学位論文 (要約)

Development of chemical post-translational modification reactions on azoline-containing peptides expressed in a reconstituted cyclodehydratase-coupled translation system

(試験管内生合成されたアゾリン含有ペプチドの化学的翻訳後修飾反応の開発)

平成 28 年 12 月　博士（理学）申請

東京大学大学院理学系研究科
化学専攻
加藤　保治

Abstract (abridged)

Backbone modifications such as azoles, azolines and azolidines are often seen in bioactive peptidic natural products. Since these heterocyclic structures would provide peptides with several characteristics such as rigid global conformation, hydrogen bonding sites with fixed orientations, improved proteolytic resistance, and cell membrane permeability, they are important motifs of the bioactive peptides and the backbone modifications would be a general strategy for organisms to develop a wide variety of bioactive peptides.

Our laboratory has previously developed an *in vitro* biosynthesis system for azoline-containing peptides by integrating the Flexible *In vitro* Translation (FIT) system and a post-translational cyclodehydratase, PatD, which is involved in the biosynthesis of patellamides. In the *in vitro* biosynthesis system, referred to as FIT–PatD system, Cys/Ser/Thr residues involved in translated peptides are modified by PatD to the corresponding azoline moieties, allowing one-pot synthesis of azoline-containing peptides. We have revealed an unprecedented substrate tolerance of PatD and demonstrated that this system can be applied for diverse azoline-containing peptides.

In order to expand structural diversity, which is accessible by *in vitro* translation and subsequent post-translational modifications, in my doctoral studies, chemical methodologies were developed, which generate peptides containing a wide range of backbone modifications by the integration of enzymatic cyclodehydration by PatD and chemical modifications.

In chapter 2 and chapter 3, the development of *in vitro* synthetic method of azole-containing peptides and azole-containing peptides are described, respectively. *(For the reasons of patent applications and future publications, details are closed in the following parts.)*

In conclusion, in this research I have developed novel methods involving the post-translational enzymatic cyclodehydration by PatD and chemical modifications on ribosomally synthesized peptides to yield peptides with various backbone modifications. The post-translational modification method developed here would provide unique structural scaffolds into peptides, which possibly leads to the development of novel bioactive peptides.

Table of contents

Chapter 1 General introduction

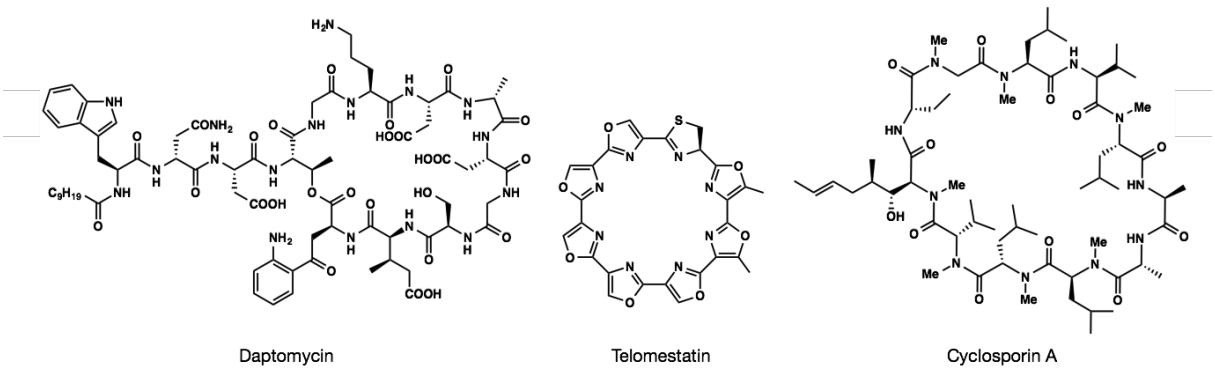
1.1 Non-standard peptides as drug candidates for protein–protein interactions (PPIs) and peptidic natural products as a source of non-standard peptides

Protein–protein interactions (PPIs) have emerged as challenging targets of great importance in chemical biology and medicinal chemistry.[1-7] It has been argued that PPIs are difficult to be targeted by small molecules, which are mainly employed in drug discovery today[8] because of larger interaction surface area involved in PPIs (1500–3000Å) compared to protein-small molecule interaction surface area (300–1000Å).[9] In addition, though antibodies can actually modulate PPIs, they cannot target intracellular proteins of therapeutic interest because they cannot penetrate cell membrane. Although intensive studies have recently provided successful examples of targeting PPIs by small molecules,[7] it is still challenging to develop small molecule PPI modulators and non-standard peptides have been getting much attention as candidates of drugs and bioactive molecules for modulation of intracellular PPIs.[10, 11]

Since detailed mechanism of PPIs can be attributed to the interactions between peptides embedded in proteins of interest, peptides would be the most natural approach to modulate PPIs by mimicking proteins.[12,13] Moreover, the fact that peptidic natural products exemplified by cyclosporin A (**Figure 1.1**), which is far from the rule of thumb on bioavailability of small molecules (or Lipinski's rule of five[14]) can penetrate cell membrane[15], encouraged the utilities of non-standard peptides as molecular scaffold for the development of novel bioactive peptides. (Note that "non-standard peptides" here denote the peptides containing "non-standard structures", which is not found in linear peptides composed of 20 kinds of proteinogenic amino acids.) Although peptides generally can not permeate cell membrane, a number of non-standard structures in cyclosporin A such as non-proteinogenic side chains, D-amino acids, *N*-methylation, and macrocyclic structure would cooperatively contribute to the membrane permeability of cyclosporin A.

In addition to above-mentioned non-standard structures found in cyclosporin A, peptidic natural products in general exhibit huge structural diversity, which is reviewed in the following part. This structural diversity must be advantageous for the development of novel bioactive peptides,

however, alternative approaches than "natural product-based drug development" would be desired for the development of peptides against some targets of therapeutic interest. Except for antimicrobial agents, the activities of peptidic natural products would have nothing to do with human diseases and the production of such molecules would not be the selective pressure in the process of evolution. The immunosuppressant activity of cyclosporin A would be just a product of chance. Thus, for the development of novel bioactive peptides with non-standard structures, peptides, which possess natural product-like structures, but as a whole, structurally different from original natural products would be required.



Figrue 1.1 Examples of peptidic natural products biosynthesized by non-ribosomal peptide synthetases (NRPS).

1.2 The development of bioactive peptides by rational design and high-throughput screening

In general, mainly two approaches can be employed for *de novo* development of bioactive peptides. First, PPI modulator can be rationally designed based on proteins of interests.[12] For example, based on co-crystallization structures, mimicry of peptide strands in either of proteins involved in targeted PPIs can be synthesized.[7] The designed peptides can interact with the targeted proteins and thus competitively inhibit the targeted PPIs. By the aid of computational analysis, which predict regions of proteins, which are critical for PPIs, constrained peptides can be also designed.[16] However, the potential problems of these approaches are; (i) crystal structures are required for the structure-based design, (ii) it would be generally difficult to obtain stronger affinity against a target protein than that of the native counterpart protein, (iii) it is difficult to design peptides, which bind to unexpected sites or exhibit PPI modulation in allosteric mechanism and (iv) it is difficult to design peptides based on a non-structured binding region, which is also prevalent in PPIs.[17]

The other approach is construction of peptide library and subsequent screening of active peptides from the library (**Figure 1.2**). This method has actually isolated (i) binding peptides against target proteins, which crystal structure was not solved,[18] (ii) binding peptides with pM affinities,[19] (iii) active peptides, which bind target proteins at unexpected sites and regulate PPIs in an allosteric manner.[20] (iv) Additionally, since this screening approach does not mimic naturally occurring binding peptide strands, no structured binding region is required.

In *de novo* development of bioactive peptides by high-throughput screening approach, the diversity of peptide libraries is highly important. Antibodies would be a good reference of such relationship between diversity and activity. Since antibodies can strongly bind to a specific target molecules, they have been widely used in biological research and clinically.[21] These characteristics are the basis of fundamental molecular detection techniques such as ELISA[22, 23] and western blotting[24-26] in biological research, and therapeutic antibodies is growing modality for drug development.[8]

In general, therapeutic antibodies have low nM order of dissociation constants (K_D). For example, K_D values are 2.6–3.1 nM for nivolumab[27, 28], 30–550 pM for adalimmub[29-31], 21 pM–9.1 nM for infliximab[29, 30], 160 nM for rituximab[32] and 2.2–16.6 nM for bevacizumab[33, 34].

On the other hand, the diversity of antibodies has been estimated[35, 36] as $>10^{10}$, suggesting that highly diverse peptide library may provide active peptides which bind to target molecules of interest with strong affinity comparable to antibodies. Actually, screening approach using genetically encoded peptide library has been yielded a number of active peptides showing antibody-like activities.[37]

In short conclusion, construction of non-standard peptide library and a subsequent screening have great advantages for the development of bioactive peptides. In order to accomplish this approach, facile synthetic methods are required for the construction of highly diverse (10^{10}–10^{11}) non-standard peptide libraries.

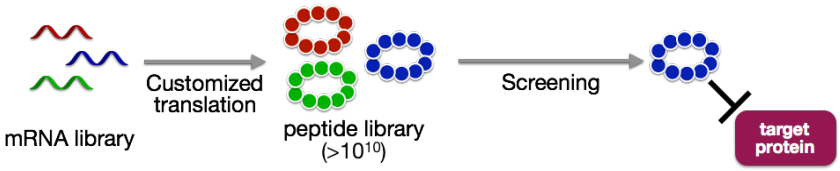


Figure 1.2 Schematic illustration of the screening for bioactive peptides. In this figure, mRNA display-mediated screening of bioactive peptides is illustrated.

1.3 Chemical synthesis and NRPS-mediated biosynthesis of non-standard peptides

Both chemical synthesis and enzymatic synthesis can be employed for the synthesis of non-standard peptides. By organic synthesis, various non-standard structures can be introduced, and structures can be controlled at atomic level. However, the diversity of peptide library is at most 10^8 even with combinatorial methods.[38] On the other hand, biosynthetic machinery can also be employed for the synthesis of non-standard peptides. A number of peptidic natural products including cyclosporin A are biosynthesized by non-ribosomal peptide synthetases (NRPS) (**Figure 1.1**). In general, peptidic natural products biosynthesized by NRPS exhibit huge structural diversity and several peptides are clinically used.[39] For example, cyclosporin A contains a number of non-standard structures, and being an orally available immunosuppressant. *Although in vitro* reconstitution and engineering of NRPS have been investigated and made success in the synthesis of natural product analogs[40, 41], the property of NRPS that several huge proteins are responsible for the polymerization of amino acids as well as modification reactions[42] has limited the diversity of natural product analogs synthesized by engineered NRPS and thus application of NRPS in the *de novo* development of bioactive peptides.

1.4 Post-ribosomal peptide synthesis (PRPS) for non-standard peptide biosynthesis

In addition to NRPS-mediated biosynthesis, many peptidic natural products are biosynthesized via ribosomal pathway. Such peptidic natural products with ribosomal origin are called "ribosomally synthesized and post-translationally modified peptides (RiPPs)" and examples are shown in **Figure 1.3**. The biosynthetic pathways of RiPPs are called "post-ribosomal peptide synthesis (PRPS)",[43] and PRPS are found in all the three domains of life, comprising universal biosynthetic pathway of peptidic natural products.[43]

Biosynthesis of RiPPs by PRPS literally initiates with ribosomal expression, or translation of a longer precursor peptide (**Figure 1.4**). Precursor peptides typically composed of 20–110 amino acid residues, and a region, which is processed to yield final natural product is named "core peptide" In some cases, multiple core peptide regions are encoded in a single precursor peptide (e.g. patellamides). In most precursor peptides of RiPPs, the core peptide is flanked by a N-terminal "leader peptide", which is usually important for the post-translational modifications and export.[44] In the case of biosynthetic pathway of bottromycins, the leader peptide is not at N-terminus but at C-terminus and thus called "follower peptide". In addition, the core peptide is followed by a C-terminal "recognition sequence" in some cases, which is recognized by a protease of macrocyclase (e.g. patellamides). In the presence of the leader peptide and several other regions, core peptides are extensively modified by a series of post-translational modifications, which is the basis of the structural diversity of RiPPs.

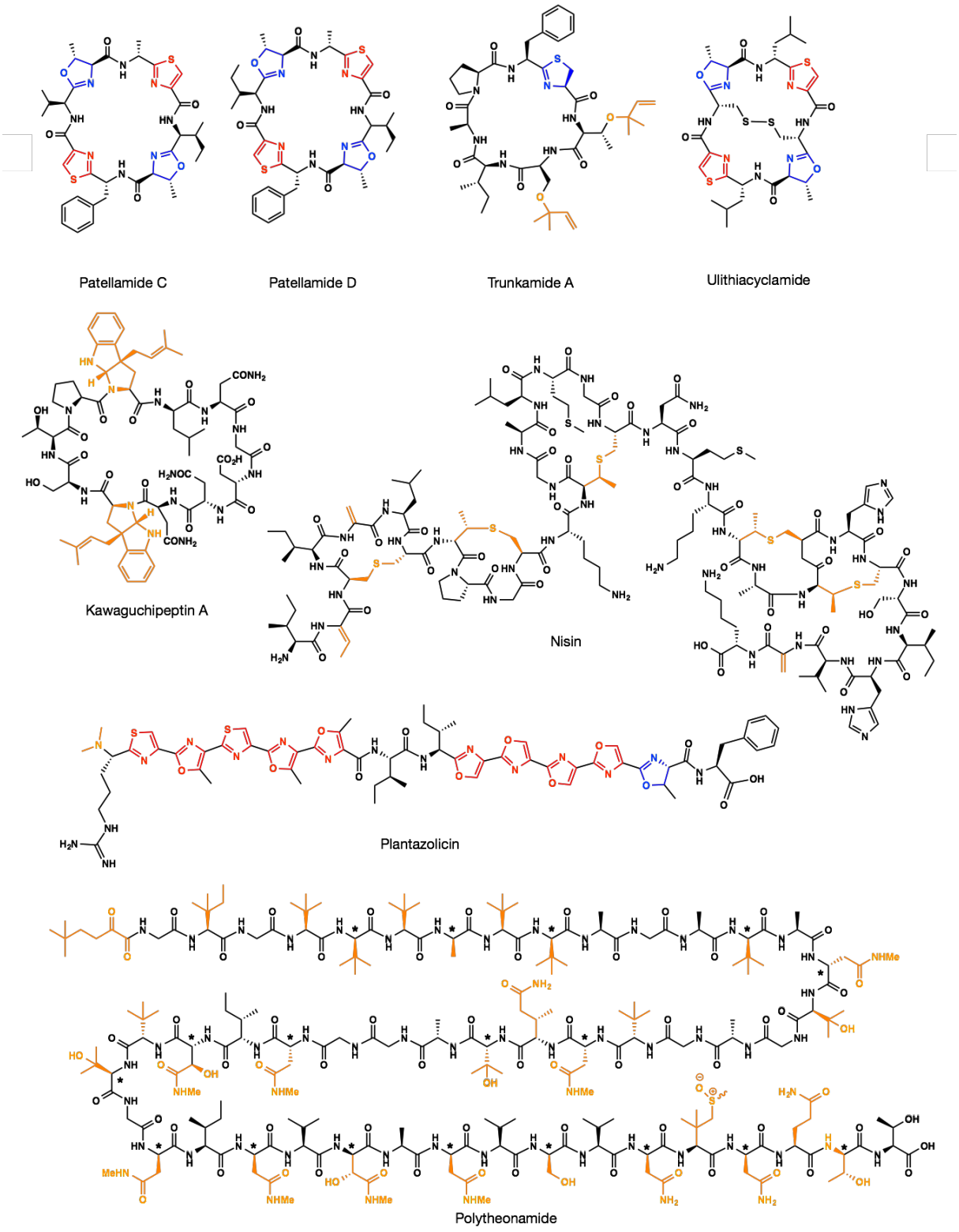


Figure 1.3 Examples of ribosomally synthesized and post-translationally modified peptides (RiPPs). Azolines, azoles and other non-standard structures are shown in blue, red and orange, respectively. In the structure of polytheonamide, amino acid residues with D-configuration are labeled with asterisk (*).

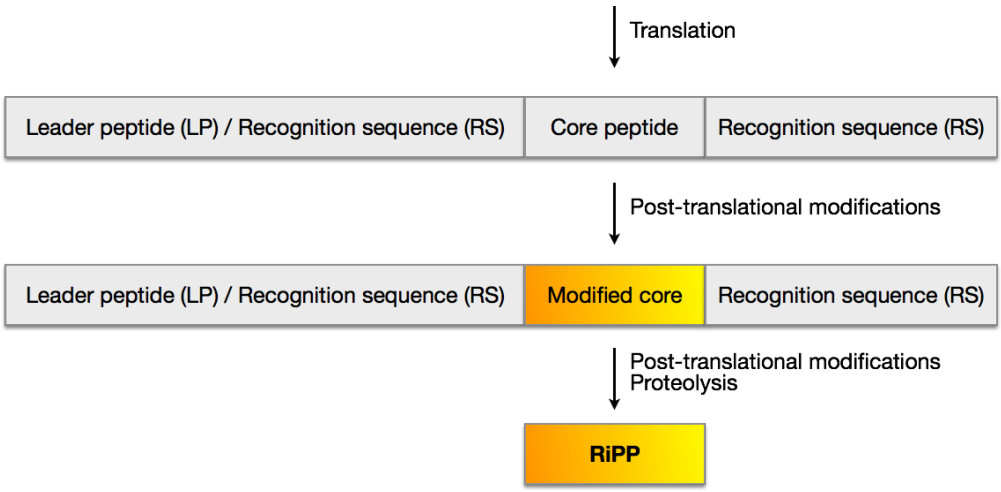


Figure 1.4 Schematic irustration of post-ribosomal peptide synthesis (PRPS): Biosynthetic pathway of ribosomally synthesized and post-translationally modified peptides (RiPPs).

1.5 Structural diversity in ribosomally synthesized and post-translationally modified peptides (RiPPs)

1.5.1 Modifications at side chain of precursor peptides

Structures found in ribosomally synthesized and post-translationally modified peptides (RiPPs) are diverse,[45] and they can be divided into modification of (i) side chains, (ii) main chains, and (iii) side chains and main chains. In the following text, structural diversity of post-translational modifications is reviewed.

Dehydration

One prevalent modification found in RiPPs is dehydration of Ser and Thr residues resulting in dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Incorporation of Dha and Dhb can contribute to structural rigidity and protease resistance of peptides.[46] In addition, Dha and Dhb endow peptides with reactivity against sulfhydryl and amine groups. For example, natural product nisin[47] (**Figure 1.3**), which exhibit antibacterial activity has Dha and Dhb residues, suggested to be michael acceptor reactive to membrane sulfhydryl groups of bacteria.[48]

Dha residues can also react with intramolecular sulfhydryl groups. Micahel addition between Dha residues and cysteine residues form lanthionine, which is defined as two alanine residues bridged by thioether bond. (In the case of Dhb, intramolecular micahel addition provide methyl lanthionine. Both of the thioether bridge can be seen in the structure on nisin shown in **Figure 1.3**) Another derivatization of dehydro amino acids is formal [4 + 2] cycloaddition, which is involved in biosynthesis of pyridine ring formation of thiopeptides. For example, that type of cycloaddition was biochemically demonstrated with TclM[49]. Additionally, michael addition of dehydro amino acid residues and oxidatively decarboxylated C-terminal cysteine residues forms similar thioether bridge with aminovinyl cysteine. Examples can be seen in epidermin[50] and cypemycin[51, 52]. Another variation is lysinoalanine, which is derived from dehydro amino acid residues and lysine residues exemplified by duramycin[53].

Epimerization

Another example of side chain modification is epimerization. For example, epimerization takes place in the biosynthesis of polytheonamides (**Figure 1.3**).[54, 55] Although epimerization itself does not provide peptides with new functional group, stereochemical conversion can contribute to protease resistance of peptides.[56] (Polytheonamides contain D-amino acids with non-proteinogenic side chains as well as D-Ala.)

O-Prenylation

Trunkamide (**Figure 1.3**) is a macrocyclic peptidic natural product isolated from *Lissoclinum patella*[57, 58] and later, production by symbiotic *Prochloron* spp. was revealed[59]. Along with macrocyclic structure and a backbone thiazoline, *O*-prenylation of Ser and Thr residues can be found as non-standard structures. Also, *O*-prenylation on Tyr residue can be seen in prenylagaramides[60] and a number of prenylation are reported in small cyclic peptides produced by cyanobacteria, or cyanobactins.[61] These *O*-prenylation is catalyzed by prenylases, exemplified by LynF enzyme.[62] LynF prenylate Ser/Thr/Tyr residues in macrocyclic peptides, unlike typical post-translational modification enzymes requiring N-terminal leader peptides.

Although *C*-prenylation on Tyr residue was also reported as seen in aestuaramides,[63] *C*-prenylation was attributed to spontaneous Claisen rearrangement.[62] Another example of C-prenylation is found on Trp residue, which is discussed in the main-chain modification part.

Disulfide formation

In general, disulfide formation between intramolecular cysteine residues can spontaneously proceed. Actually, for biosynthetic gene cluster of ulithiacyclamide[59, 64] (**Figure 1.3**), which is a macrocyclic peptidic natural product with disulfide bond linkage as well as backbone heterocycles, no oxidase has been reported to be involved in the disulfide formation. However, in some cases, enzymatic oxidation would be involved in biosynthesis of disulfide linkage. For example, some conotoxins are very difficult to synthesize chemically,[65] which suggests assistance by some mechanisms exist to correctly and efficiently fold the precursor peptide into the mature product

in vivo. This assumption is consistent with the report that in the case of thermophilin 9, the disruption of disulfide oxidase altered inhibitory spectrum of the producing strain[66] and that protein disulfide isomerases (PDIs) was major soluble proteins in *Conus* venom duct extracts[67]. This enzymatic assistance would be, at least in some cases, necessary for the biosynthesis of conotoxins. It would be worth noting, however, that for bioactivities of conotoxins, constrained structures rather than disulfide bonds themselves would be important based on the report that the replacement of disulfide forming cysteines by allyl glycines retained the bioactivity of leucocin, which is attributable to hydrophobic intermolecular interactions of the diallyl side chains.[68]

1.5.2 Modifications at main chain of precursor peptides

Probably, most prevalent main-chain modification is cleavage of leader peptide since almost all precursor peptides of RiPPs have leader peptides. It would be necessary for RiPPs producers to append leader peptides to precursor peptides in order to protect peptides/proteins from unnecessary modifications. And also in some cases, leader peptides would provide the producers with immunity since some RiPPs are inactive before the cleavage of the N-terminal leader peptides.[44] N to C macrocyclization is also common main-chain modification in RiPPs biosynthesis. A number of bioactive peptides have macrocyclic structures.[43]

C-prenylation

C-prenylation of tryptophan residue is also example of main chain modification. (*O*-prenylation is mentioned above.) Kawaguchipeptins are macrocyclic peptidic natural products, which are isolated from *Microcystis aeruginos*a NIES-88.[69, 70] Kawaguchipeptin A contains two prenylated tryptophan residues as well as a D-leucine residue[69] unlike kawaguchipeptin B, which is composed of proteinogenic amino acid residues (**Figure 1.3**).[70] A post-translational modification enzyme, KgpF catalyzes the prenylation at γ-position resulting in a tricyclic structure containing newly introduced pyrrolidine ring and KgpF can modify linear peptides and macrocyclic peptides[71] as well as Fmoc-Trp-OH[72]. *C*-prenylation of tryptophan residue can also be seen in ComX phormone.[73-75] C-prenylation in ComX phormone catalyzed by ComQ and in Kawaguchipeptin A

catalyzed by KgpF share the same scaffold with opposite stereochemistry.[72]

Backbone heterocycles such as azolines and azoles

Peptidic backbone heterocycles would rigidify global structure of the peptides and would endow peptides with protease resistance. Moreover, there are diverse interactions known between heterocycle-containing peptidic natural products and proteins/nucleic acids/metal ions.[76] Moreover, patellamide C (**Figure 1.3**), one of the macrocyclic peptidic natural products containing backbone heterocycles exhibited membrane permeability even higher than cyclosporin A, which is a clinically used and orally available peptidic natural product, by the parallel artificial membrane permeability assay (PAMPA).[77] PAMPA is a cell-free membrane permeability assay, which has been widely used for small molecules[78] and peptides[15] in order to evaluate passive membrane diffusion behavior of the compounds. The high membrane permeability should be attributed to backbone heterocycles as well as macrocyclic structure of patellamide C, demonstrating potential advantage of backbone heterocycles in the development of bioactive peptides.

The heterocyclic structures such as azolines and azoles are post-translationally introduced into peptidic backbone by cyclodehydratases and dehydrogenases, respectively. The cyclodehydratases introduce thiazoline, oxazoline, and methyloxazoline from Cys, Ser and Thr, respectively. The cyclodehydratases are YcaO domain-containing proteins and the cyclodehydration is dependent on ATP and magnesium ion[79] with two proposed intermediates for the mechanism of backbone amide activation, phosphorylated hemiorthoamide[80, 81].

The resulting azolines sometimes further derivatized by flavin mononucleotide (FMN)-dependent dehydrogenase to form thiazole, oxazole, and methyloxazole from thaizoline, oxazoline and methyloxazoline, respectively.[79] In some cases these heterocycle-related post-tranlsational modification enzymes cooperatively catalyze the reaction exemplified by the case of microcin B17 biosynthesis, where three proteins, the cyclodehydratase McbB, oxidase McbC, and putative docking protein McbD were required for the oxidation as well as cyclodehydration.[82]

1.6 Biosynthetic pathway of patellamdies, as an example of PRPS-mediated biosynthesis

From the technical point of view, the major advantage of the synthesis of non-standard peptides in a post-translational manner would be its compatibility with high-throughput screening system such as mRNA display system as long as post-translational modification enzymes have enough wide substrate scope, or promiscuity. In this context, we focused on post-translational introduction of backbone heterocycles because of (i) the abovementioned properties of heterocycles, which would be advantageous for the development of bioactive peptides and (ii) difficulties in direct incorporation of backbone heterocycles into translated peptides.

Based on such assumption, we have previously focused on the biosynthetic pathway of patellamides. Patellamides were isolated from *Lissoclinum patella.*[83] and later structures were revised[84-87]. They were revealed to be RiPPs, produced by *Prochloron spp*., which is cyanobacterial symbionts.[88] As for patellamide D, anti-multi drug resistance activity was reported.[89] In addition, patellamide C had high membrane permeability.[15]

Biosynthetic genes of patellamides are composed of patA–patG[88], of which patA, patD, patE, and patG were denoted as essential[90] (with a citation[88]). patE encodes precursor peptide, which is composed of N-terminal leader peptide (LP), core peptides (CP) flanked by upstream and downstream recognition sequences (uRS and dRS), aligned as LP-uRS-CP-dRS/uRS-CP-dRS, where the sequences of two CPs are not exactly the same, corresponding to the two different patellamides as final products (**Figure 1.5**).

PatA is a protease, which cleaves amide bonds at the N-terminal side of the core peptides (CPs) with recognition sequences G(L/V)E(A/P)S. Protease domain of PatA was fully active similar to the full-length PatA and more stable than the full-length PatA.[91]

PatD is a YcaO domain-containing cyclodehydratase,[79] which modify Cys, Thr and Ser residues into thiazoline, oxazoline and methyloxazoline respectively. The X-ray crystal structure of PatD itself has not been reported, but structure of TruD, a homolog of PatD was reported.[81] TruD activates amide bond to be modified by adenylation using ATP.[81] In addition, ATP binding residues conserved among YcaO domains were revealed on the basis of X-ray crystal structure and biochemical studies,[92] but catalytic mechanism of the cyclodehydration is still elusive.

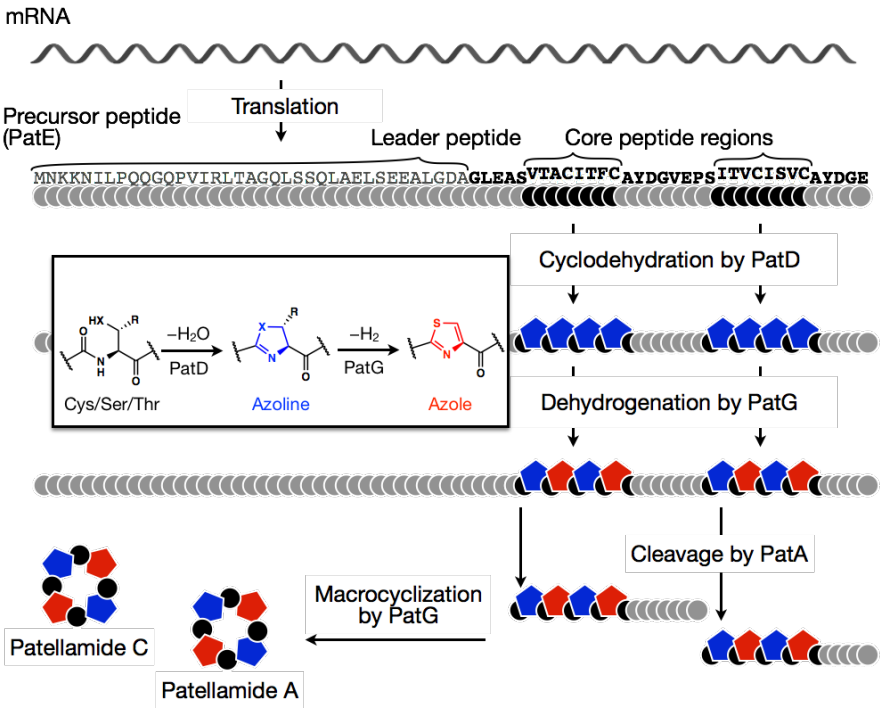


Figure 1.5 Biosynthetic pathway of patellamides.

PatG is composed of three domains, an N-terminal dehydrogenase domain, a central domain with unknown function, and a C-terminal protease (macro cyclization) domain. The macrocyclization reaction between N-terminal amine and C-terminal residues, which have downstream sequences, AYDG(E/V) was reconstituted *in vitro* and the synthesis of a wide variety of macrocyclic peptides was demonstrated.[93, 94] X-ray crystal structure of the macrocyclase domain of PatG was solved and structural basis for the macrocyclization was proposed.[95]

PatF was predicted as a prenylase and required for the production of patellamides,[90] But there is no prenyl group in known patellamides, and thus mysterious protein. The lack of prenyl group in patellamides was rationalized by mutations in catalytic residues in PatF.[96] Involvement of PatF as well as PatB and C in patellamide biosynthesis is unclear at this point, because to the best of my knowledge, reconstitution or heterologous expression of total biosynthetic pathway of patellamides has not been clearly demonstrated.

Collectively, proposed biosynthetic pathway is depicted in **Figure 1.5**. First, the precursor peptide PatE is ribosomally expressed. In the presence of N-terminal leader peptide, the cyclodehydratase PatD and the dehydrogenase domain of PatG install thiazole and (methyl)oxazoline. PatA liberate α-amino group at the N-terminal end of the core peptides, which is subsequently macrocyclized by the macrocyclase domain of PatG. Note that the cognate substrate of PatG oxidase is still elusive and oxidation of thiazolines into thiazoles after macrocyclization is also possible.

1.7 FIT–PatD system; integration of a customized cell-free translation system and a post-translational cyclodehydratase PatD

*This section is based on the publication from our laboratory; "One-pot synthesis of azoline-containing peptides in a cell-free translation system integrated with a posttranslational cyclodehydratase", Yuki Goto, Yumi Ito, Yasuharu Kato, Shotaro Tsunoda, Hiroaki Suga, *Chem. Biol.*, **2014**, 21, 766-774.

We have previously devised an *in vitro* biosynthetic system for azoline-containing peptides[97] by integrating the post-translational cyclodehydratase PatD, which is involved in patellamide biosynthesis[88] and a reconstituted cell-free translation system[98]. The *in vitro* biosynthetic system enabled one-pot synthesis of a wide variety of azoline-containing peptides in a template DNA dependent manner. In addition, the *in vitro* biosynthetic system enabled extensive mutagenesis studies, unveiling recognition determinants by which modification reaction is governed and unexpectedly high substrate tolerance of the cyclodehydratase PatD (**Figure 1.6**). In brief, (i) dRS can be truncated. (ii) uRS tolerates mutations and the length from leader peptide dictates core peptide region. (iii) The C-terminal region of the leader peptide (26L–37A) is necessary for the efficient cyclodehydration and peptides bearing that region at N-terminus can be efficiently modified by PatD. (iv) Precursor peptides without leader peptides were modified to some extent in the presence of a separate leader peptide. This "*in trans* modification" suggested "PatD-activation" mechanism by the leader peptide. In addition, (a) PatD can heterocyclize all of the Cys/Thr/Ser residues, with higher modification efficiency in this order. (b) PatD can accept various artificial core peptide sequences with (b-1) various amino acid length, (b-2) various amino acid compositions including hydrophilic residues and (b-3) consecutive cysteine residues to be modified, which are all rare in naturally-occurring core peptide sequences. Collectively, unprecedented substrate tolerance of post-translational cyclodehydratase PatD was demonstrated and these observations were the basis of the present studies, which are described in the following chapters.

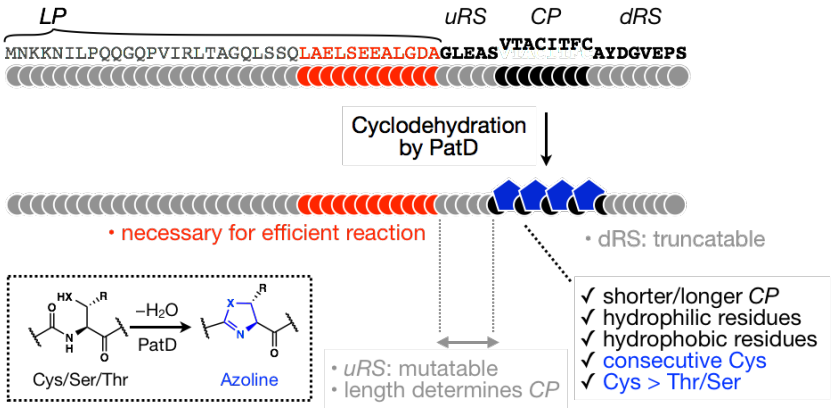


Figure 1.6 Summary of the mutagenesis experiments in our previous study, which is related to the experimental design in the present thesis.

1.8 Technological backgrounds

1.8.1 Translation reaction as peptide synthetic method and its molecular mechanism

Translation is a biosynthetic reaction of proteins, in which ribosome[99] polymerize amino acids in a mRNA template dependent manner. Since peptide synthesis by translation reaction proceeds rapidly (12–22 amino acids/sec)[100, 101] and precisely (error rate ~1/1000)[102], translation is a powerful tool as peptide synthetic method. Relationship between the sequence of peptides and the sequence of template mRNAs (nucleotides) is governed by codons, sequences of three nucleotides.[103] For example, the codon UUU is corresponding to phenylalanine, and thus poly U sequence direct the synthesis of poly phenylalanine sequence.[104] In principle there are 64 kinds of codons (= 4^3), and the relationship between codons and amino acids are summarized as the genetic code.[105]

As above mentioned, relationship between codons and amino acids are strictly controlled. In molecular level, this control can be attributed to the adopter molecule, tRNA, and the relationship can be rationalized from two points (**Figure 1.7**), (i) paring between codons and tRNAs and (ii) paring between tRNAs and amino acids. (i) First, every tRNA has the sequence called anticodon, which is a complementary sequence to the corresponding codon, and thus base paring between codons and anticodons can link codons and tRNAs properly. (ii) Aminoacylation reactions of amino acids on tRNAs are catalyzed by aminoacyl tRNA synthetases (ARSs), which selectively catalyze the reaction using corresponding tRNAs and amino acids. For example, Trptophanyl aminoacyl tRNA synthetase (TrpRS) catalyze aminoacylation of $tRNA^{Trp}_{CCA}$ by tryptophan selectively, and the substrate specificities of ARSs can link tRNAs and amino acids properly. Note that $tRNA^{Trp}_{CCA}$ denotes that the tRNA is corresponding to tryptophan and its anticodon sequence is 5'-CCA-3', which is complementary to tryptophan codon 5'-UGG-3'.

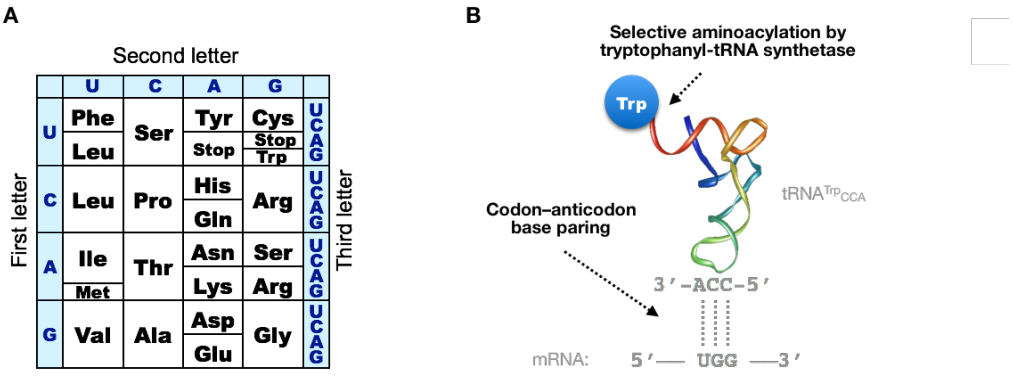


Figure 1.7 The genetic code and molecular mechanism of sequence dependency of translation.

1.8.2 Ribosomal synthesis of non-standard peptides by mis-acylated tRNAs

The mechanism for fidelity control in translation reaction is consistent with the fact that ribosome must tolerate 20 kinds of proteinogenic amino acids as the building blocks for peptide synthesis. Although translation system, as a whole, can only synthesize canonical peptides, the property that ribosome does not recognize side chains of amino acids charged onto tRNAs is the basis of the engineering of translation system discussed in the following text.

Based on the background, synthetic methods of misacylated tRNA with non-proteinogenic amino acids have been investigated. Early examples are, albeit not non-proteinogenic, the replacement of cysteine by alanine upon the Raney-nickel mediated desulfurization on Cys-tRNA^{Cys},[106] being an early indication of the successful incorporation of a wide variety of non-proteinogenic amino acids by the "misacylation strategy". For the synthetic methods of misacylated tRNAs, semi-enzymatic synthesis, in which a chemically synthesized aminoacyl-dinucleotide and tRNA body are ligated by T4 RNA ligase [107, 108] and artificially evolved aminoacyl tRNA synthetase[109] have been mainly used, but these methods limit the variety of non-proteinogenic amino acids, which may be tested for the ribosomal incorporation and subsequent biological or chemical events.

Promiscuity of the aminoacyl tRNA syntheases, though they were basically specific enzymes, can also be utilized for the introduction on non-proteinogenic amino acids.[110] ARS can

aminoacylate tRNA with non-proteinogenic amino acids structurally-relevant to the original proteinogenic amino acid. Although this method require only a cell free translation system depleted with a specific amino acid and addition of the corresponding non-proteinogenic amino acid, the major limitation is the structural variety of non-proteinogenic amino acids are limited to the original proteinogenic amino acid-like structures. In addition, since ARS recognize tRNA species, it is hard to "reasign" a proteinogenic amino acid into codons different from the original codon. For example, assignment of cysteine at tryptophan codon (UGG), which is the key technological manipulation in the chapter 2, would be difficult with such approaches.

1.8.3 Potential of flexizymes for preparations of mis-acylated tRNAs.

Flexizymes, an artificially evolved ribozyme, which have previously developed in our laboratory[111, 112], enabled facile aminoacylation of tRNAs with non-proteinogenic amino acids (**Figure 1.8**). Several flexizymes have been developed, which catalyze aminoacylation of tRNA bearing 3' CCA end by aminoacyl substrates activated with leaving groups with different substrate selectivity for each flexizyme. The flexizyme, eFx recognize aromatic moieties in side chain of amino acid derivatives activated with cyanomethyl ester, whereas the flexizyme dFx recognize aromatic dinitrobenzyl group in the leaving group itself. Combined with other variants, virtually any amino acids can be acylated onto tRNAs.

The versatility of the flexizyme-mediated aminoacylation reaction enabled the utilization of a wide variety of non-proteinogenic amino acids, including ones with reactive moieties for post-translational chemical modification reactions. Such non-proteinogenic amino acids include D-amino acids[113-115], β-aminoacids[116], *N*-methyl/*N*-substituted amino acids[117, 118] and α-hydroxy acids[119, 120], and amino acids containing chloroacetyl group[121, 122], which is reactive with intramolecular thiol, azido-/alkyne- containing amino acids[123], and 5-hydroxy tryptophan/benzyl amine-containing amino acids[124].

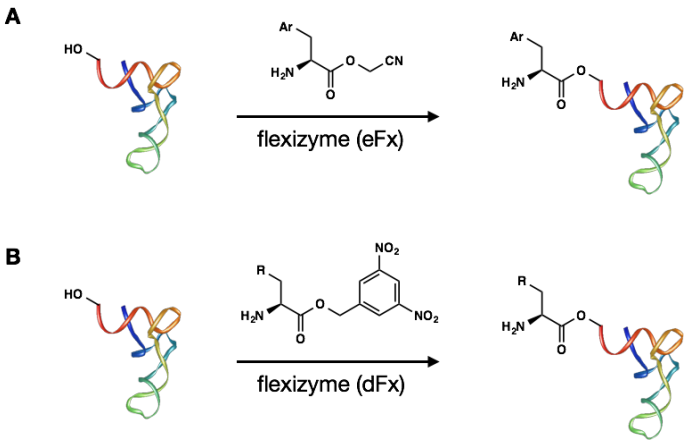


Figure 1.8 Aminoacylation of tRNAs by activated amino acids catalyzed by flexizymes.

1.8.4 Orthogonal tRNA for customized cell-free translation of non-standard peptides

Another important aspect in the customized translation system is orthogonality of tRNAs against aminoacyl tRNA synthetases since undesired aminoacylation by one of the 20 aminoacyl tRNA synthetases of liberated tRNAs originally charged with non-proteinogenic amino acids would cause contamination at the specific codon to be reprogrammed. To circumvent such undesired aminoacylation, *in vitro* transcribed $tRNA^{AsnE2}$, which was previously developed in our laboratory[119] was utilized for the flexizyme-mediated aminoacylation reactions in the present study.

Chapter 2 Development of *in vitro* synthetic method of azole-containing peptides (abridged)

Parts of this chapter were published in: "Attempts at in Vitro Reconstitution of a Post-translational Dehydrogenase toward Synthesis of Azole-containing Peptides", Yasuharu Kato, Yuki Goto, Hiroaki Suga, *Peptide Science 2014*, **2015**, 137-138. and "Laser-induced oxidation of a peptide-embedded thiazoline by an assistance of adjacent thiazoline", Yasuharu Kato, Yuki Goto, Hiroaki Suga, *Peptide Science 2015,* **2016**, 27-28

(For the reasons of patent applications and future publications, details are closed in the following parts.)

Chapter 3 Development of *in vitro* synthetic method of Ψ[CH_2NH]-containing peptides (abridged)

(For the reasons of patent applications and future publications, details are closed in the following parts.)

Chapter 4 General conclusion (abridged)

In conclusion, I have developed novel methods involving the posttranslational enzymatic cyclodehydration by PatD and chemical modifications such as oxidation and reduction on *in vitro* expressed peptides to synthesize backbone-modified peptides inspired by naturally-occurring structural diversity in peptidic natural products, which were reviewed in chapter 1.

In chapter 2 and chapter 3, the development of *in vitro* synthetic method of azole-containing peptides and azole-containing peptides are described, respectively. *(For the reasons of patent applications and future publications, details are closed in the following parts.)*

In conclusion, in this research I have developed novel methods involving the post-translational enzymatic cyclodehydration by PatD and chemical modifications on ribosomally synthesized peptides to yield peptides with various backbone modifications. The post-translational modification method developed here would provide unique structural scaffolds into peptides, which possibly leads to the development of novel bioactive peptides.

List of accomplishments

【Publications】

1. "Laser-induced oxidation of a peptide-embedded thiazoline by an assistance of adjacent thiazoline", Yasuharu Kato, Yuki Goto, Hiroaki Suga, *Peptide Science 2015,* **2016**, 27-28
2. "One-pot synthesis of azoline-containing peptides in a cell-free translation system integrated with a posttranslational cyclodehydratase", Yuki Goto, Yumi Ito, Yasuharu Kato, Shotaro Tsunoda, Hiroaki Suga, *Chem. Biol.*, **2014**, 21, 766-774.
3. "Attempts at *in Vitro* Reconstitution of a Post-translational Dehydrogenase toward Synthesis of Azole-containing Peptides.", Yasuharu Kato, Yuki Goto, Hiroaki Suga, *Peptide Science 2014*, **2015**, 137-138

【Oral presentations】

4. "翻訳後修飾による複素環含有ペプチドの in vitro 生合成法の開発", 加藤保治, 後藤佑樹, 菅裕明, サントリー生物有機科学研究所報告会, 大阪, 2015 年 3 月
5. "Development of post-translational modification reactions toward in vitro synthesis of peptides with heterocyclic backbones", Yasuharu Kato, Yuki Goto, Hiroaki Suga, 日本化学会第 95 春季年会, 千葉, 2015 年 3 月
6. "Oxidation of azolines assisted by an adjacent azoline moiety toward in vitro synthesis of azole-containing peptides", Yasuharu Kato, Yuki Goto, Hiroaki Suga, 第 52 回ペプチド討論会,平塚, 2015 年 11 月
7. "In vitro biosynthesis of backbone-modified peptides by post-translational modification reactions", Yasuharu Kato, Yuki Goto, Hiroaki Suga, 日本化学会第 96 春季年会, 京都, 2016 年 3 月, (年会ハイライト講演)
8. "アゾリン含有ペプチドの in vitro 合成法の確立とその応用", 加藤保治, 伊藤悠美、角田翔太郎、後藤 佑樹, 菅裕明, 日本薬学会第 136 年会, 横浜, 2016 年 3 月, (大学院生シンポジウム招待講演)
9. "翻訳後修飾による主鎖骨格修飾ペプチド合成法の開発", 加藤保治, 後藤佑樹, 菅裕明, 第 10 回バイオ関連化学シンポジウム, 金沢, 2016 年 9 月
10. "Combination of enzymatic and chemical post-translational modifications for synthesis of various backbone-modified peptides", Yasuharu Kato, Yuki Goto, Hiroaki Suga, 日本化学会第 97 春季年会, 横浜, 2017 年 3 月
11. "翻訳後修飾による主鎖修飾ペプチド合成法の開発とその応用", 加藤保治, 後藤佑樹, 菅裕明, 日本薬学会第 137 年会, 仙台, 2016 年 3 月, (大学院生シンポジウム招待講演)

【Poster presentations】

12. “翻訳後修飾によるアゾール含有ペプチド合成法の開発”, 加藤保治, 後藤佑樹, 菅裕明, 第 14 回東京大学生命科学シンポジウム, 東京, 2014 年 4 月
13. “主鎖骨格にアゾールを有するペプチドの翻訳合成法の開発” 加藤保治, 後藤佑樹, 菅裕明, 日本ケミカルバイオロジー学会第 9 回年会, 大阪, 2014 年 6 月
14. “翻訳後修飾によるアゾール含有ペプチド人工生合成法の開発”, 加藤保治, 後藤佑樹, 菅裕明, 新規素材探索研究会第 13 回セミナー, 横浜, 2014 年 6 月
15. “翻訳後修飾によるアゾール含有ペプチド合成法の開発”, 加藤保治, 後藤佑樹, 菅裕明, 第 2 回バイオ関連化学シンポジウム若手フォーラム, 岡山, 2014 年 9 月
16. “翻訳後修飾によるアゾール含有ペプチド合成法の開発”, 加藤保治, 後藤佑樹, 菅裕明, 第 8 回バイオ関連化学シンポジウム, 岡山, 2014 年 9 月
17. “Artificial post-translational modifications toward synthesis of azole-containing peptides”, 加藤保治, 後藤佑樹, 菅裕明, 第 51 回ペプチド討論会, 徳島, 2014 年 10 月
18. “Development of post-translational modification reactions for the synthesis of Ψ(CH_2NH)-containing peptides”, Yasuharu Kato, Yuki Goto, Hiroaki Suga, The 21st ZESTY Network Seminar, 東京, 2015 年 7 月
19. “ヘテロ環骨格含有ペプチドの合成にむけた新規翻訳後修飾反応の開発”, 加藤保治, 後藤佑樹, 菅裕明, 日本化学会第 95 春季年会アドバンスト・テクノロジー・プログラム, 千葉, 2015 年 3 月
20. “In vitro synthesis of azoline-containing peptides and its applications for various backbone-modified peptides”, Yasuharu Kato, Yuki Goto, Hiroaki Suga, The 15th Tateshina Conference on organic Chemistry,長野, 2015 年 11 月
21. Development of post-translational modification reactions for the synthesis of peptides with Ψ(CH_2NH) structures, Yasuharu Kato, Yuki Goto, Hiroaki Suga, The 2015 International Chemical Congress of Pacific Basin Societies (Pacfichem 2015), Hawaii, 2015 年 12 月
22. “Development of post-translational modifications toward novel bioacitive peptides”, Yasuharu Kato, Yuki Goto, Hiroaki Suga, 日本化学会第 96 春季年会アドバンスト・テクノロジー・プログラム, 京都, 2016 年 3 月
23. “In vitro synthesis of various backbone-modified peptides by post-translational modification reactions”, Yasuharu Kato, Yuki Goto, Hiroaki Suga, 日本化学会第 97 春季年会アドバンスト・テクノロジー・プログラム, 横浜, 2017 年 3 月

References

1. Chothia, C. & Janin, J. Principles of protein-protein recognition. *Nature* **256**, 705-708 (1975).
2. Jones, S. & Thornton, J.M. Principles of protein-protein interactions. *Proceedings of the National Academy of Sciences* **93**, 13-20 (1996).
3. Bogan, A.A. & Thorn, K.S. Anatomy of hot spots in protein interfaces. *Journal of molecular biology* **280**, 1-9 (1998).
4. Arkin, M.R. & Wells, J.A. Small-molecule inhibitors of protein–protein interactions: progressing towards the dream. *Nature reviews Drug discovery* **3**, 301-317 (2004).
5. Wells, J.A. & McClendon, C.L. Reaching for high-hanging fruit in drug discovery at protein–protein interfaces. *Nature* **450**, 1001-1009 (2007).
6. Arkin, M.R., Tang, Y. & Wells, J.A. Small-molecule inhibitors of protein-protein interactions: progressing toward the reality. *Chemistry & biology* **21**, 1102-1114 (2014).
7. Scott, D.E., Bayly, A.R., Abell, C. & Skidmore, J. Small molecules, big targets: drug discovery faces the protein-protein interaction challenge. *Nature Reviews Drug Discovery* **15**, 533-550 (2016).
8. Rask-Andersen, M., Masuram, S. & Schiöth, H.B. The druggable genome: evaluation of drug targets in clinical trials suggests major shifts in molecular class and indication. *Annual review of pharmacology and toxicology* **54**, 9-26 (2014).
9. Nevola, L. & Giralt, E. Modulating protein–protein interactions: the potential of peptides. *Chemical Communications* **51**, 3302-3315 (2015).
10. Gao, M., Cheng, K. & Yin, H. Targeting Protein-Protein Interfaces Using Macrocyclic Peptides. *Biopolymers* **104**, 310-316 (2015).
11. Nevola, L. & Giralt, E. Modulating protein-protein interactions: the potential of peptides. *Chemical Communications* **51**, 3302-3315 (2015).
12. Pelay-Gimeno, M., Glas, A., Koch, O. & Grossmann, T.N. Structure-Based Design of Inhibitors of Protein-Protein Interactions: Mimicking Peptide Binding Epitopes. *Angewandte*

Chemie-International Edition **54**, 8896-8927 (2015).

13. Araghi, R.R. & Keating, A.E. Designing helical peptide inhibitors of protein-protein interactions. *Current Opinion in Structural Biology* **39**, 27-38 (2016).
14. Lipinski, C.A., Lombardo, F., Dominy, B.W. & Feeney, P.J. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* **23**, 3-25 (1997).
15. Ahlbach, C.L. et al. Beyond cyclosporine A: conformation-dependent passive membrane permeabilities of cyclic peptide natural products. *Future medicinal chemistry* **7**, 2121-2130 (2015).
16. Siegert, T.R., Bird, M.J., Makwana, K.M. & Kritzer, J.A. Analysis of Loops that Mediate Protein-Protein Interactions and Translation into Submicromolar Inhibitors. *Journal of the American Chemical Society* **138**, 12876-12884 (2016).
17. London, N., Raveh, B. & Schueler-Furman, O. Druggable protein–protein interactions–from hot spots to hot segments. *Current opinion in chemical biology* **17**, 952-959 (2013).
18. Tanaka, Y. et al. Structural basis for the drug extrusion mechanism by a MATE multidrug transporter. *Nature* **496**, 247-251 (2013).
19. Yamagishi, Y. et al. Natural product-like macrocyclic N-methyl-peptide inhibitors against a ubiquitin ligase uncovered from a ribosome-expressed de novo library. *Chemistry & biology* **18**, 1562-1570 (2011).
20. Matsunaga, Y., Bashiruddin, N.K., Kitago, Y., Takagi, J. & Suga, H. Allosteric Inhibition of a Semaphorin 4D Receptor Plexin B1 by a High-Affinity Macrocyclic Peptide. *Cell Chemical Biology* **23**, 1341-1350 (2016).
21. Ecker, D.M., Jones, S.D. & Levine, H.L. in MAbs 9-14 (Taylor & Francis, 2015).
22. Van Weemen, B. & Schuurs, A. Immunoassay using antigen—enzyme conjugates. *FEBS letters* **15**, 232-236 (1971).
23. Engvall, E. & Perlmann, P. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry* **8**, 871-874 (1971).
24. Eisenstein, M. Westward expansion. *Nature Methods* **2**, 796-796 (2005).

25. Renart, J., Reiser, J. & Stark, G.R. Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proceedings of the National Academy of Sciences* **76**, 3116-3120 (1979).

26. Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences* **76**, 4350-4354 (1979).

27. Brahmer, J.R. et al. Phase I study of single-agent anti–programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *Journal of Clinical Oncology* **28**, 3167-3175 (2010).

28. Wang, C. et al. In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates. *Cancer immunology research* **2**, 846-856 (2014).

29. Schafer, P. et al. Apremilast, a cAMP phosphodiesterase‐4 inhibitor, demonstrates anti‐inflammatory activity in vitro and in a model of psoriasis. *British journal of pharmacology* **159**, 842-855 (2010).

30. Song, M.-Y. et al. Characterization of a novel anti-human TNF-α murine monoclonal antibody with high binding affinity and neutralizing activity. *Experimental & molecular medicine* **40**, 35-42 (2008).

31. Magnenat, L. et al. in mAbs 127-139 (Taylor & Francis, 2017).

32. Ernst, J.A. et al. Isolation and characterization of the B-cell marker CD20. *Biochemistry* **44**, 15150-15158 (2005).

33. Yu, Y. et al. A humanized anti-VEGF rabbit monoclonal antibody inhibits angiogenesis and blocks tumor growth in xenograft models. *PLoS One* **5**, e9072 (2010).

34. Liang, W.-C. et al. Cross-species vegf-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal vegf. *Journal of Biological Chemistry* (2005).

35. Glanville, J. et al. Precise determination of the diversity of a combinatorial antibody library

gives insight into the human immunoglobulin repertoire. *Proceedings of the National Academy of Sciences* **106**, 20216-20221 (2009).

36. Georgiou, G. et al. The promise and challenge of high-throughput sequencing of the antibody repertoire. *Nature biotechnology* **32**, 158-168 (2014).
37. Passioura, T., Katoh, T., Goto, Y. & Suga, H. Selection-based discovery of druglike macrocyclic peptides. *Annual review of biochemistry* **83**, 727-752 (2014).
38. Gray, B.P. & Brown, K.C. Combinatorial peptide libraries: mining for cell-binding peptides. *Chemical reviews* **114**, 1020-1081 (2013).
39. Strieker, M., Tanović, A. & Marahiel, M.A. Nonribosomal peptide synthetases: structures and dynamics. *Current opinion in structural biology* **20**, 234-240 (2010).
40. Winn, M., Fyans, J., Zhuo, Y. & Micklefield, J. Recent advances in engineering nonribosomal peptide assembly lines. *Natural product reports* **33**, 317-347 (2016).
41. Kries, H. Biosynthetic engineering of nonribosomal peptide synthetases. *Journal of Peptide Science* **22**, 564-570 (2016).
42. Fischbach, M.A. & Walsh, C.T. Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. *Chemical reviews* **106**, 3468-3496 (2006).
43. Arnison, P.G. et al. Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Natural product reports* **30**, 108-160 (2013).
44. Oman, T.J. & Van Der Donk, W.A. Follow the leader: the use of leader peptides to guide natural product biosynthesis. *Nature chemical biology* **6**, 9-18 (2010).
45. McIntosh, J.A., Donia, M.S. & Schmidt, E.W. Ribosomal peptide natural products: bridging the ribosomal and nonribosomal worlds. *Natural product reports* **26**, 537-559 (2009).
46. Humphrey, J.M. & Chamberlin, A.R. Chemical synthesis of natural product peptides: coupling methods for the incorporation of noncoded amino acids into peptides. *Chemical Reviews* **97**, 2243-2266 (1997).
47. Knerr, P.J. & van der Donk, W.A. Discovery, biosynthesis, and engineering of lantipeptides.

Annual review of biochemistry **81**, 479-505 (2012).

48. Morris, S.L., Walsh, R.C. & Hansen, J. Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. *Journal of Biological Chemistry* **259**, 13590-13594 (1984).
49. Wever, W.J. et al. Chemoenzymatic synthesis of thiazolyl peptide natural products featuring an enzyme-catalyzed formal [4+ 2] cycloaddition. *Journal of the American Chemical Society* **137**, 3494-3497 (2015).
50. Allgaier, H., Jung, G., Werner, R.G., Schneider, U. & Zähner, H. Elucidation of the structure of epidermin, a ribosomally synthesized, tetracyclic heterodetic polypeptide antibiotic. *Angewandte Chemie International Edition in English* **24**, 1051-1053 (1985).
51. Komiyama, K. et al. A new antibiotic, cypemycin. Taxonomy, fermentation, isolation and biological characteristics. *The Journal of antibiotics* **46**, 1666-1671 (1993).
52. Minami, Y. et al. Structure of cypemycin, a new peptide antibiotic. *Tetrahedron letters* **35**, 8001-8004 (1994).
53. HAYASHI, F. et al. The structure of PA48009: the revised structure of duramycin. *The Journal of antibiotics* **43**, 1421-1430 (1990).
54. Hamada, T., Sugawara, T., Matsunaga, S. & Fusetani, N. Polytheonamides, unprecedented highly cytotoxic polypeptides, from the marine sponge theonella swinhoei: 1. Isolation and component amino acids. *Tetrahedron letters* **35**, 719-720 (1994).
55. Hamada, T., Matsunaga, S., Yano, G. & Fusetani, N. Polytheonamides A and B, Highly Cytotoxic, Linear Polypeptides with Unprecedented Structural Features, from the Marine Sponge, Theonella s winhoei. *Journal of the American Chemical Society* **127**, 110-118 (2005).
56. Tugyi, R. et al. Partial D-amino acid substitution: Improved enzymatic stability and preserved Ab recognition of a MUC2 epitope peptide. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 413-418 (2005).
57. Carroll, A.R. et al. Patellins 1-6 and Trunkamide A: Novel Cyclic Hexa-, Hepta-and Octa-peptides From Colonial Ascidians, Lissoclinum sp. *Australian journal of chemistry* **49**,

659-667 (1996).

58. Wipf, P. & Uto, Y. Total synthesis and revision of stereochemistry of the marine metabolite trunkamide A. *The Journal of organic chemistry* **65**, 1037-1049 (2000).

59. Donia, M.S., Ravel, J. & Schmidt, E.W. A global assembly line to cyanobactins. *Nature chemical biology* **4**, 341 (2008).

60. Donia, M.S. & Schmidt, E.W. Linking chemistry and genetics in the growing cyanobactin natural products family. *Chemistry & biology* **18**, 508-519 (2011).

61. Sivonen, K., Leikoski, N., Fewer, D.P. & Jokela, J. Cyanobactins—ribosomal cyclic peptides produced by cyanobacteria. *Applied microbiology and biotechnology* **86**, 1213-1225 (2010).

62. McIntosh, J.A., Donia, M.S., Nair, S.K. & Schmidt, E.W. Enzymatic basis of ribosomal peptide prenylation in cyanobacteria. *Journal of the American Chemical Society* **133**, 13698-13705 (2011).

63. McIntosh, J.A., Lin, Z., Tianero, M.D.B. & Schmidt, E.W. Aestuaramides, a natural library of cyanobactin cyclic peptides resulting from isoprene-derived Claisen rearrangements. *ACS chemical biology* **8**, 877-883 (2013).

64. Ireland, C. & Scheuer, P.J. Ulicyclamide and ulithiacyclamide, two new small peptides from a marine tunicate. *Journal of the American Chemical Society* **102**, 5688-5691 (1980).

65. Bulaj, G. et al. Synthetic μO-conotoxin MrVIB blocks TTX-resistant sodium channel NaV1. 8 and has a long-lasting analgesic activity. *Biochemistry* **45**, 7404-7414 (2006).

66. Fontaine, L. & Hols, P. The inhibitory spectrum of thermophilin 9 from Streptococcus thermophilus LMD-9 depends on the production of multiple peptides and the activity of BlpGSt, a thiol-disulfide oxidase. *Applied and environmental microbiology* **74**, 1102-1110 (2008).

67. Bulaj, G. et al. Efficient oxidative folding of conotoxins and the radiation of venomous cone snails. *Proceedings of the National Academy of Sciences* **100**, 14562-14568 (2003).

68. Derksen, D.J., Stymiest, J.L. & Vederas, J.C. Antimicrobial leucocin analogues with a disulfide bridge replaced by a carbocycle or by noncovalent interactions of allyl glycine

residues. *Journal of the American Chemical Society* **128**, 14252-14253 (2006).

69. Ishida, K., Matsuda, H., Murakami, M. & Yamaguchi, K. Kawaguchipeptin A, a novel cyclic undecapeptide from cyanobacterium Microcystis aeruginosa (NIES-88). *Tetrahedron* **52**, 9025-9030 (1996).

70. Ishida, K., Matsuda, H., Murakami, M. & Yamaguchi, K. Kawaguchipeptin B, an antibacterial cyclic undecapeptide from the cyanobacterium Microcystis aeruginosa. *Journal of natural products* **60**, 724-726 (1997).

71. Parajuli, A. et al. A Unique Tryptophan C-Prenyltransferase from the Kawaguchipeptin Biosynthetic Pathway. *Angewandte Chemie-International Edition* **55**, 3596-3599 (2016).

72. Okada, M. et al. Stereospecific prenylation of tryptophan by a cyanobacterial post-translational modification enzyme. *Organic & Biomolecular Chemistry* **14**, 9639-9644 (2016).

73. Magnuson, R., Solomon, J. & Grossman, A.D. Biochemical and genetic characterization of a competence pheromone from B. subtilis. *Cell* **77**, 207-216 (1994).

74. Okada, M. et al. Structure of the Bacillus subtilis quorum-sensing peptide pheromone ComX. *Nature chemical biology* **1**, 23-24 (2005).

75. Okada, M. et al. Chemical structure of posttranslational modification with a farnesyl group on tryptophan. *Bioscience, biotechnology, and biochemistry* **72**, 914-918 (2008).

76. Roy, R.S., Gehring, A.M., Milne, J.C., Belshaw, P.J. & Walsh, C.T. Thiazole and oxazole peptides: biosynthesis and molecular machinery. *Natural product reports* **16**, 249-263 (1999).

77. White, T.R. et al. On-resin N-methylation of cyclic peptides for discovery of orally bioavailable scaffolds. *Nature chemical biology* **7**, 810-817 (2011).

78. Kansy, M., Senner, F. & Gubernator, K. Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. *Journal of medicinal chemistry* **41**, 1007-1010 (1998).

79. Czekster, C.M., Ge, Y. & Naismith, J.H. Mechanisms of cyanobactin biosynthesis. *Current Opinion in Chemical Biology* **35**, 80-88 (2016).

80. Dunbar, K.L., Melby, J.O. & Mitchell, D.A. YcaO domains use ATP to activate amide backbones during peptide cyclodehydrations. *Nature chemical biology* **8**, 569-575 (2012).

81. Koehnke, J. et al. The cyanobactin heterocyclase enzyme: a processive adenylase that operates with a defined order of reaction. *Angewandte Chemie International Edition* **52**, 13991-13996 (2013).

82. Li, Y.-M., Milne, J.C., Madison, L.L., Kolter, R. & Walsh, C.T. From peptide precursors to oxazole and thiazole-containing peptide antibiotics: microcin B17 synthase. *Science* **274**, 1188 (1996).

83. Leikoski, N. et al. Analysis of an inactive cyanobactin biosynthetic gene cluster leads to discovery of new natural products from strains of the genus Microcystis. *PLoS One* **7**, e43002 (2012).

84. Hamada, Y., Shibata, M. & Shioiri, T. New methods and reagents in organic synthesis. 55. Total syntheses of patellamides B and C, cytotoxic cyclic peptides from a tunicate 1. Their proposed structures should be corrected. *Tetrahedron letters* **26**, 5155-5158 (1985).

85. Hamada, Y., Shibata, M. & Shioiri, T. New methods and reagents in organic synthesis. 56. Total syntheses of patellamides B and C, cytotoxic cyclic peptides from a tunicate 2. Their real structures have been determined by their syntheses. *Tetrahedron letters* **26**, 5159-5162 (1985).

86. Hamada, Y., Shibata, M. & Shioiri, T. New methods and reagents in organic synthesis. 58.: A synthesis of patellamide a, a cytotoxic cyclic peptide from a tunicate. Revision of its proposed structure. *Tetrahedron letters* **26**, 6501-6504 (1985).

87. Schmidt, U. & Griesser, H. Total synthesis and structure determination of patellamide B. *Tetrahedron letters* **27**, 163-166 (1986).

88. Schmidt, E.W. et al. Patellamide A and C biosynthesis by a microcin-like pathway in Prochloron didemni, the cyanobacterial symbiont of Lissoclinum patella. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 7315-7320 (2005).

89. Williams, A.B. & Jacobs, R.S. A marine natural product, patellamide D, reverses multidrug resistance in a human leukemic cell line. *Cancer letters* **71**, 97-102 (1993).

90. Donia, M.S. et al. Natural combinatorial peptide libraries in cyanobacterial symbionts of marine ascidians. *Nature chemical biology* **2**, 729-735 (2006).

91. Agarwal, V., Pierce, E., McIntosh, J., Schmidt, E.W. & Nair, S.K. Structures of cyanobactin maturation enzymes define a family of transamidating proteases. *Chemistry & biology* **19**, 1411-1422 (2012).

92. Dunbar, K.L. et al. Discovery of a new ATP-binding motif involved in peptidic azoline biosynthesis. *Nature chemical biology* **10**, 823-829 (2014).

93. Lee, J., McIntosh, J., Hathaway, B.J. & Schmidt, E.W. Using marine natural products to discover a protease that catalyzes peptide macrocyclization of diverse substrates. *Journal of the American Chemical Society* **131**, 2122-2124 (2009).

94. McIntosh, J.A. et al. Circular logic: nonribosomal peptide-like macrocyclization with a ribosomal peptide catalyst. *Journal of the American Chemical Society* **132**, 15499-15501 (2010).

95. Koehnke, J. et al. The mechanism of patellamide macrocyclization revealed by the characterization of the PatG macrocyclase domain. *Nature structural & molecular biology* **19**, 767-772 (2012).

96. Bent, A.F. et al. Structure of PatF from Prochloron didemni. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications* **69**, 618-623 (2013).

97. Goto, Y., Ito, Y., Kato, Y., Tsunoda, S. & Suga, H. One-pot synthesis of azoline-containing peptides in a cell-free translation system integrated with a posttranslational cyclodehydratase. *Chemistry & biology* **21**, 766-774 (2014).

98. Shimizu, Y. et al. Cell-free translation reconstituted with purified components. *Nature biotechnology* **19**, 751-755 (2001).

99. Schmeing, T.M. & Ramakrishnan, V. What recent ribosome structures have revealed about the mechanism of translation. *Nature* **461**, 1234-1242 (2009).

100. Young, R. & Bremer, H. Polypeptide-chain-elongation rate in Escherichia coli B/r as a function of growth rate. *Biochemical Journal* **160**, 185-194 (1976).

101. Bilgin, N., Claesens, F., Pahverk, H. & Ehrenberg, M. Kinetic properties of Escherichia coli

ribosomes with altered forms of S12. *Journal of molecular biology* **224**, 1011-1027 (1992).

102. Bouadloun, F., Donner, D. & Kurland, C. Codon-specific missense errors in vivo. *The EMBO journal* **2**, 1351 (1983).

103. Crick, F., Barnett, L., Brenner, S. & Watts-Tobin, R. General Nature of the Genetic Code for Proteins. *Nature* **192**, 1227-1232 (1961).

104. Nirenberg, M.W. & Matthaei, J.H. The dependence of cell-free protein synthesis in E. coli upon naturally occurring or synthetic polyribonucleotides. *Proceedings of the National Academy of Sciences* **47**, 1588-1602 (1961).

105. Nirenberg, M. Historical review: Deciphering the genetic code–a personal account. *Trends in biochemical sciences* **29**, 46-54 (2004).

106. Chapeville, F. et al. On the role of soluble ribonucleic acid in coding for amino acids. *Proceedings of the National Academy of Sciences* **48**, 1086-1092 (1962).

107. Ellman, J.A., Mendel, D. & Schultz, P.G. Site-specific incorporation of novel backbone structures into proteins. *Science* **255**, 197 (1992).

108. Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C. & Schultz, P.G. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* **244**, 182 (1989).

109. Wang, L., Brock, A., Herberich, B. & Schultz, P.G. Expanding the genetic code of Escherichia coli. *Science* **292**, 498-500 (2001).

110. Josephson, K., Hartman, M.C. & Szostak, J.W. Ribosomal synthesis of unnatural peptides. *Journal of the American Chemical Society* **127**, 11727-11735 (2005).

111. Goto, Y., Katoh, T. & Suga, H. Flexizymes for genetic code reprogramming. *Nature protocols* **6**, 779-790 (2011).

112. Murakami, H., Ohta, A., Ashigai, H. & Suga, H. A highly flexible tRNA acylation method for non-natural polypeptide synthesis. *Nature Methods* **3**, 357 (2006).

113. Goto, Y., Murakami, H. & Suga, H. Initiating translation with D-amino acids. *RNA* **14**, 1390-1398 (2008).

114. Fujino, T., Goto, Y., Suga, H. & Murakami, H. Reevaluation of the D-amino acid compatibility with the elongation event in translation. *Journal of the American Chemical*

Society **135**, 1830-1837 (2013).

115. Katoh, T., Tajima, K. & Suga, H. Consecutive Elongation of D-Amino Acids in Translation. *Cell Chemical Biology* (2016).

116. Fujino, T., Goto, Y., Suga, H. & Murakami, H. Ribosomal Synthesis of Peptides with Multiple β-Amino Acids. *Journal of the American Chemical Society* **138**, 1962-1969 (2016).

117. Kawakami, T., Murakami, H. & Suga, H. Messenger RNA-programmed incorporation of multiple N-methyl-amino acids into linear and cyclic peptides. *Chemistry & biology* **15**, 32-42 (2008).

118. Kawakami, T., Murakami, H. & Suga, H. Ribosomal Synthesis of Polypeptoids and Peptoid − Peptide Hybrids. *Journal of the American Chemical Society* **130**, 16861-16863 (2008).

119. Ohta, A., Murakami, H., Higashimura, E. & Suga, H. Synthesis of polyester by means of genetic code reprogramming. *Chemistry & biology* **14**, 1315-1322 (2007).

120. Ohta, A., Murakami, H. & Suga, H. Polymerization of α - Hydroxy Acids by Ribosomes. *ChemBioChem* **9**, 2773-2778 (2008).

121. Goto, Y. et al. Reprogramming the translation initiation for the synthesis of physiologically stable cyclic peptides. *ACS chemical biology* **3**, 120-129 (2008).

122. Sako, Y., Goto, Y., Murakami, H. & Suga, H. Ribosomal synthesis of peptidase-resistant peptides closed by a nonreducible inter-side-chain bond. *ACS chemical biology* **3**, 241-249 (2008).

123. Sako, Y., Morimoto, J., Murakami, H. & Suga, H. Ribosomal synthesis of bicyclic peptides via two orthogonal inter-side-chain reactions. *Journal of the American Chemical Society* **130**, 7232-7234 (2008).

124. Yamagishi, Y., Ashigai, H., Goto, Y., Murakami, H. & Suga, H. Ribosomal synthesis of cyclic peptides with a fluorogenic oxidative coupling reaction. *ChemBioChem* **10**, 1469-1472 (2009).

125. Melby, J.O., Nard, N.J. & Mitchell, D.A. Thiazole/oxazole-modified microcins: complex natural products from ribosomal templates. *Current opinion in chemical biology* **15**, 369-378 (2011).

126. Carmeli, S., Moore, R.E., Patterson, G.M., Corbett, T.H. & Valeriote, F.A. Tantazoles, unusual cytotoxic alkaloids from the blue-green alga Scytonema mirabile. *Journal of the American Chemical Society* **112**, 8195-8197 (1990).

127. Carmeli, S., Moore, R.E. & Patterson, G.L. Mirabazoles, minor tantazole-related cytotoxins from the terrestrial blue-green alga Scytonema mirabile. *Tetrahedron letters* **32**, 2593-2596 (1991).

128. Gaumont, A.-C., Gulea, M. & Levillain, J. Overview of the Chemistry of 2-Thiazolines. *Chemical reviews* **109**, 1371-1401 (2009).

129. Charette, A.B. & Chua, P. Mild method for the synthesis of thiazolines from secondary and tertiary amides. *The Journal of Organic Chemistry* **63**, 908-909 (1998).

130. Raman, P., Razavi, H. & Kelly, J.W. Titanium (IV)-mediated tandem deprotection− cyclodehydration of protected cysteine N-amides: biomimetic syntheses of thiazoline-and thiazole-containing heterocycles. *Organic letters* **2**, 3289-3292 (2000).

131. Numajiri, Y., Takahashi, T., Takagi, M., Shin-ya, K. & Doi, T. Total synthesis of largazole and its biological evaluation. *Synlett* **2008**, 2483-2486 (2008).

132. Sakakura, A., Kondo, R., Umemura, S. & Ishihara, K. Dehydrative cyclization of serine, threonine, and cysteine residues catalyzed by molybdenum (VI) oxo compounds. *Tetrahedron* **65**, 2102-2109 (2009).

133. Seebeck, F.P. & Szostak, J.W. Ribosomal synthesis of dehydroalanine-containing peptides. *Journal of the American Chemical Society* **128**, 7150-7151 (2006).

134. Hofmann, F.T., Szostak, J.W. & Seebeck, F.P. In vitro selection of functional lantipeptides. *Journal of the American Chemical Society* **134**, 8038-8041 (2012).

135. Pace, C.N., Vajdos, F., Fee, L., Grimsley, G. & Gray, T. How to measure and predict the molar absorption coefficient of a protein. *Protein science* **4**, 2411-2423 (1995).

136. Zipperer, A. et al. Human commensals producing a novel antibiotic impair pathogen colonization. *Nature* **535**, 511-516 (2016).

137. Drechsel, H. et al. Structure elucidation of yersiniabactin, a siderophore from highly virulent Yersinia strains. *European Journal of Organic Chemistry* **1995**, 1727-1733 (1995).

138. Cox, C.D., Rinehart, K.L., Moore, M.L. & Cook, J.C. Pyochelin: novel structure of an iron-chelating growth promoter for Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences* **78**, 4256-4260 (1981).

139. Miller, D.A., Luo, L., Hillson, N., Keating, T.A. & Walsh, C.T. Yersiniabactin synthetase: a four-protein assembly line producing the nonribosomal peptide/polyketide hybrid siderophore of Yersinia pestis. *Chemistry & biology* **9**, 333-344 (2002).

140. Patel, H.M. & Walsh, C.T. In vitro reconstitution of the Pseudomonas aeruginosa nonribosomal peptide synthesis of pyochelin: characterization of backbone tailoring thiazoline reductase and N-methyltransferase activities. *Biochemistry* **40**, 9023-9031 (2001).

141. Malins, L.R. & Payne, R.J. Synthetic amino acids for applications in peptide ligation–desulfurization chemistry. *Australian Journal of Chemistry* **68**, 521-537 (2015).

142. Dawson, P.E., Miur, T.W., Clark-Lewis, I. & Kent, S.B. Synthesis of proteins by native chemical ligation. *Science* **266**, 776-780 (1994).

143. Kent, S. et al. Through the looking glass–a new world of proteins enabled by chemical synthesis. *Journal of Peptide Science* **18**, 428-436 (2012).

144. Yan, L.Z. & Dawson, P.E. Synthesis of peptides and proteins without cysteine residues by native chemical ligation combined with desulfurization. *Journal of the American Chemical Society* **123**, 526-533 (2001).

145. Crich, D. & Banerjee, A. Native chemical ligation at phenylalanine. *Journal of the American Chemical Society* **129**, 10064-10065 (2007).

146. Haase, C., Rohde, H. & Seitz, O. Native chemical ligation at valine. *Angewandte Chemie International Edition* **47**, 6807-6810 (2008).

147. Chen, J., Wan, Q., Yuan, Y., Zhu, J. & Danishefsky, S.J. Native chemical ligation at valine: a contribution to peptide and glycopeptide synthesis. *Angewandte Chemie* **120**, 8649-8652 (2008).

148. Yang, R., Pasunooti, K.K., Li, F., Liu, X.-W. & Liu, C.-F. Dual native chemical ligation at lysine. *Journal of the American Chemical Society* **131**, 13592-13593 (2009).

149. Ajish Kumar, K., Haj‐Yahya, M., Olschewski, D., Lashuel, H.A. & Brik, A. Highly

efficient and chemoselective peptide ubiquitylation. *Angewandte Chemie International Edition* **48**, 8090-8094 (2009).

150. Chen, J., Wang, P., Zhu, J., Wan, Q. & Danishefsky, S.J. A program for ligation at threonine sites: application to the controlled total synthesis of glycopeptides. *Tetrahedron* **66**, 2277-2283 (2010).

151. Harpaz, Z., Siman, P., Kumar, K. & Brik, A. Protein synthesis assisted by native chemical ligation at leucine. *ChemBioChem* **11**, 1232-1235 (2010).

152. Shang, S., Tan, Z., Dong, S. & Danishefsky, S.J. An advance in proline ligation. *Journal of the American Chemical Society* **133**, 10784-10786 (2011).

153. Siman, P., Karthikeyan, S.V. & Brik, A. Native chemical ligation at glutamine. *Organic letters* **14**, 1520-1523 (2012).

154. Malins, L.R., Cergol, K.M. & Payne, R.J. Peptide ligation–desulfurization chemistry at arginine. *ChemBioChem* **14**, 559-563 (2013).

155. Thompson, R.E., Chan, B., Radom, L., Jolliffe, K.A. & Payne, R.J. Chemoselective peptide ligation–desulfurization at aspartate. *Angewandte Chemie International Edition* **52**, 9723-9727 (2013).

156. Cergol, K.M., Thompson, R.E., Malins, L.R., Turner, P. & Payne, R.J. One-Pot Peptide Ligation–Desulfurization at Glutamate. *Organic letters* **16**, 290-293 (2013).

157. Malins, L.R., Cergol, K.M. & Payne, R.J. Chemoselective sulfenylation and peptide ligation at tryptophan. *Chemical Science* **5**, 260-266 (2014).

158. Sayers, J., Thompson, R.E., Perry, K.J., Malins, L.R. & Payne, R.J. Thiazolidine-Protected beta-Thiol Asparagine: Applications in One-Pot Ligation-Desulfurization Chemistry. *Organic Letters* **17**, 4902-4905 (2015).

159. Wan, Q. & Danishefsky, S.J. Free‐radical‐based, specific desulfurization of cysteine: a powerful advance in the synthesis of polypeptides and glycopolypeptides. *Angewandte Chemie International Edition* **46**, 9248-9252 (2007).

160. Choudhary, A. & Raines, R.T. An Evaluation of Peptide‐Bond Isosteres. *ChemBioChem* **12**, 1801-1807 (2011).

161. Szelke, M. et al. Potent new inhibitors of human renin. (1982).

162. Oh, H.-S., Ko, S.-S., Cho, H. & Lee, K.-H. Design and synthesis of antibacterial pseudopeptides with a potent antibacterial activity and more improved stability from a short cationic antibacterial peptide. *Bull. Korean Chem. Soc* **26**, 161-164 (2005).

163. Kim, S.-M., Kim, J.-M., Joshi, B.P., Cho, H. & Lee, K.-H. Indolicidin-derived antimicrobial peptide analogs with greater bacterial selectivity and requirements for antibacterial and hemolytic activities. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics* **1794**, 185-192 (2009).

164. Xu, G.G., Zhang, Y., Mercedes-Camacho, A.Y. & Etzkorn, F.A. A reduced-amide inhibitor of Pin1 binds in a conformation resembling a twisted-amide transition state. *Biochemistry* **50**, 9545-9550 (2011).

165. Geyer, A., Mueller, G. & Kessler, H. Conformational Analysis of a Cyclic RGD Peptide Containing a .psi.[CH2-NH] Bond: A Positional Shift in Backbone Structure Caused by a Single Dipeptide Mimetic. *Journal of the American Chemical Society* **116**, 7735-7743 (1994).

166. Zivec, M., Jakopin, Z. & Gobec, S. Recent advances in the synthesis and applications of reduced amide pseudopeptides. *Current medicinal chemistry* **16**, 2289-2304 (2009).

167. Assem, N., Natarajan, A. & Yudin, A.K. Chemoselective peptidomimetic ligation using thioacid peptides and aziridine templates. *Journal of the American Chemical Society* **132**, 10986-10987 (2010).

168. Kourouklis, D., Murakami, H. & Suga, H. Programmable ribozymes for mischarging tRNA with nonnatural amino acids and their applications to translation. *Methods* **36**, 239-244 (2005).

169. Chen, H., O'Connor, S., Cane, D.E. & Walsh, C.T. Epothilone biosynthesis: assembly of the methylthiazolylcarboxy starter unit on the EpoB subunit. *Chemistry & biology* **8**, 899-912 (2001).

170. Emtenäs, H., Alderin, L. & Almqvist, F. An enantioselective ketene− imine cycloaddition method for synthesis of substituted ring-fused 2-pyridinones. *The Journal of organic*

chemistry **66**, 6756-6761 (2001).

171. Koniev, O. et al. Selective irreversible chemical tagging of cysteine with 3-arylpropiolonitriles. *Bioconjugate chemistry* **25**, 202-206 (2014).

172. Liao, W. et al. 1, 2, 3-Triazole-boranes: stable and efficient reagents for ketone and aldehyde reductive amination in organic solvents or in water. *Chemical Communications*, 6436-6438 (2009).

Acknoledgement

本研究を遂行し、学位論文をまとめるにあたり、ご指導ご鞭撻をいただいた菅裕明教授に心より感謝申し上げます。菅研究室の研究環境は十分すぎると言ってもいいほどで、研究の律速段階が自分自身であるという環境は、自己を鍛錬する場として最適なものだったと思います。菅先生のもとに集まった研究室のメンバーは国籍から研究のバックグラウンドにいたるまで多種多様であり、刺激的な研究生活・学生生活を送ることができました。ミーティングでの議論を通して、実験の細かい点を追求するだけでなく、研究を大局的に見る視点、そして何より研究を通して新しい世界を切り開こうという姿勢を少しでも会得できていたらと思います。菅研究室での 5 年間は、これから研究者として生きて行く上で欠かせない経験だったと確信しています。

本研究を進める上で、直接的に実験の指導をしていただきました後藤佑樹准教授に心より感謝申し上げます。翻訳後修飾の研究テーマを示していただいてから今日にいたるまで数え切れないほどのご指導をいただきました。研究がうまく進まず、苦しい中であっても実験データと向き合って方向性を探り続けることの困難さと重要さを学びました。5 年間ご指導をいただく中で、目指すべき研究者像の輪郭を描くことができました。

研究発表に関してご助言をいただきました、狩野直和准教授に感謝申し上げます。専門分野の異なる視点からご指摘をいただくことで、自分自身の研究を客観的に見る機会を得ることができました。また、研究に関するご助言および研究室運営を通して研究をサポートいただきました、加藤敬行助教に感謝申し上げます。おかげさまで研究に全力投球することができました。

研究を進めるにあたり、様々なご指導、ご助言をいただきました研究室の先輩方に感謝申し上げます。特に、分子生物学の研究をしていた私に有機化学の実験法をご指導いただきました、樋口岳博士、山田光博博士に感謝申し上げます。また、修士課程および博士課程で研究室生活を共にした、石橋正成氏、井関めぐみ氏、岩根由彦氏、高辻諒氏、角田翔太郎氏、西尾洸祐氏、村上直央氏に感謝申し上げます。特に岩根氏、高辻氏、西尾氏とは 5 年間、学生生活を共に過ごしました。互いに切磋琢磨し、支え合える同期がいてくれたおかげでなんとか卒業を迎えることができたと思っています。加えて、研究室の後輩の皆様に感謝申し上げます。研究で煮詰まったときでも無駄話に付き合ってもらうことで、元気を分けていただきました。

なお、本研究を遂行するにあたり、日本学術振興会より 2 年間、特別研究員奨励費の助成をいただきました。感謝申し上げます。

最後に、これまでの学生生活を暖かく見守ってくれた両親に感謝の意を表し、本論文の末尾の言葉とさせていただきます。