DOI: 10.1002/cbic.200900058

# Ribosomal Synthesis of Peptides with C-Terminal Lactams, Thiolactones, and Alkylamides

Eiji Nakajima,[a] Yuki Goto,[a] Yusuke Sako,[a] Hiroshi Murakami,[b] and Hiroaki Suga*[a, b]

The C terminus of a peptide expressed by the translation apparatus generally ends in a carboxylate group. On the other hand, the C termini of some naturally occurring peptides have amide moieties instead of carboxylates, which are believed to give better biostability. Here, we describe a new strategy for the ribosomal synthesis of peptides featuring C-terminal lactam, thiolactone, and alkylamide units. The method was based on the concept of genetic code reprogramming involving the flexizymes (flexible tRNA acylation ribozymes) and the PURE (peptide synthesis using recombinant elements) system, in which vacant codons are reassigned to nonproteinogenic amino acids; this enabled us to convert the C termini of peptides into the above functionalities. We have also applied this method to the synthesis of a macrocyclic peptide closed by an amide bond formed between a lysine side chain and the peptide C terminus. This method thus offers us new opportunities to express various peptides with C-terminal modifications as well as macrocyclic peptides using the translation apparatus, and potentially to accelerate the discovery of peptidic drugs designed for various therapeutic targets.

## Introduction

The C terminus of a peptide expressed by the translation apparatus generally ends in a carboxylate group, which is generated by ribosome-catalyzed hydrolysis of the carboxy ester bond between the C-terminal amino acid and the 3′-OH of peptidyl-tRNA and triggered by binding of the release factors to the ribosome A site.[1,2] On the other hand, it is known that the C termini of some naturally occurring peptides are modified by an amide instead of the carboxylate,[3–5] and potentially contribute to their biostability.[6–8] These C-terminal amide groups are generated by enzymatic oxidative cleavage at the α positions of the subsequent amino acids in the parent peptides.[9] More recently, intein, known as a protein-splicing enzyme, has been engineered to trap the thioester intermediate, which subsequently reacts with an externally added amine to form the C-amidated peptide.[10] Notably, this methodology has been applied to C-terminal modifications featuring not only simple amides, but also alkylamides.[11,12]

We have been engaged in a research program directed towards the development of new methodologies for the ribosomal synthesis of nonstandard peptides. Since the translation apparatus is restricted to the use of the 20 standard proteinogenic amino acids assigned by triplet nucleotides (codons) on mRNA, the expressed peptides generally consist of combinations of these amino acids. However, by breaking the "natural" assignments between codons and proteinogenic amino acids, it is possible to express nonstandard peptides containing multiple nonproteinogenic amino acids. We refer to this "artificial" codon reassignment as genetic code reprogramming.[13–22] Despite the simplicity of the concept behind this approach, there have been two major technical obstacles to its achievement: 1) how the natural assignment of the codon or codon box to the proteinogenic amino acid can be broken, that is, how can the codon or codon box be made vacant for the reassignment; and 2) how the nonproteinogenic amino acid(s) can be charged with a tRNA bearing the anticodon corresponding to the vacant codon.

We have utilized two systems to overcome these inconveniences. To assist with point (1), we have taken advantage of the manipulatability of a reconstituted *E. coli* cell-free translation system, often referred to as the PURE (peptide synthesis using recombinant elements) system.[23–25] The most important feature of this translation system is that certain amino acids, and, if necessary, aminoacyl-tRNA synthetases, can be withdrawn from the translation elements. Use of such a withdrawn PURE system, termed *w*PURE, allows us to create desired vacant codons.

To assist with point (2), we have developed an enzyme system, named flexizyme system, to facilitate the preparation of tRNAs charged with various nonproteinogenic amino acids. It consists of two flexizymes, dFx and eFx, that can be used depending on the choice of leaving group in the substrates: dFx for a 3,5-dinitrobenzyl ester (DBE) and eFx either for a 4-chlorobenzyl thioester (CBT) or for a cyanomethyl ester (CME). The use of a combination of these two systems has thus allowed us to reprogram the genetic code, and enabled ribosomal

[a] E. Nakajima, Dr. Y. Goto, Dr. Y. Sako, Prof. H. Suga
Department of Chemistry and Biotechnology
Graduate School of Engineering, The University of Tokyo
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)
Fax: (+81) 35-452-5495
E-mail: hsuga@rcast.u-tokyo.ac.jp

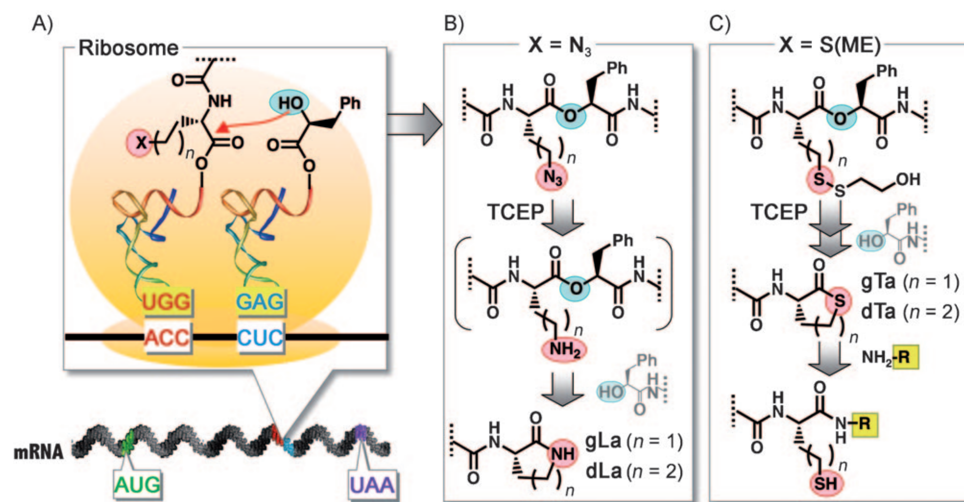
[b] Dr. H. Murakami, Prof. H. Suga
Research Center for Advanced Science and Technology
The University of Tokyo
4-6-1 Komaba, Meguro-ku, Tokyo 153-8904 (Japan)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900058.

 InterScience © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 

synthesis of nonstandard peptides, the sequences and compositions of which are encoded in the mRNA sequences.[26–36]

As a new application of this genetic code reprogramming, here we report a methodology for the ribosomal synthesis of peptides featuring C-terminal modifications involving the site-specific incorporations of γ-azidohomoalanine (Aha) or 2-mercaptoethanol-masking homocysteine ((ME)Hcy) followed by phenyllactic acid ($F^{lac}$) under the reprogrammed genetic code (Scheme 1 A). The resulting peptides contain a peptide bond originating from the above nonproteinogenic amino acid, followed by a single ester bond originating from $F^{lac}$. Subsequent treatment of such peptides with tris(2-carboxyethyl)phosphine (TCEP) triggers the conversion of the terminal residue either into a γ-lactam (Scheme 1 B, gLa) or into a γ-thiolactone (gTa) that can be converted into a variety of alkylamides (Scheme 1 C). Moreover, when a lysine residue is incorporated upstream of the C-terminal gTa, macrocyclization takes place to afford a cyclic peptide. This methodology thus enables us to prepare a wide variety of C-terminal-modified peptides using the mRNA-directed translation platform.




**Scheme 1.** A strategy for ribosomal synthesis of nonstandard peptides containing lactam, thiolactone, and alkylamide moieties. A) Schematic presentation of successive incorporation of a nonproteinogenic amino acid (Xaa) and phenyllactic acid ($F^{lac}$) in a ribosome. Xaa-$tRNA^{Asn-E1}_{GGU}$ and $F^{lac}$-$tRNA^{Asn-E1}_{GAG}$ decode ACC and CUC codons, respectively, on the mRNA and result in the Xaa-$F^{lac}$ sequence, linked by an ester bond. B) Post-translational TCEP reduction of the azide side chain, which triggers the formation of γ/δ-lactams at the C terminus of the peptide; TCEP: tris(2-carboxyethyl)phosphine. C) Post-translational TCEP reduction of the ME-protected sulfhydryl side chain, which triggers the formation of a γ/δ-thiolactone, followed by inter- or intramolecular amidation at the C terminus of the peptide; ME: 2-mercaptoethanol.

## Results and Discussion

### Incorporation of the unnatural substrates and synthesis of C-terminally cyclized peptides

To construct the foundation for the above methodology, we first examined the incorporation of a nonproteinogenic amino acid bearing a nucleophilic side chain, such as a γ-amino or thio group, followed by $F^{lac}$ into a peptide chain at specific sites; the expressed peptide should then self-rearrange to afford the corresponding γ-lactam or thiolactone. Unfortunately, initial attempts making direct use either of γ-aminohomoalanine or of Hcy did not succeed in activation of the carboxyl group by DBE for tRNA aminoacylation (vide infra) because of the occurrence of rapid self-cyclization. We therefore revised the strategy and masked the amino group in γ-aminohomoalanine by replacing it with a γ-azide moiety (Figure 1 A, Aha). In the case of Hcy, its sulfhydryl group was masked by a disulfide bond with 2-mercaptoethanol (ME; Figure 1 A, (ME)Hcy). Conveniently, both masking groups could be converted into the corresponding nucleophiles by exposure to TCEP; thereby the system was primed to trigger the desired spontaneous C-terminal cyclization.

To incorporate Aha or (ME)Hcy (for convenience, we refer to these nonproteinogenic amino acids as Xaa) and $F^{lac}$ successively into a peptide chain at the designated positions, threonine (T; ACC) and leucine (L; CUC) codons were chosen for the reassignment of Xaa and $F^{lac}$, respectively. The reassignment was achieved by the combination of the appropriate flexizyme and *w*PURE systems. Xaa and $F^{lac}$ were esterified with DBE and CME groups, respectively, and thus were made suitable substrates for dFx and eFx (Figure 1 A). To make the ACC and CUC codons vacant, we prepared a *w*PURE system in which amino acids T and L were withdrawn. The reassignment of Xaa and $F^{lac}$ to these vacant codons was achieved by preparation of the corresponding acyl-tRNAs bearing the anticodons. Specifically, $tRNA^{Asn-E1}_{GGU}$[27] was charged with Aha and (ME)Hcy by using dFx, while $tRNA^{Asn-E1}_{GAG}$ was charged with $F^{lac}$ by using eFx. Similarly, $tRNA^{Asn-E1}_{GGU}$ was charged with δ-azidonorvaline (Anv) and δ-(ME)mercaptonorvaline ((ME)Mnv; Figure 1 A, $n=2$).[27] The acylation efficiency of each substrate was confirmed by using a tRNA analogue: microhelix RNA (Figure S1 in the Supporting Information). These acyl-tRNAs were thus used to express the desired nonstandard peptides.

To observe the successive incorporations of Xaa followed by $F^{lac}$ into the peptide chain, we designed an mRNA template expressing a model peptide (Figure 1 B, mR1 and P1-Xaa-$F^{lac}$). Note that in this model peptide the FLAG sequence was upstream of Xaa-$F^{lac}$. This allowed us to purify the peptide using anti-FLAG antibody. In this way, the whole translated products could be isolated for the detection not only of the expected full-length peptide but also of truncated peptides generated by potential failures of the codon reassignments. When

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 

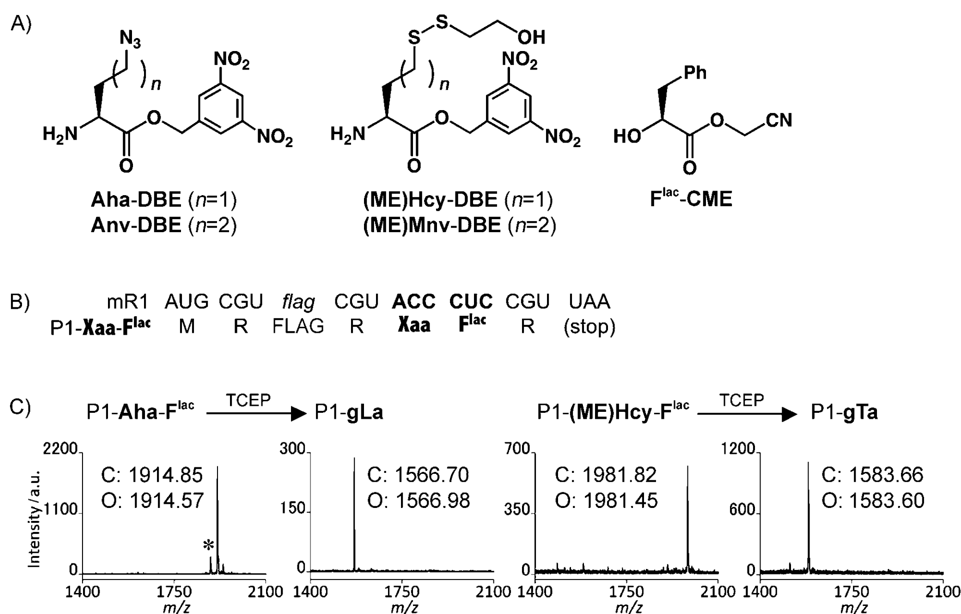



**Figure 1.** Ribosomal synthesis of the C-terminal γ/δ-lactam- and γ/δ-thiolactone-containing peptides, P1-gLa and P1-gTa, respectively. A) Structures of the nonproteinogenic amino acid DBEs and phenyllactic acid CME; Aha: azidohomoalanine; Anv: azidonorvaline; (ME)Hcy: 2-mercaptoethanol-protected homocysteine; (ME)Mnv: 2-mercaptoethanol-protected mercaptonorvaline; DBE: 3,5-dinitrobenzyl ester; CME: cyanomethyl ester. B) Sequences of mR1 and P1-Xaa-$F^{lac}$; *flag* indicates the RNA sequence coding the FLAG peptide (DYKDDDDK). Xaa and $F^{lac}$ are assigned to codons ACC and CUC, respectively. C) MALDI-TOF analysis of the expressed peptides. The calculated (C) and observed (O) molecular masses for singly charged species, $[M+H]^+$, of the peptide are shown in each spectrum. TCEP reduction of P1-Aha-$F^{lac}$ and P1-(ME)Hcy-$F^{lac}$ resulted in the formation of γ-lactam and γ-thiolactone, respectively, at the peptide C terminus. The asterisk (*) denotes a peak of P1-Aha-$F^{lac}$ in which Aha was presumably reduced by DTT present in the translation mixture; calcd: 1890.02, found: 1890.71.

translation was executed in the presence of Xaa-$tRNA^{Asn-E1}_{GGU}$ and $F^{lac}$-$tRNA^{Asn-E1}_{GAG}$ in the *w*PURE system, and the FLAG-isolated product obtained from the experiment was analyzed by MALDI-TOF, a single major peak was observed (Figure 1C, P1-Aha-$F^{lac}$ and P1-(ME)Hcy-$F^{lac}$). In all cases, the major peak was consistent with the expected molecular mass of the full-length peptide, whereas a minor peak observed in the P1-Aha-$F^{lac}$ spectrum (Figure 1C, indicated by asterisk) was assigned to the full-length peptide with the amino group, and was presumably generated by the dithiothreitol reduction of the azide side chain.[37] The peptide expression directed by the reprogrammed codons thus successfully yielded the expected full-length peptide.

Subsequent TCEP reduction of the masking group triggered the self-cyclization of the Xaa residue, and released the $F^{lac}$-R dipeptide. MALDI-TOF analysis of the product generated from the P1-Aha-$F^{lac}$ peptide gave a single major peak that was consistent with the expected MS value for P1-gLa (Figure 1C). Similarly, TCEP reduction of P1-(ME)Hcy-$F^{lac}$ afforded the corresponding thiolactone, P1-gTa. The same procedure was also employed for P1-Anv-$F^{lac}$ and P1-(ME)Mnv-$F^{lac}$ to yield the corresponding C-terminal δ-lactam and thiolactone peptides (Figure S2 in the Supporting Information). These data clearly demonstrate that the C-terminal rearrangement from Xaa-$F^{lac}$ to the γ/δ-lactam or thiolactone cleanly took place in these model peptides expressed with the reprogrammed codons.

To verify the versatility of this methodology, we translated another mRNA (mR2) encoding a longer and more complex peptide sequence: P2-Xaa-$F^{lac}$ (Figure 2A). For this 16-mer peptide, mR2 was translated in the presence of [$^{14}$C]-Asp, so that the expression of the corresponding radiolabeled product could be monitored by appropriate PAGE analysis (Figure S3 in the Supporting Information). Notably, high expression levels were observed only in the presence of both Xaa-$tRNA^{Asn-E1}_{GGU}$ and $F^{lac}$-$tRNA^{Asn-E1}_{GAG}$, and the observed expression level of P2-Xaa-$F^{lac}$ fell to 33–58% of that of wild-type P2-Thr-Leu, the quantity of which was determined to be approximately 3 pmol $\mu L^{-1}$. As in the case of the P1-Xaa-$F^{lac}$, C-terminal γ-lactam and γ-thiolactone formation of P2-Xaa-$F^{lac}$ was triggered by TCEP reduction. Because the purification tag was not implanted in this set of peptides, the product was simply desalted by using a resin-modified micropipette tip. Even after such a simple workup, MALDI-TOF analysis both of

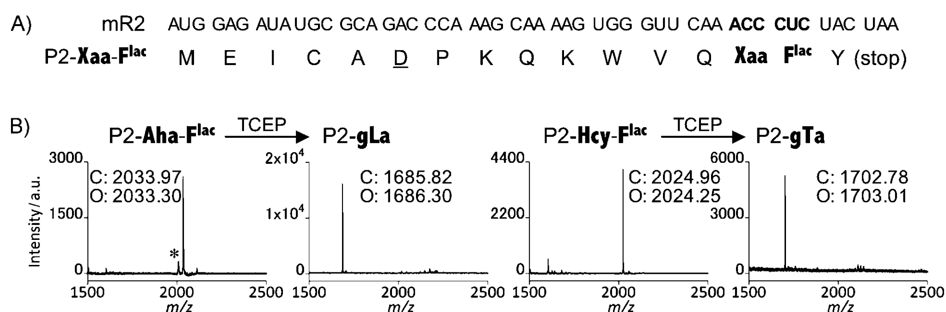



**Figure 2.** Ribosomal synthesis of the C-terminal γ/δ-lactam- and γ/δ-thiolactone-containing peptides, P2-gLa and P2-gTa, respectively. A) Sequences of mR2 and P2-Xaa-$F^{lac}$. Xaa and $F^{lac}$ are assigned to codons ACC and CUC, respectively. B) MALDI-TOF analysis of the peptides. Calculated (C) and observed (O) molecular masses for the singly charged species, $[M+H]^+$, of the peptide are shown in each spectrum. TCEP reduction of P2-Aha-$F^{lac}$ and P2-(ME)Hcy-$F^{lac}$ resulted in the formation of γ-lactam and γ-thiolactone, respectively, at the peptide C termini. The asterisk (*) denotes a peak of P2-Aha-$F^{lac}$ in which Aha was presumably reduced by DTT present in the translation mixture; calcd: 2008.02, found: 2008.54.

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 

the precursor peptide (Figure 2 B, P2-Aha-$F^{lac}$ or P2-Hcy-$F^{lac}$) and of the peptide containing C-terminal γ-lactam or γ-thiolactone moieties (Figure 2 B, P2-gLa and P2-gTa) showed clean single major peaks consistent with the expected MS values. Likewise, expression and TCEP conversion of the peptides into the corresponding C-terminal δ-lactam and δ-thiolactone peptides was also successfully executed (Figure S4 in the Supporting Information, P2-dLa and P2-dTa).

### Synthesis of C-terminally modified peptides and of a macrocyclized peptide with the γ-thiolactones

Because a C-terminal thiolactone might be reactive toward amine nucleophiles,[38,39] we were interested in converting it into various C-terminal amide groups (Figure 3 A). P1-gTa was accordingly treated with thirteen different amines, from simple ammonium hydroxide to a fluorescent-labeled alkylamine (Figure 3 B, **1**–**13**). On addition of an excess amount of the individual amine to the P1-gTa, the desired C-terminal amidation of P1-gTa occurred cleanly, and occasionally along with oxidation of the liberated sulfhydryl group with ME (Figure 3 C). It should be noted that ME was one of the standard reagents included both in the ordinary PURE and in the *w*PURE systems, and such ME adducts were presumably generated by oxidation by air during the amidation process. In addition, P2-gTa was also subjected to C-terminal amidation with the three chosen amines, **1**, **9**, and **13**, to yield the desired C-amide peptides.

Lastly, we attempted macrocyclization of a peptide, through linking between the C terminus and an amino group in a lysine (K) side chain from the corresponding γ-thiolactone peptide intermediate. We designed a peptide, P3-Hcy-$F^{lac}$, containing a single K residue upstream of Hcy-$F^{lac}$, and expressed it from the parent mRNA (mR3; Figure 4 A). The expressed peptide was treated with TCEP to induce the C-terminal γ-thiolactone formation, and this was followed by incubation with ME under basic conditions to trigger the desired macrocyclization (Figure 4 B). MALDI-TOF analyses both of the intermediate peptide (P3-gTa) and of the macrocyclic peptide (mcP3-(ME)Hcy) confirmed the desired conversion (Figure 4 C). This demonstrates that the amidation methodology developed here is applicable to both intermolecular and intramolecular reactions, and has enabled us to prepare a wide array of C-terminal peptide-alkylamides.

## Conclusions

We have developed a new methodology for the ribosomal synthesis of peptides incorporating C-terminal lactam, thiolactone, and alkylamide moieties. The key technical development involves the ribosomal incorporation of a nonproteinogenic amino acid (Xaa), in which the amino or thio nucleophile in the side chain is masked (e.g., Aha or (ME)Hcy), followed by $F^{lac}$, at the C-terminal region of a peptide by means of genetic code reprogramming. TCEP reduction of the masking group exposes the amino or sulfhydryl nucleophile, and triggers C-terminal γ-lactam (gLa) or thiolactone (gTa) formation. Moreover, the C-terminal γ-thiolactone can be amidated by treatment with various alkylamines. Intramolecular amidation between a lysine residue and the C-terminal gTa yields a macrocyclic peptide. Since such a modification confers biostability on the peptide, the bioactivity of the peptide can be enhanced.[6–8] Most importantly, the ribosomal synthesis of the peptides is mRNA encoded, which facilitates the preparation of peptide libraries.[29] Because C-terminal amide modification of linear and cyclic peptides should enhance their physiological stability, this methodology gives us an opportunity to explore the sequence space of such peptides and could potentially yield new peptidic drugs in the future.

## Experimental Section

**Preparation of aminoacyl-tRNAs with flexizymes**: Aminoacylation reactions were performed by the following procedure: $tRNA^{Asn\text{-}E1}$ (40 μм) in Tris-HCl (0.2 м, pH 8.0, 6.25 μL) was heated at 95 °C for 1 min, and the system was then allowed to cool to room temperature over 5 min. $MgCl_2$ (3 м, 2.5 μL) and dFx (200 μм, 1.25 μL) were then added and the solution was incubated at room temperature for 5 min. Substrate in DMSO (25 mм, 2.5 μL) was then added and the mixture was incubated on ice for 2 h. The acylation reaction was quenched by addition of sodium acetate (0.6 м, pH 5.0, 40 μL), and the RNA was recovered by ethanol precipitation. The pellet was rinsed twice with ethanol (70 %) containing sodium acetate (0.1 м, pH 5.0), and once with ethanol (70 %). The pellet was dried and stored at −80 °C.

**Ribosomal synthesis of wild-type peptides and of peptides containing nonproteinogenic amino acids/hydroxy acids**: The ordinary PURE system was reconstituted with ribosome, 20 aminoacyl RNA synthetases, protein factors, necessary organic and inorganic components, and the 20 proteinogenic amino acids (0.2 mм each); for tricine SDS-PAGE analysis, [$^{14}$C]-Asp (50 μм) was used in place of Asp, as reported elsewhere.[29] Control wild-type peptide was translated with this PURE system in the presence of DNA template (40 nм) at 37 °C for 1 h, in a total volume of 5 μL. The *w*PURE system was reconstituted with the same components as the PURE system, except that only the necessary proteinogenic amino acids (0.2 mм each) and acyl-tRNAs (50 μм each) were added to the mixture, depending upon the peptide being translated. For the synthesis of P1, which contained the nonproteinogenic amino acids Aha, Anv, (ME)Hcy or (ME)Mnv, and phenyllactic acid ($F^{lac}$), the *w*PURE system in the presence of DNA template D1 (40 nм), Met, Arg, Tyr, Lys, and Asp (0.2 mм each), and Aha-$tRNA^{Asn\text{-}E1}_{GGU}$, Anv-$tRNA^{Asn\text{-}E1}_{GGU}$, (ME)Hcy-$tRNA^{Asn\text{-}E1}_{GGU}$, or (ME)Mnv-$tRNA^{Asn\text{-}E1}_{GGU}$ (50 μм each), and $F^{lac}$-$tRNA^{Asn\text{-}E1}_{GAG}$ (50 μм) were used. For the synthesis of P2, which contained the nonproteinogenic amino acids Aha, Anv, (ME)Hcy, or (ME)Mnv, and phenyllactic acid ($F^{lac}$), the above *w*PURE system in the presence of DNA template D2 (40 nм), Met, Glu, Ile, Cys, Ala, Pro, Lys, Gln, Trp, Val, Arg, Tyr, Asp (0.2 mм each; Asp was replaced with 50 μм [$^{14}$C]-Asp for tricine SDS-PAGE analysis), and Aha-$tRNA^{Asn\text{-}E1}_{GGU}$ or Anv-$tRNA^{Asn\text{-}E1}_{GGU}$, (ME)Hcy-$tRNA^{Asn\text{-}E1}_{GGU}$, or (ME)Mnv-$tRNA^{Asn\text{-}E1}_{GGU}$ (50 μм each), and $F^{lac}$-$tRNA^{Asn\text{-}E1}_{GAG}$ (50 μм) were used. For tricine SDS-PAGE analysis the reaction mixture (2.5 μL) was mixed with loading buffer (2.5 μL; 0.9 м Tris-HCl, pH 8.5, 30 % glycerol, and 8 % SDS) and analyzed by 15 % tricine SDS-PAGE.

**Mass spectra measurements of peptides**: For the mass analysis a sample (5 μL) of the reaction mixture was mixed with 2×TBS (5 μL; 100 mм Tris-HCl, pH 8.0, 300 mм NaCl) and incubated in prewashed FLAG-M2 agarose (Sigma) for 1 h. The resin was then washed once with TBS (50 mм Tris-HCl, pH 8.0, 150 mм NaCl; 10 μL), and

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 

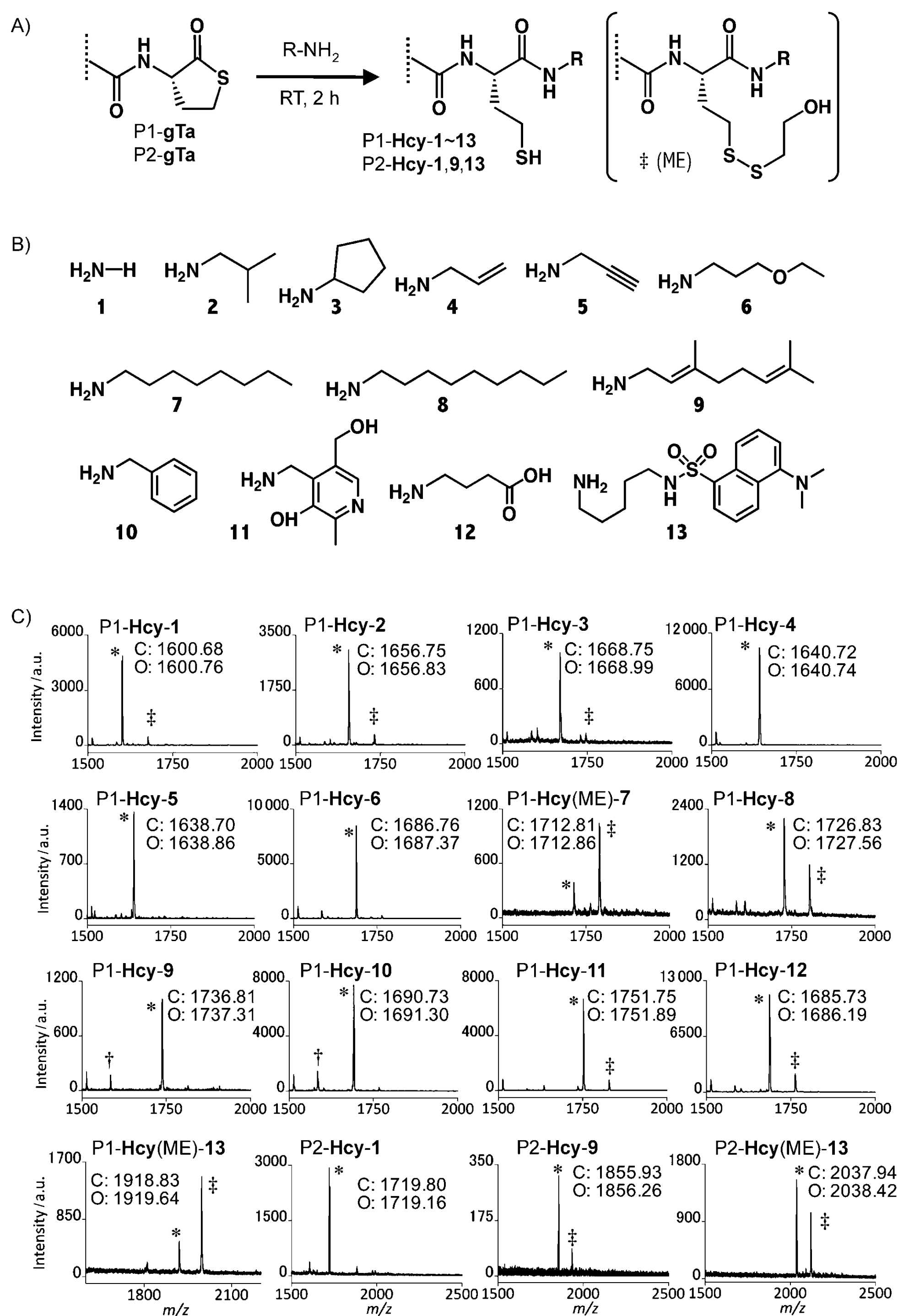



**Figure 3.** Amidation of P1-gTa and P2-gTa with a variety of amines. A) Schematic representation of C-terminal amidation with γ-thiolactones. P1-gTa and P2-gTa were incubated with amines that opened their γ-thiolactone moieties to yield the alkylamides. The products in parentheses represent the oxidation of the sulfhydryl group of Hcy with ME. B) Primary amines used in this study: ammonia (**1**), isobutylamine (**2**), cyclopentylamine (**3**), allylamine (**4**), propargylamine (**5**), 3-ethoxypropylamine (**6**), octylamine (**7**), nonylamine (**8**), geranylamine (**9**), benzylamine (**10**), pyridoxine (**11**), 4-aminobutanoic acid (**12**), and dansylcadaverine (**13**). The C terminus of P1-gTa was subjected to amidation with all of the amines, whereas that of P2-gTa was subjected to **1**, **9**, or **13**. C) MALDI-TOF MS spectra of the C-terminally modified P1 and P2. Calculated (C) and observed (O) molecular masses for the singly charged species, $[M+H]^+$, of the peptide are shown in each spectrum. Asterisks (*) indicate peaks corresponding to the peptide modified with each amine at the C terminus. Daggers (†) indicate unreacted P1-gTa, and double daggers (‡) indicate products with sulfhydryl groups oxidized with ME, in which the molecular mass of P1-Hcy-X (X = **1**–**13** is increased by approximately 76.

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 

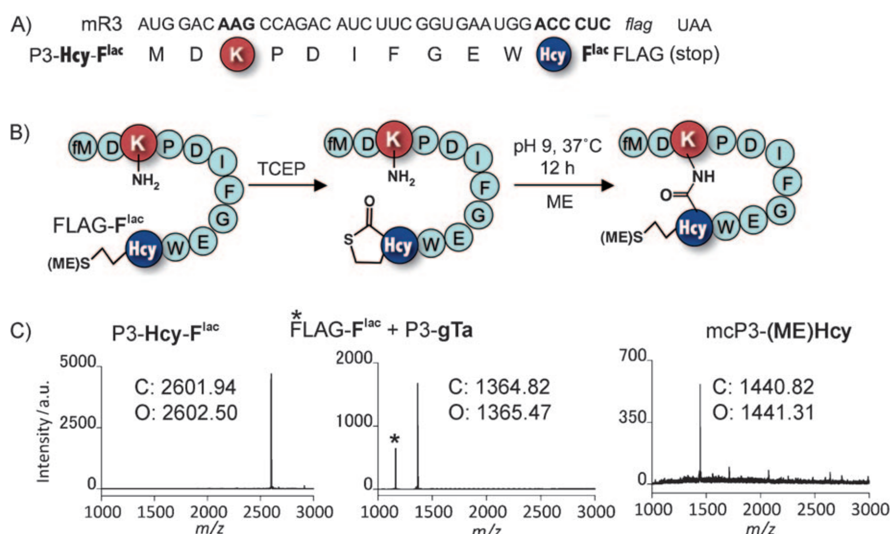



**Figure 4.** Macrocyclization of P3 with γ-thiolactone. A) Sequences of mR3 and P3-Xaa-F$^{lac}$. (ME)Hcy and F$^{lac}$ are assigned to codons ACC and CUC, respectively. B) Schematic representation of the macrocyclization with P3-gTa. TCEP reduction of P3-Hcy-F$^{lac}$ induced the formation of P3-gTa, which was incubated with ME (10 mM) at pH 9.0, 37 °C for 12 h. These procedures afforded mcP3-(ME)Hcy through linkage between the C terminus and the side chain of the K residue. C) MALDI-TOF analysis of the corresponding peptides. Calculated (C) and observed (O) molecular masses for the singly charged species, $[M+H]^+$, of the peptide are shown in each spectrum. The asterisk (*) denotes a peak corresponding to liberated F$^{lac}$-FLAG from P3-Hcy-F$^{lac}$; calcd: 1162.13, found: 1163.44.

the immobilized peptide was eluted with TFA (0.2%, 5 μL). The resulting peptide was desalted with a C18 micro-ZipTip (Millipore), and eluted with aq. acetonitrile (50%, 1 μL, 0.1% TFA), saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid. Mass measurements were performed by MALDI-TOF (autoflex TOF/TOF, Bruker).

**Synthesis of C-terminal γ/δ-lactams or thiolactones**: For the C-terminally cyclized peptides, translation product (5 μL) was mixed with TCEP (100 mM, 0.5 μL) and bicine-KOH (1 M, pH 9.0, 0.5 μL) at room temperature for 2 h. For the confirmation of cyclization, the mixture was purified on FLAG resin, eluted with TFA (0.2%, 5 μL), and analyzed by MALDI-TOF.

**C-terminal modification with various alkylamides**: For C-terminal modification with various alkylamides, the C-terminal γ-thiolactone peptide was prepared as described above, in 5 μL total volume. The mixture was incubated with alkylamine (2 M, 0.5 μL, ammonia, isobutylamine, cyclopentylamine, allylamine, propargylamine, 3-ethoxypropylamine, octylamine, nonylamine, geranylamine, benzylamine, pyridoxine, 4-aminobutanoic acid, or dansylcadaverine, as shown in Figure 3 B) at 25°C for 2 h. These alkylamines were purchased from Kanto Chemicals (Tokyo, Japan), Sigma–Aldrich (Japan), or Nacalai Tesque (Kyoto, Japan). For the confirmation of modification, the mixture was purified on FLAG resin, eluted with TFA (0.2%, 2 μL), and analyzed by MALDI-TOF. Note that ME (1 mM) was included in the *w*PURE system as one of the standard reagents, and occasionally formed a disulfide bond with the free sulfhydryl group in residues such as Hcy and Cys by air oxidation.

**Peptide macrocyclization**: For the macrocyclization of the peptide, the C-terminal γ-thiolactone peptide was synthesized as described above in a total volume of 5 μL, and purified on FLAG resin and eluted with TFA (0.2%, 5 μL). The eluent was incubated with 2-mercaptoethanol (100 mM, 0.5 μL) and bicine-KOH (1 M, pH 9.0, 0.5 μL) at 37 °C for 12 h to induce the macrocyclization between the C terminus and the lysine side chain.

## Acknowledgements

*This work was supported by Technology Program grants from the New Energy and Industrial Technology Development Organization (NEDO) and the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (S) (16101007) to H.S., and from the Global COE Program "Chemistry Innovation through Cooperation of Science and Engineering", MEXT, Japan to E.N.*

**Keywords:** genetic code · peptidomimics · protein engineering · ribosomal synthesis · ribozymes

[1] L. L. Kisselev, R. H. Buckingham, *Trends Biochem. Sci.* **2000**, *25*, 561.
[2] S. Petry, A. Weixlbaumer, V. Ramakrishnan, *Curr. Opin. Struct. Biol.* **2008**, *18*, 70.
[3] D. J. Merkler, *Enzyme Microb. Technol.* **1994**, *16*, 450.
[4] A.-J. Ren, Z.-F. Guo, Y.-K. Wang, L. Lin, X. Zheng, W.-J. Yuan, *Peptides* **2009**, *30*, 439.
[5] V. Sanchez-Margalet, M. Lucas, R. Goberna, *J. Mol. Endocrinol.* **1996**, *16*, 1.
[6] L. Hilsted, K. Hint, J. Christiansen, J. F. Rehfeld, *Gastroenterology* **1988**, *94*, 96.
[7] K. R. Shoemaker, P. S. Kim, D. N. Brems, S. Marqusee, E. J. York, I. M. Chaiken, J. M. Stewart, R. L. Baldwin, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 2349.
[8] A. Wettergren, L. Pridal, M. Wojdemann, J. J. Holst, *Regul. Pept.* **1998**, *77*, 83.
[9] A. F. Bradbury, D. G. Smyth, *Biosci. Rep.* **1987**, *7*, 907.
[10] I. R. Cottingham, A. Millar, E. Emslie, A. Colman, A. E. Schnieke, C. McKee, *Nat. Biotechnol.* **2001**, *19*, 974.
[11] T. J. Tolbert, C.-H. Wong, *J. Am. Chem. Soc.* **2000**, *122*, 5421.
[12] J. Mukhopadhyay, A. N. Kapanidis, V. Mekler, E. Kortkhonjia, Y. W. Ebright, R. H. Ebright, *Cell* **2001**, *106*, 453.
[13] B. Zhang, Z. Tan, L. G. Dickson, M. N. Nalam, V. W. Cornish, A. C. Forster, *J. Am. Chem. Soc.* **2007**, *129*, 11316.
[14] Z. Tan, A. C. Forster, S. C. Blacklow, V. W. Cornish, *J. Am. Chem. Soc.* **2004**, *126*, 12752.
[15] Z. Tan, S. C. Blacklow, V. W. Cornish, A. C. Forster, *Methods* **2005**, *36*, 279.
[16] A. O. Subtelny, M. C. Hartman, J. W. Szostak, *J. Am. Chem. Soc.* **2008**, *130*, 6131.
[17] C. Merryman, R. Green, *Chem. Biol.* **2004**, *11*, 575.
[18] K. Josephson, M. C. Hartman, J. W. Szostak, *J. Am. Chem. Soc.* **2005**, *127*, 11727.
[19] M. C. Hartman, K. Josephson, C. W. Lin, J. W. Szostak, *PLoS ONE* **2007**, *2*, e972.
[20] A. Frankel, S. W. Millward, R. W. Roberts, *Chem. Biol.* **2003**, *10*, 1043.
[21] A. C. Forster, H. Weissbach, S. C. Blacklow, *Anal. Biochem.* **2001**, *297*, 60.
[22] A. C. Forster, Z. Tan, M. N. Nalam, H. Lin, H. Qu, V. W. Cornish, S. C. Blacklow, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6353.
[23] Y. Shimizu, Y. Kuruma, B. W. Ying, S. Umekage, T. Ueda, *FEBS J.* **2006**, *273*, 4133.
[24] Y. Shimizu, T. Kanamori, T. Ueda, *Methods* **2005**, *36*, 299.
[25] Y. Shimizu, A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa, T. Ueda, *Nat. Biotechnol.* **2001**, *19*, 751.


© 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[26] H. Murakami, A. Ohta, H. Ashigai, H. Suga, *Nat. Methods* **2006**, *3*, 357.
[27] A. Ohta, H. Murakami, E. Higashimura, H. Suga, *Chem. Biol.* **2007**, *14*, 1315.
[28] Y. Goto, H. Murakami, H. Suga, *RNA* **2008**, *14*, 1390.
[29] Y. Goto, A. Ohta, Y. Sako, Y. Yamagishi, H. Murakami, H. Suga, *ACS Chem. Biol.* **2008**, *3*, 120.
[30] T. J. Kang, H. Suga, *Biochem. Cell Biol.* **2008**, *86*, 92.
[31] T. J. Kang, S. Yuzawa, H. Suga, *Chem. Biol.* **2008**, *15*, 1166.
[32] T. Kawakami, H. Murakami, H. Suga, *J. Am. Chem. Soc.* **2008**, *130*, 16861.
[33] A. Ohta, H. Murakami, H. Suga, *ChemBioChem* **2008**, *9*, 2773.
[34] Y. Sako, Y. Goto, H. Murakami, H. Suga, *ACS Chem. Biol.* **2008**, *3*, 241.
[35] Y. Sako, J. Morimoto, H. Murakami, H. Suga, *J. Am. Chem. Soc.* **2008**, *130*, 7232.
[36] T. Kawakami, H. Murakami, H. Suga, *Chem. Biol.* **2008**, *15*, 32.
[37] L. Cartwright, D. W. Hutchinson, V. W. Armstrong, *Nucleic Acids Res.* **1976**, *3*, 2331.
[38] C. E. Hop, R. Bakhtiar, *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1049.
[39] J. Perła-Kaján, T. Twardowski, H. Jakubowski, *Amino Acids* **2007**, *32*, 561.

Received: February 4, 2009
Published online on April 15, 2009

 © 2009 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 