7 mg, 23%).

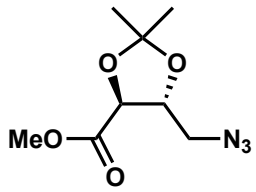
^{1}H NMR (300 MHz, $CDCl_3$): δ. 4.48 (d, *J*=7.71 Hz, 1H), 4.25 (m, *J*=2.41 Hz, 1H), 3.97 (ddd, J=3.02, 4.52, 12.16 Hz, 1H), 3.81 (s, 3H), 3.78 (d, J=4.92 Hz, 1H), 1.50 (s, 3H), 1.46 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.36, 111.57, 79.28, 75.08, 61.98, 52.62, 26.93, 25.79.



(4*S*, 5*R*)-methyl 2,2-dimethyl-5-toluenesulfonyl-1,3-dioxolane-4-carboxylate (8d)

Compound **8d** (149.7 mg, 0.787 mmol) was dissolved in 5 mL of dry pyridine. The mixture was cooled to 0℃, then tosyl chloride (300.1 mg, 1.574 mmol) was added. This solution was stirred at 0℃ for overnight. 1M HCl was added and the water layer was extracted with chloroform three times. The combined organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 1), afforded compound **8d** (242.0 mg, 84%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.81 (d, *J*=8.37 Hz, 2H), 7.36 (d, *J*=8.01 Hz, 2H), 4.34 (dd, *J*=2.97, 6.81 Hz, 3H), 4.18 (*d*, J=11.68 Hz, 1H), 3.78 (s, 3H), 2.46 (s, 3H), 1.40 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.38, 145.24, 132.84, 130.03, 128.19, 112.36, 76.32, 75.21, 68.51, 52.78. 26.80, 25.94, 21.81. HR-ESI-MS: Calculated for $C_{15}H_{20}O_7S$ $[M+Na]^+$ 367.0822, Observed: 367.0832.

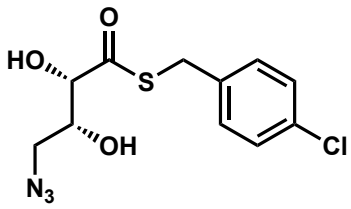


(4*S*,5R)-methyl 5-azidomethyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate (8e)[143]

Compound **8d** (242.0 mg, 0.703 mmol) was dissolved in 3.5 mL of DMF. NaN_3 (137.1 mg, 2.11 mmol) was added and stirred for 14 h at 85℃. The solvent was evaporated under reduced pressure. Water was added and the water layer was extracted with EtOAc twice. The combined organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 1), afforded compound **8e** (132.9 mg, 88%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 4.44 (d, *J*=7.53 Hz, 1H), 4.32 (m, 1H), 3.71 (dd, *J*=3.03, 13.33 Hz, 1H), 3.36 (dd, *J*=4.38, 13.36 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.63,

111.98, 77.90, 75.63, 52.60, 51.59, 26.67, 25.72. HR-ESI-MS: Calculated for $C_8H_{13}N_3O_4$ $[M+Na]^+$ 238.0798, Observed: 238.0791.

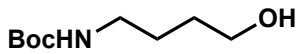


4-azide-(2*S*, 3*R*)-dihydroxy butanoic acid CBT ester (8-CBT)

Compound **8e** (6.5 mg, 29.8 μmol) was dissolved in the mixture of 300 μL of water and 600 μL of THF. LiOH monohydride (3.8 mg, 89.4 μmol) was added and stirred for 15 min at room temperature. The mixture was evaporated and 1 mL of TFA was added. The mixture was stirred for 40 min at room temperature. Water and $NaHCO_3$ were added until the mixture was neutralized and the water layer was washed with EtOAc twice. The water layer was acidified by 1M HCl and extracted with EtOAc five times. The combined organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was used for next step without further purification.

The crude product and 4-chlorobenzyl mercaptan (3.87 μL, 29.8 μmol) were dissolved in 250 μL of 1,4-dioxane. DCC (5.2 mg, 24.8 μmol) in 500 μL of EtOAc was added to this solution, then stirred for 24 h. The reaction mixture was filtered with celite, then the solvent was evaporated under reduced pressure. EtOAc was added, and the organic layer was washed with sat. $NaHCO_3$ aq. twice, 1M HCl twice, and brine. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 1), afforded compound **8-CBT** (4.0 mg, 45% in 3 steps).

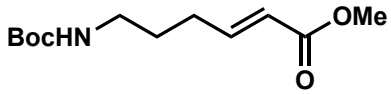
^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29-7.22 (m, 4H), 4.23 (m, 1H), 4.18 (m, 1H), 4.11 (s, 2H), 3.58-3.44 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 201.46, 135.63, 133.37, 130.37, 129.02, 77.36, 71.28, 53.44, 32.49. HR-ESI-MS: Calculated for $C_{11}H_{12}N_3O_3ClS$ $[M-H]^-$ 300.0205, Observed: 300.0205.



4-(Boc-amino)-1-butanol (10b)[134]

4-amino-1-butanol (**10a**) (1.00 mL, 11.69 mmol) was dissolved in the mixture of 3 mL of water and 3 mL of THF. *N*,*N*-diisopropyl ethyl amine (2.44 mL, 14.02 mmol) and Boc_2O (3.83 g, 17.53 mmol) were added and stirred for overnight at room temperature. 1M HCl was added and the water layer was extracted with EtOAc six times. The combined organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 2 : 1), afforded compound **10b** (2.07 g, 94%).

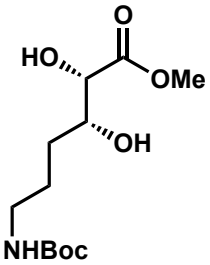
^{1}H NMR (300 MHz, $CDCl_3$): δ 4.73 (s, 1H), 3.66 (t, *J*=5.78 Hz, 2H), 3.15 (d, *J*=5.91 Hz, 2H), 2.24 (s, 1H), 1.58 (m, 4H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.19, 79.18, 62.31, 40.29, 29.70, 28.42, 26.59. HR-ESI-MS: Calculated for $C_9H_{19}NO_3$ $[M+Na]^+$ 212.1257, Observed: 212.1267.



(2E)-6-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hexenoic acid methyl ester (10c)

$(COCl)_2$ (765.6 μL, 8.93 mmol) was dissolved in 2.5 mL of dry DCM at -78 °C. To this solution, DMSO (792.5 μL, 11.16 mmol) in 3 mL dry DCM at -78 °C was added dropwise. After stirring 2 min, the solution of compound **10b** (844.7 mg, 4.46 mmol) in 5 mL of dry DCM at -78 °C was added dropwise. This solution was stirred further 15 min at -78 °C, then Et_3N (4.05 mL, 29.01 mmol) was added. After stirring 5 min, the yellow solution was allowed to warm up to room temperature. Methyl (triphenylphosphoranylidene) acetate (2.99 g, 8.93 mmol) was added to this solution, then stirred overnight. Water was added, and the water layer was extracted with chloroform three times. The organic layer was washed with brine, dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 1), afforded compound **10c** (852.6 mg, 78%).

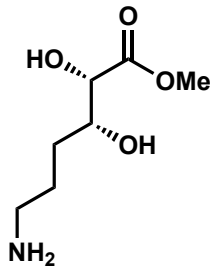
^{1}H NMR (300 MHz, $CDCl_3$): δ 6.95 (td, *J*=7.37, 14.74 Hz, 1H), 5.84 (d, *J*=15.67, 1H), 4.62 (s, 1H), 3.72 (s, 3H), 3.14 (d, *J*=6.33 Hz, 2H), 2.24 (dd, *J*=7.31, 14.60 Hz, 2H), 1.65 (dd, *J*=7.27, 14.54 Hz, 2H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 167.03, 156.04, 148.41, 121.58, 79.35, 51.52, 40.14, 29.56, 28.67, 28.50. HR-ESI-MS: Calculated for $C_{11}H_{21}NO_4$ $[M+Na]^+$ 266.1363, Observed: 266.1372.



6-[[(1,1-dimethylethoxy)carbonyl]amino]-(2*S*, 3*R*)-dihydroxy hexanoic acid methyl ester (10d)

1.87 g of ad-mix-β (containing, 4.01 mmol of $K_3Fe(CN)_6$, 4.01 mmol of K_2CO_3, 0.013 mmol of $(DHDQ)_2PHAL$, and 0.006 mmol of $K_2OsO_4•2H_2O$) was dissolved in the mixture of 3 mL of water and 3 mL of tBuOH. The solution was stirred for 30 min. Methansulfonamide (256.6 mg, 2.70 mmol) was added and cooled to 0 °C. Compound **10c** (306.6 mg, 1.34 mmol) in the mixture of 6 mL of water and 6 mL of tBuOH was added to this solution, then stirred for 30 hours at 0 °C. $Na_2S_2O_3$ (2.78 g) was added and stirred for a while. EtOAc was added and washed with NaOH two times. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure, afforded compound **10d** (145.0 mg, 66%).

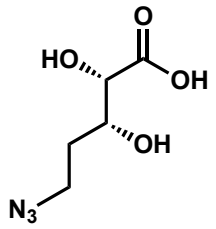
^{1}H NMR (300 MHz, $CDCl_3$): δ 4.81 (s, 1H), 4.09 (d, *J*=2.25 Hz, 1H), 3.91 (d, *J*=2.76 Hz, 1H), 3.14 (d, *J*=6.06 Hz, 2H), 1.63 (m, 4H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.87, 156.33, 79.34, 73.57, 72.30, 52.70, 40.30, 30.55, 28.43, 26.49. HR-ESI-MS: Calculated for $C_{12}H_{23}NO_6$ $[M+Na]^+$ 300.1418, Observed: 300.1430.



6-amino-(2*S*, 3*R*)-dihydroxy hexanoic acid methyl ester (10e)

Compound **10d** (60.5 mg, 0.230 mmol) was dissolved in 2 mL of 4N HCl in EtOAc. The solution was stirred for 3 h, the solvent was evaporated under reduced pressure, afforded crude compound **10e** as a HCl salt (41.8 mg, 91%).

^{1}H NMR (300 MHz, D_2O): δ 4.35 (d, *J*=2.55 Hz, 1H), 4.05 (dt, *J*=2.09 Hz, 3.54, 1H), 3.83 (s, 3H), 3.09 (t, *J*=7.3 Hz2, 2H), 1.90-1.65 (m, 4H). ^{13}C NMR (75 MHz, D_2O): δ 174.67, 73.39, 73.13, 71.56, 52.75, 39.33, 29.22, 29.09, 23.43. HR-ESI-MS: Calculated for $C_7H_{15}NO_4$ $[M+H]^+$ 178.1074, Observed: 178.1076.



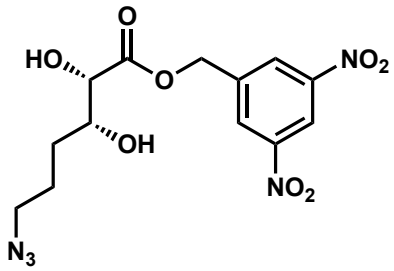
6-azide-(2*S*, 3*R*)-dihydroxy hexanoic acid (10)

TfN_3 solution in DCM (0.52mmol) was prepared as previously described.

Compound **10e** (41.8 mg, 0.21 mmol), K_2CO_3 (43.4 mg, 0.31 mmol), and $CuSO_4$•H_2O (0.5 mg, 2.09 μmol) were dissolved in 5 mL of water and 5 mL of MeOH. To this solution, freshly prepared TfN_3 solution (0.52 mmol) was added and stirred vigorously for 1 week. The additional TfN_3 solution (0.66 mmol) was added and stirred for 1 day. The solution was evaporated until MeOH was vaporized. DCM was added and the organic layer was washed with 1M HCl twice. The water layer was back extracted with DCM twice and EtOAc five times. The combined organic layer was dried over Na_2SO_4, filtrated, and evaporated under

reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 2), afforded compound **10** (15.1 mg, 38%).

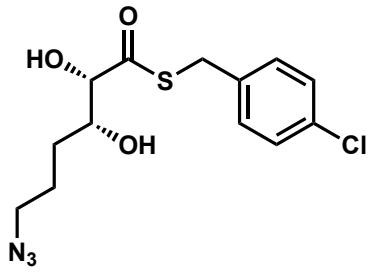
^{1}H NMR (300 MHz, CD_3OD): δ 4.04 (s, 1H), 3.88 (m, 1H), 3.75 (s, 1H), 3.34 (t, *J*=6.24 Hz, 2H), 1.90-1.65 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 175.01, 75.06, 73.28, 52.51, 31.67, 26.58. HR-ESI-MS: Calculated for $C_6H_{11}N_3O_4$ $[M-H]^-$ 188.0677, Observed: 188.0682.



6-azide-(2*S*, 3*R*)-dihydroxy hexanoic acid DBE ester (10-DBE)

Compound **10** (6.4 mg, 33.8 μmol) was dissolved in 1 mL of dry DMF. *N*,*N*-diisopropyl ethyl amine (8.8 μL, 50.8 μmol) and 2,4-dinitrobenzyl chloride (8.8 mg, 40.6 μmol) were added to this solution and stirred for 12 h. 1 M HCl and diethyl ether were added and the water layer was extracted with diethyl ether twice. The combined organic layer was washed with 1 M HCl followed by brine. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 2 : 1), afforded compound **10-DBE** (4.8 mg, 38%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 9.02 (d, *J*=2.01 Hz, 1H), 8.59 (d, *J*=1.95 Hz, 2H), 5.47 (dd, *J*=13.51, 26.47 Hz, 1H), 4.24 (q, *J*=2.37 Hz, 1H), 4.03 (d, *J*=6.30 Hz, 1H), 3.38 (t, *J*=6.32 Hz, 2H), 3.03 (d, *J*=5.52 Hz, 1H), 2.10 (d, *J*=8.85 Hz, 1H), 1.76 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 201.46, 135.63, 133.37, 130.37, 129.02, 77.36, 71.28, 53.44, 32.49. HR-ESI-MS: Calculated for $C_{13}H_{15}N_5O_8$ $[M-H]^-$ 368.0848, Observed: 368.0860.



6-azide-(2*S*, 3*R*)-dihydroxy hexanoic acid CBT ester (10-CBT)

Compound **10** (9.5 mg, 50.2 μmol) and 4-chlorobenzyl mercaptan (6.53 μL, 50.2 μmol) were dissolved in 400 μL of 1,4-dioxane. DCC (8.6 mg, 41.9 μmol) in 800 μL of EtOAc was added to this solution, then stirred for overnight. The reaction mixture was filtered with celite, then the solvent was evaporated under reduced pressure. EtOAc was added, and the organic layer was washed with 1M HCl twice, sat. $NaHCO_3$ aq. twice, and brine. The organic layer was dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude product was chromatographed on silica gel (hexane / EtOAc = 1 : 1), afforded compound **10-CBT** (1.8 mg, 11%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.26 (m, 4H), 4.12 (d, *J*=3.45, 3.54, 2H), 4.03 (m, 1H), 3.36 (m, 2H), 1.71 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 202.34, 135.69, 133.53, 130.37, 129.00, 79.90, 72.28, 51.40, 32.44, 30.91, 25.39. HR-ESI-MS: Calculated for $C_{13}H_{16}N_3O_3ClS$ $[M-H]^-$ 328.0528, Observed: 328.0519.

Chapter 4 Total synthesis of a peptide inhibitor containing a statine

4.1. Introduction

Aspartic proteases, which catalyze hydrolytic cleavage of peptides/proteins, play important roles in many biological functions, including cancer cell proliferation and virus infection. Therefore they are regarded as therapeutic targets[144,145]. Among all, β-secretase protein BACE1 is a famous protease related to Altzheimer's disease[146–148]. The amyloid precursor protein is processed by BACE1 to produce amyloid-beta peptide. The aggregation of overexpressed peptides stimulates the signaling cascade that causes neuritic injury. Based on this mechanism, studies to inhibit BACE1 are considered to lead the development of drugs toward Altzheimer's disease.

In Chapter 3, I have demonstrated that the developed post-translational chemical modifications could produce various γ-peptide moieties including statine and its analogue in ribosomally synthesized peptides. The results promoted us to design and synthesize a peptide inhibitor for the demonstration of versatility of the established reactions. Here, I aimed at the total synthesis of P10-P4'statV peptide, which comprises of 13 proteinogenic α-amino acids, a statine residue, and free N-terminus[149]. This peptide was designed based on the substrate peptide of BACE1, where the amino acid exist in the cleavable site was substituted with the statine residue. P10-P4'statV is known as a strong inhibitor of BACE1 protease with IC_{50} ~30 nM. This peptide was initially synthesized by the solid-phage peptide synthesis method,

When it comes to the synthesis of P10-P4'statV via *in vitro* translation, however, not only the incorporation of statine but also the production of free N-terminus are problematic. Because *in vitro* translation system utilizes translational proteins derived from *E. coli.*, the initiation event of translation requires the N-acylated moiety to start translation[85,150]. The FIT system was utilized for the investigation of substrate acceptability at the initiator position, but all of the results demonstrated the necessity of the N-acylated substrates. To date, although the FIT system achieved the production of free N-terminus by combining an

enzymatic reaction, substrate preference of the enzyme did not allow us to synthesize free Lys[87].

Therefore, another strategy for the synthesis of free N-terminus of P10-P4'statV is essential. In this study, I introduced a *p*-nitro benzyl protecting (*p*NZ-) group into the peptide N-terminus, and conducted the water-friendly posttranslational deprotection with sodium hydrosulfite after translation[151] (**Figure 4.1**). Sodium hydrosulfite selectively reduced the nitro group of *p*NZ, followed by the deletion of a qunonimine methide and a carbon dioxide. After producing the free N-terminus, the developed posttranslational chemical modification reactions convert **2** to statine. In this chapter, I demonstrated that the introduction of *p*NZ protection achieved the total synthesis of P10-P4'statV peptide.

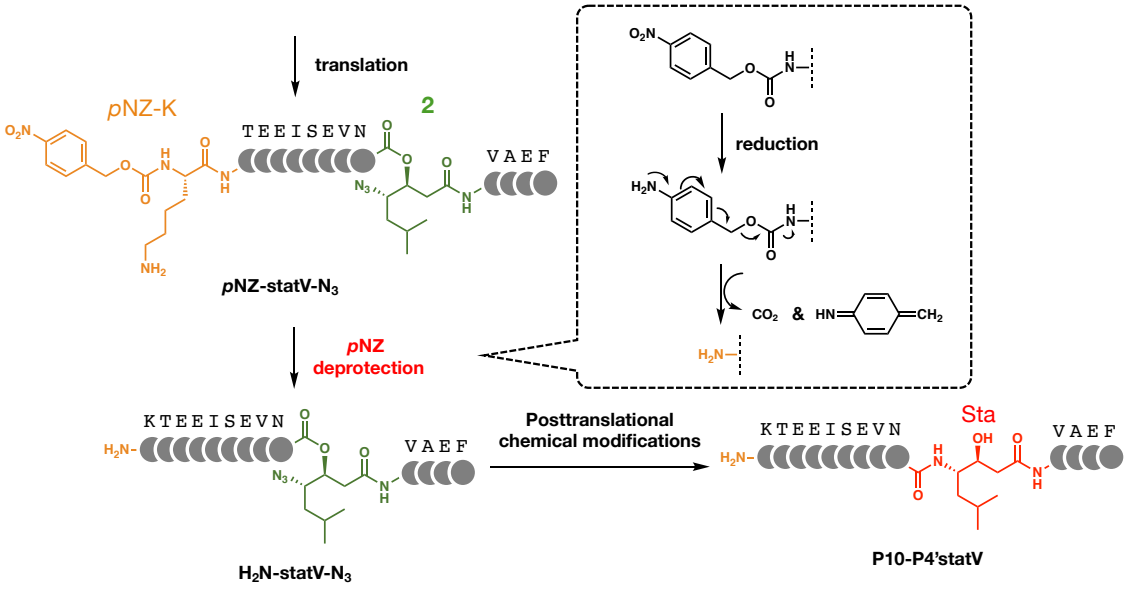


Figure 4.1 | Strategy for total synthesis of P10-P4'statV peptide

4.2. Results and discussion

4.2.1. Synthesis of P10-P4'statV

I prepared template DNA based on the sequence of P10-P4'statV, and assigned a *p*NZ-protected lysine as an initiated amino acid, and an azide-statine (**2**) at the position of statine in the P10-P4'statV peptide respectively (**Figure 4.2a**). After translation by the FIT system, the LC-MS spectrum detected the peak corresponding to the mass of full-length peptide ***p*NZ-statV-N_3** (**Figure 4.2b**). With this peptide, the N-terminal *p*NZ group was firstly deprotected by incubating with sodium hydrosulfite. XIC spectra after the reaction showed that the deprotection completed within 30 min to produce N-terminal free peptide **H_2N-statV-N_3** without producing any expected byproducts. This peptide was further applied to chemical modification reactions without any purifications, resulting in the generation of a peptide bearing the same mass with P10-P4'statV. To confirm the synthesis of the desired peptide, a commercially available P10-P4'statV as a positive control was also analyzed in the same conditions. These XIC spectra showed the identical retention time, indicating the synthesis of P10-P4'statV peptide was achieved via the *p*NZ deprotection and chemical modification reactions.

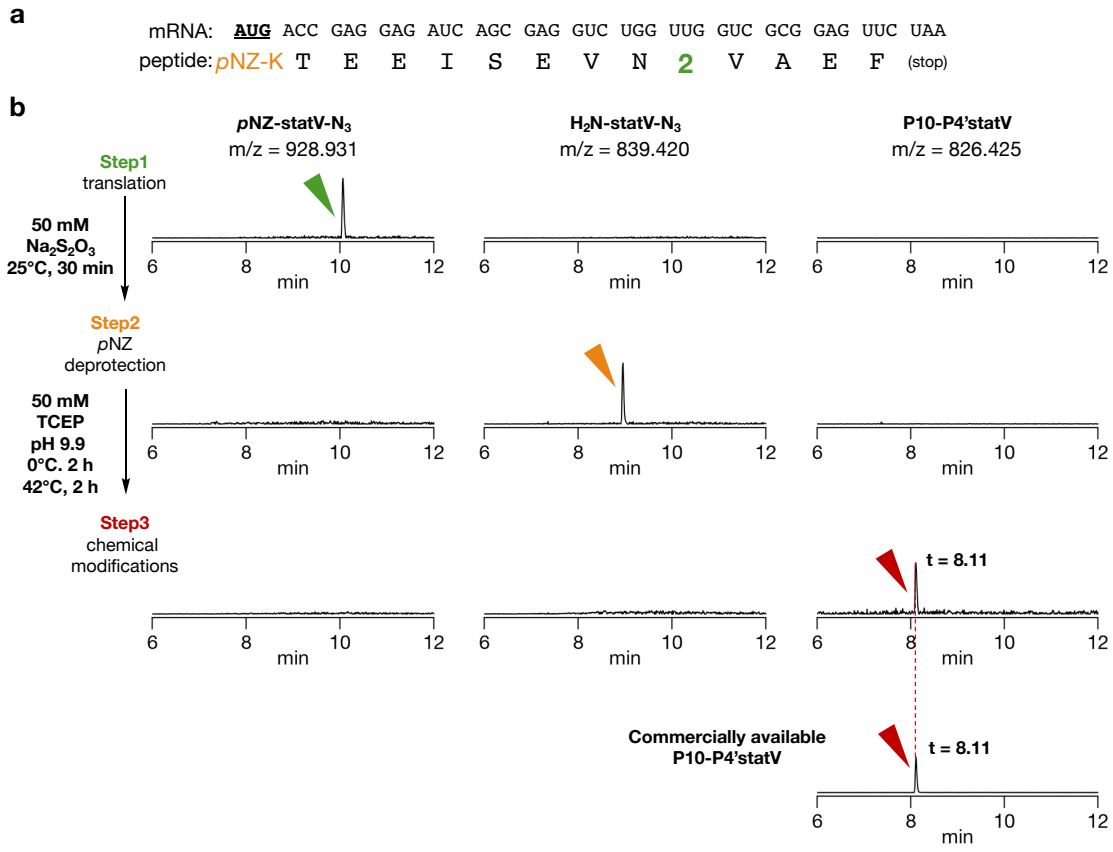


Figure 4.2 | Total synthesis of P10-P4'statV by *p*NZ deprotection and chemical modifications

(a) mRNA sequence and correspond peptide sequence for synthesis of P10-P4'statV.

(b) XIC spectra of peptides at each reaction steps. The rows show each reaction steps and the columns show the expected peptides.

4.2.2. LC-MS/MS analysis of synthesized P10-P4'statV

The synthesized peptide and the positive control shown in the XIC of **Figure 4.2** were further subjected to LC-MS/MS study to obtain further evidences that our reactions could achieve the total synthesis of P10-P4'statV. LC-MS/MS spectra were obtained from both of the synthesized peptide and the positive control, showing the completely identical patterns (**Figure 4.3a-c**). The fragments containing a statine residue were well-assigned, indicating that the peptide had the desired sequence including statine residue.

Furthermore, the most characteristic peak was the unique a-ion fragments, which are cleaved between Val and Ala next to statine residue. It was reported that the MS/MS spectrum of pepstatin A also showed the a-ion fragmentation next to the statine residue (**Figure 4.3d**)[152]. The fragmentation pattern was plausibly derived from the structure of statine, where a β-hydroxy group stabilized the fragmented a-ion (**Figure 4.3e**). Such a-ion fragmentation also strongly supported the existence of a statine residue in the synthesized peptide. All the results shown here demonstrated that the total synthesis of P10-P4'statV was achieved by combining the translation system, the posttranslational modifications, and the deprotection.

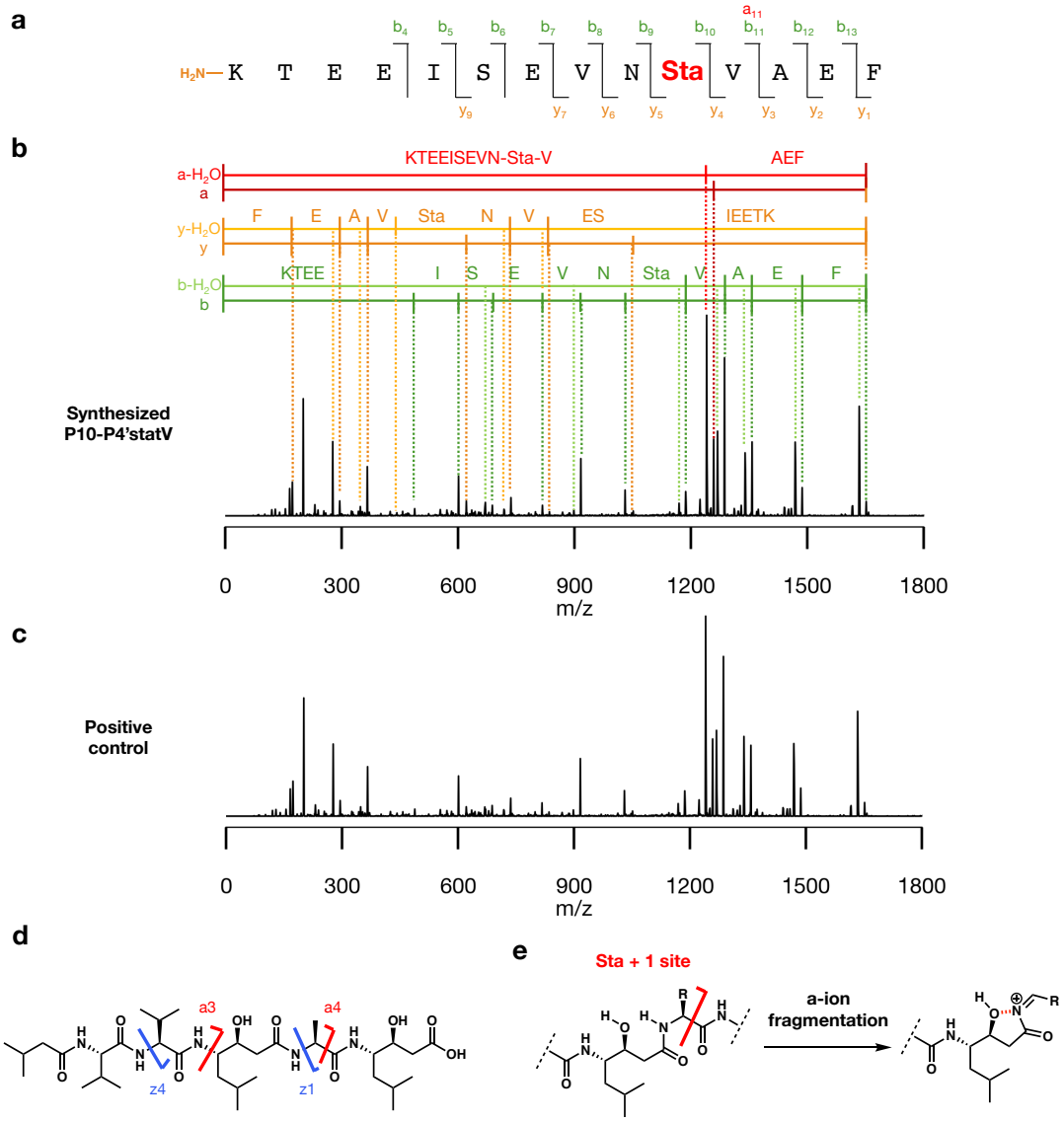


Figure 4.3 | LC-MS/MS analysis of P10-P4'statV peptides

(a) Summary of observed fragmentation patterns.

(b) LC-MS/MS spectrum of the synthesized peptide and peak assignments.

(c) LC-MS/MS spectrum of the commercially available P10-P4'statV.

(d) Reported a- and z-ion fragmentation patterns in the LC-MS/MS study of pepstatin A.

(d) Plausible mechanism to fragmentate a-ion cleaved at statine + 1 site.

4.3. Conclusion

In this study, to synthesize P10-P4'statV, *p*NZ protecting group and posttranslational deprotection strategy were introduced. The comparison of LC-MS and LC-MS/MS spectra between the synthesized peptide and the positive control was conducted. All of the results shown in this chapter demonstrated that the total synthesis of P10-P4'statV was achieved by means of *in vitro* translation system, posttranslational deprotection of *p*NZ group, and chemical modification reactions to generate a statine residue.

This study showed that the developed posttranslational chemical modifications were compatible with the total synthesis of the peptide inhibitors bearing a statine residue. The development of peptide inhibitors including P10-P4'statV often utilize the chemical synthesis, which is laborious and difficult to derivatize the products. Since the synthesis described here utilized the translation system, the derivatization of the products, or the modification of peptide sequences are easily achieved by just changing the mRNA template and the codon assignments. The studies carried out here would be the milestone for the rational synthesis of various peptide inhibitors. This study is also valuable to suggest that *in vitro* translation system is applicable for total synthesis of peptidic compounds, where chemical synthesis is mainly utilized.

4.4. Material and methods

・Materials

All chemicals and reagents were purchased from Tokyo Chemical Industry, Kanto Chemical, Nacalai Tesque, Sigma-Aldrich Japan, Watanabe chemical Industry, or Fujifilm Wako Pure Chemical Industries. All DNA oligomers were purchased from Eurofin Genomics or GeneDesign. P10-P4'statV peptide was purchased from Peptide Institute. *p*NZ-protected lysine is the gift from Dr. Yichao Huang.

・List of DNA oligomers

Primer	Sequence
P10-P4statV_WL_a.R51	GAACTCCGCGACCAACCAGACCTCGCTGATCTCCTCGGTCATGTTTTTCTC
P10-P4statV_WL_b.R33	CGAAGCTTAGAACTCCGCGACCAACCAGACCTC
Pro1E2#3CAA_Leu.R62	TGGCGGGTGATAGGGGATTCGAACCCCTGACCCCTTCGTTTTGAGCGAAGTGCGCTACCAGG

・Combination of DNA primers

Name	Extension	1st PCR	2nd PCR
DNA template	T7eSD6M.F46 P10-P4statV_WL_a.R51	-	T7ex5.F22 P10-P4statV_WL_b.R33
$tRNA^{Pro1E2\#3}_{caa}$	Pro1E.F50 Pro1E2#3CAA_Leu.R62	-	T7ex5.F22 Pro1E2#3-OMe.R20

・**Preparation of *p*NZ-Lys-tRNA^{ini}, Asn-tRNA$^{Pro1E2\#3}_{cca}$, and 2-tRNA$^{Pro1E2\#3}_{caa}$**

tRNA$^{Pro1E2\#3}_{caa}$ was prepared in the same manner as tRNA$^{Pro1E2\#3}_{cug}$.

*p*NZ-Lys-tRNA^{ini} was prepared in the same manner as ClAc-L-Tyr-tRNA^{ini}. The acylation reaction was conducted for 24 h in pH 10.0 with 300 mM of $MgCl_2$. Asn-tRNA$^{Pro1E2\#3}_{cca}$ was prepared by using 25 mM of Asn-DBE, dFx and tRNA$^{Pro1E2\#3}_{cca}$. The acylation reaction was conducted for 2 h in pH 7.5.

・**Translation**

In addition to translation mixture (10% (v/v) DNA template, 0.5 mM amino acid mixture (T, E, I, S, V, A, and F), 10.9% (v/v) solA, 12% (v/v) solB, 25 μM acylated-tRNAs, and 200 μM NaOAc), 5 μM EF-P was added to this mixture. The mixture was incubated at 37°C for 30 min.

・**Posttranslational deprotection of *p*NZ group and chemical modification reactions**

To the 2.5 μL of translation mixture, 2.5 μL of 100 mM $Na_2S_2O_3$ was added. The reaction mixture was incubated at 25℃ for 30 min. The reactants were used for the next steps without further purifications. The samples for LC-MS study were prepared by adding 15 μL of 5% TFA aq..

The reaction mixture was incubated on ice for 1 min. To the mixture, the cooled solution containing 1 vol. of 250 mM TCEP in pH 9.9 and 4 vol. of 500 mM Na_2HPO_4-NaOH buffer at pH 9.9 was added. The reaction mixture was incubated at 0℃ for 2 h, and 42℃ for 2 h.

・**LC-MS and LC-MS/MS analysis**

5 μL of the each samples was injected to LC-MS. LC-MS study was conducted with the gradient (0.01% formic acid in water : 0.1% formic acid in acetonitrile) = 99:1 to 40:60 within 10 min. The same samples were further subjected to MS/MS analysis. To the targeted peaks, the peptides were fragmented by 25V of collision energy and the daughter ions were detected. The MS/MS spectra were deconvolutied and the spectra were further analyzed by

the software (MassLynx).

As the control sample, commercially available P10-P4'statV was dissolved in water to adjust the concentration to 200 nM. To 1 μL of solution, 19 μL of 5% TFA aq. was added (f.c. 10 nM) and 5 μL of the sample was analyzed by both of LC-MS and LC-MS/MS.

Chapter 5 General conclusion

In summary, I have developed a novel synthetic methodology to produce various γ- and δ-peptides including statines in ribosomally expressed peptides by combining Staudinger reaction and *O*-*N* acyl transfer reaction. As described in Chapter 1, these structures synthesized in my studies are important building blocks for the discovery of novel bioactive peptides.

In Chapter 2, I demonstrated the posttranslational chemical modification reactions are able to convert the γ-azide-β-hydroxy acid structure into the correspond γ-peptide. During the development of reactions, although I also found the formation of byproduct which is quite specific to my reactions, I succeeded to suppress the byproduct formation with almost quantitative conversion yield.

The investigation of the substrate scope was conducted in Chapter 3. I found not only the statine and phenylstatine structure but also various γ- and δ-peptides are incorporated into ribosomally expressed peptides, demonstrating the generality of developed reactions. Because statine and phenylstatine are interesting structure for their inhibitory contribution, the developed reactions are useful for the synthesis of newly designed bioactive peptides. In addition, the ester exchange methodology is proven to be the good strategy to improve the conversion yield. The results implied me that the posttranslational chemical modification reactions are also applicable to synthesize polyketide-like hydrophilic carbon chains.

Finally, total synthesis of a peptide inhibitor P10-P4'statV was achieved in Chapter 4 by introducing the protecting group to generate free N-terminus. I demonstrated that the two steps reactions worked as expected, and produce P10-P4'statV peptide from the precursor peptide. The total synthesis in this study suggested that our methodology enables the rational design of statine-containing peptides. Because the developed reactions utilized translation system, changing the mRNA template would produce another statine containing peptides. Therefore, the reactions developed in this study would enable the rational design of peptide inhibitor and accelerate the discovery of novel bioactive peptides. Furthermore, our reactions would be applicable for construction of natural peptide library which randomly contains statine moieties. The combination to display technology would lead the easily discover of bioactive peptides.

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List of achievements

【Publication(s) related to the thesis】

1. ” Studies of Intramolecular *O*-to-*N* Acyl Transfer Reaction toward Production of Peptides Containing γ-Peptide Moieties”, Tomohiro Kuroda, Yuki Goto, Hiroaki Suga, *Peptide Science 2017*, **2018**, 52-53

【Publication(s) not related to the thesis】

1. 黒田 知宏, 後藤 佑樹, 菅 裕明 「遺伝暗号リプログラミングによる人工翻訳系の創製」 人工細胞の創製とその応用 第 4 章 第 3 節 シーエムシー出版 P.162－P.171.
2. “Chemoenzymatic posttranslational modification reactions for the synthesis of ψ[CH_2NH]-containing peptides”, Yasuharu Kato, Tomohiro Kuroda, Yichai Huang, Risa Ohta, Yuki Goto, Hiroaki Suga, *Angew. Chem. Int. Ed.,* **2019**, *58*, 1-6.

【Oral presentation】

1. “Attempt for the formation of carbon–carbon bond in ribosome” Tomohiro Kuroda, Ryo Takatsuji, Yuki Goto, Hiroaki Suga, 日本化学会第 96 回春季年会, 京都, 2016 年 3 月
2. “*in vitro* biosynthesis of peptides containing alkyl–backbone via post-translational acyl–transfer reaction” Tomohiro Kuroda, Yuki Goto, Hiroaki Suga, 日本化学会第 97 回春季年会, 神奈川, 2017 年 3 月
3. “*In vitro* synthesis of peptides containing carbon–backbone via post-translational acyl transfer reaction” Tomohiro Kuroda, Yuki Goto, Hiroaki Suga, 第 54 回ペプチド討論会, 大阪, 2017 年 11 月

【Poster presentation】

1. “リボソームによる炭素-炭素結合形成を指向した新規翻訳基質の開発”, 黒田 知宏, 高辻 諒, 後藤 佑樹, 菅 裕明, 日本化学会 第 96 春季年会, 京都, 2016 年 3 月
2. “Development of post-translational acyl-transfer reactions toward formation of peptides with alkyl-backbone”, Tomohiro Kuroda, Yuki Goto, Hiroaki Suga, 日本化学会 第 97 春季年会, 神奈川, 2017 年 3 月
3. “炭素主鎖骨格含有ペプチドの *in vitro* 合成を指向した翻訳後アシル転位反応の開発”, 黒田 知宏, 後藤 佑樹, 菅 裕明, 第 2 回生合成リデザイン若手シンポジウム, 群馬, 2017 年 8 月
4. “翻訳後アシル転移反応による炭素主鎖骨格含有ペプチドの合成”, 黒田 知宏, 後藤 佑樹, 菅 裕明, 第 5 回バイオ関連化学シンポジウム若手フォーラム, 東京, 2017 年 9 月

5. “炭素主鎖骨格含有ペプチドを合成する翻訳後アシル転位反応の開発”, 黒田 知宏, 後藤 佑樹, 菅 裕明, 第 11 回バイオ関連化学シンポジウム, 東京, 2017 年 9 月
6. “Development of post–translational acyl transfer reaction toward *in vitro* synthesis of peptides with carbon–backbone”, Tomohiro Kuroda, Yuki Goto, Hiroaki Suga, 8th Annual Indian Scientists Association in Japan (ISAJ) Symposium, Tokyo, December 2017
7. “Post-translational acyl transfer reaction on the backbone of peptide”, Tomohiro Kuroda, Yuki Goto, Hiroaki Suga, ETH Zurich－The University of Tokyo Joint Symposium, Zurich, Switzerland, January 2018
8. “Post-translational acyl transfer reaction on the backbone of peptide”, Tomohiro Kuroda, Yuki Goto, Hiroaki Suga, The 26th ZESTY network seminar, Tokyo, July 2018
9. “Development of post-translational acyl transfer reactions for generation of γ/δ-peptide linkages in ribosomally synthesized peptides”, Tomohiro Kuroda, Yuki Goto, Hiroaki Suga, 10th international peptide symposium, Kyoto, December 2018
10. “翻訳後化学修飾反応によるオキサゾール環含有ペプチド合成法の開発”, 堤見 遥, 黒田 知宏, 後藤 佑樹, 菅 裕明, 日本ケミカルバイオロジー学会第 14 回年会, 名古屋, 2019 年 6 月

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