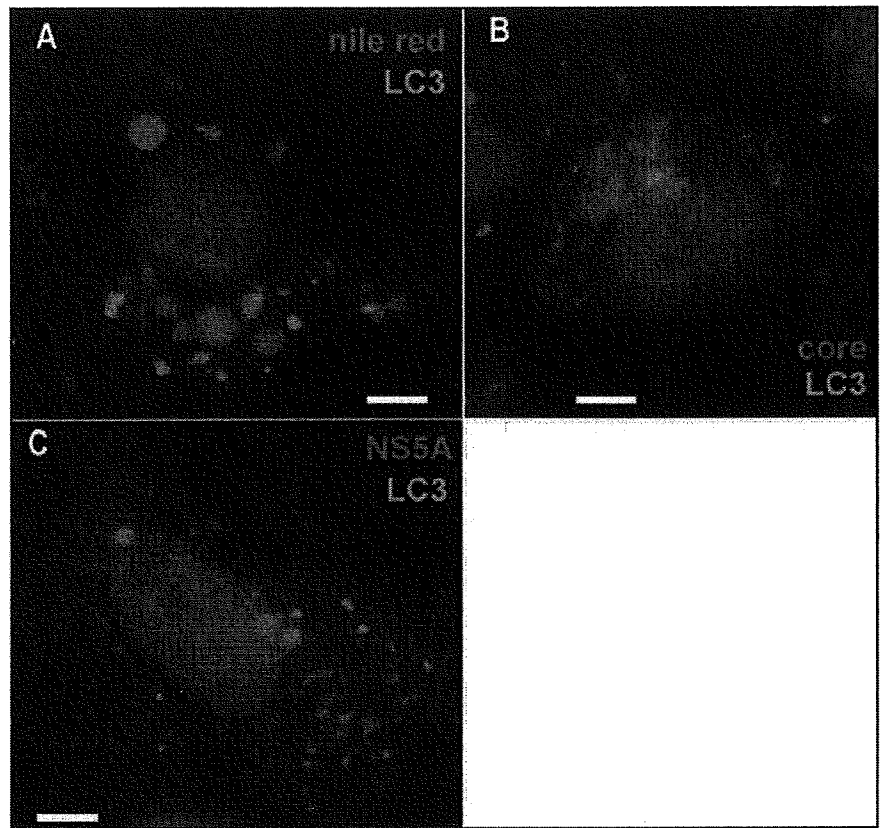


Figure 7. Minor colocalization of LC3 with lipid droplets, HCV core or HCV NS5A proteins. Lipid droplets in HCV-infected cells were stained Nile red (A, pseudo color is red), HCV core proteins were stained with mouse anti-HCV core antibody and Alexa594-conjugated goat anti-mouse IgG (B, pseudo color is red), and HCV NS5A proteins were stained with mouse anti-HCV NS5A antibody and Alexa594-conjugated goat anti-mouse IgG (C, pseudo color is red). Pseudo color of LC3 is green in (A–C). Merged images are shown. Bars indicate 5 μ m.



G4000). Total RNA was prepared by a RNeasy plus mini kit (Qiagen, 74134). Quantitative RT-PCR of mRNA was performed with a Lightcycler480 using a Lightcycler RNA Master SYBR Green I kit (Roche, 3064760).

Acknowledgements

This study was supported in part by grants-in-aid from the Ministry of Health, Labor and Welfare of Japan, and by a grant from the Mochida Memorial Foundation for Medical and Pharmaceutical Research (to I.T.), and by Grants-in-Aid for Scientific Research on Priority Areas "Proteolysis in the Regulation of Biological Processes" from the Ministry of Education, Science, Sports and Culture of Japan (to I.T.).

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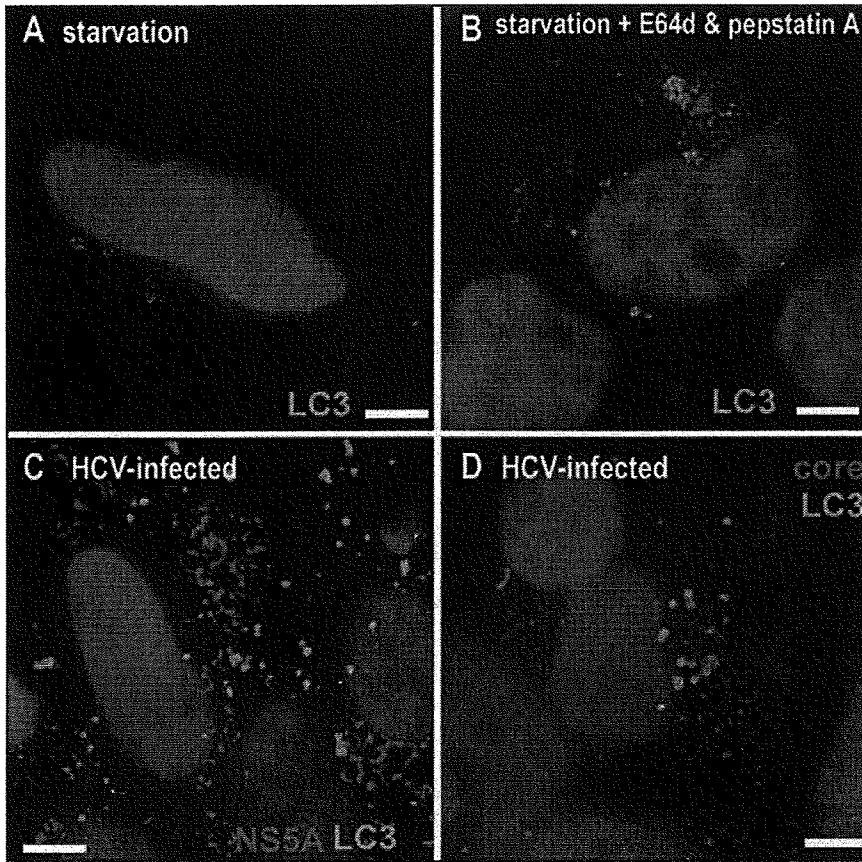


Figure 8. Deconvoluted images of LC3, core and NS5A in HCV-infected Huh7.5.1 cells. The fluorescence images of endogenous LC3 (A–D, pseudo color is green), NS5A (C, pseudo color is red), and core (D, pseudo color is red) were obtained by fluorescent microscopy. The images were deconvoluted with ImageJ program (<http://rsbweb.nih.gov/ij/>) and a plug-in for iterative deconvolution (<http://www.optinav.com/Iterative-Deconvolution.htm>). Pseudo color for DAPI staining is blue. Merged images are shown. Note that the size of LC3-puncta in HCV-infected cells (C and D) were larger than under starvation conditions in the absence (A) or presence (B) of E64d and pepstatin A.

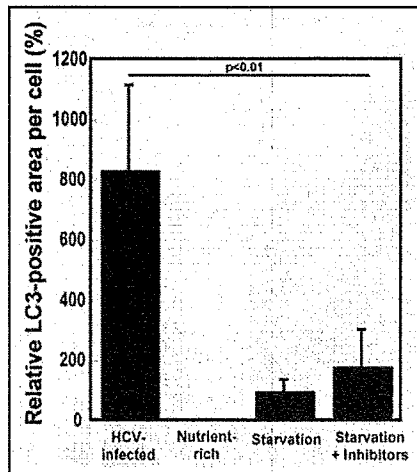


Figure 9. Increase of the fluorescence of LC3 in HCV-infected cells. The images of fluorescence of LC3 were analyzed with an ImageJ program (<http://rsbweb.nih.gov/ij/>) and plug-ins for iterative deconvolution (<http://www.optinav.com/Iterative-Deconvolution.htm>) and tophat-filter (<http://rsb.info.nih.gov/ij/plugins/lipschitz/>). The fluorescent images of over 200 cells were analyzed, and relative intensity was calculated; the average intensity of LC3 in Huh7.5.1 cell under starvation conditions [starvation] in the absence of E64d and pepstatin A was set to 100%.

Table 1 Little mRNAs of interferons were activated by ATG7- and BECN1-RNAi

	<i>IFNA1</i>	<i>IFNA2</i>	<i>IFNB1</i>
	(copies of mRNA/100 ng total RNA)		
Uninfected Huh7	5.6 ± 1.5	0.8 ± 0.1	0.4 ± 0.1
HCV-infected/scrambled RNA	5.8 ± 5.0	1.8 ± 0.1	4.9 ± 3.6
HCV-infected/ <i>ATG7</i> RNAi	0.6 ± 0.1	1.3 ± 0.3	0.9 ± 0.5
HCV-infected/ <i>BECN1</i> RNAi	0.3 ± 0.1	1.1 ± 0.2	2.1 ± 1.3

HCV was infected into Huh7.5.1 cells, and RNA interference was performed at day 1 and day 3 post-infection (HCV-infected). At day 5 post-infection, total RNA was prepared with a RNeasy plus kit. As a negative control for RNAi, scrambled RNA was employed. As a negative control for HCV-infection, Huh7 cells were incubated for 5 days (Uninfected Huh7), and total RNA was prepared. Quantitative RT-PCR of mRNA was performed with a Lightcyder480 (Roche) with appropriate primer-sets for each genes.

Diverse backbone-cyclized peptides via codon reprogramming

Takashi Kawakami^{1,4}, Atsushi Ohta^{1,4}, Masaki Ohuchi², Hiroshi Ashigai¹, Hiroshi Murakami³ & Hiroaki Suga¹⁻³

We report a methodology for the ribosomal synthesis of backbone-cyclized peptides involving genetic code reprogramming to introduce one or more nonproteinogenic amino acids. Expression of linear peptides bearing a cysteine-proline dipeptide sequence followed by glycolic acid results in self-rearrangement to a C-terminal diketopiperazine-thioester, which non-enzymatically generates a cyclized peptide. We demonstrate the ribosomal synthesis of several naturally occurring backbone-cyclized peptides and a library based on a bicyclic scaffold, and we identify bioactive sequences by screening and deconvolution.

One of the common backbone frameworks found in many naturally occurring short peptides is a cyclic structure that connects the N and C termini (Fig. 1a). Even though such cyclic peptides are initially synthesized as linear peptides by ribosomes¹ or nonribosomal peptide synthetases², the enzymatic cyclization of the precursor linear peptides at the N and C termini grants them unique biological properties

that their precursor peptides do not have, such as proteolytic stability³ and high affinity toward their binding partners or targets⁴. This study reports a new enabling methodology that generates backbone-cyclized peptides amenable to the multiple introduction of non-natural amino acids in a reconstituted translation system.

A recent study reported the solid-phase synthesis of peptides containing a Cys-Pro-glycolic acid (^HO₂G) sequence in which these residues self-rearrange to form a diketopiperazine-thioester (dkp-thioester) derived from the conjugation of cysteine and proline via the non-enzymatic nitrogen to sulfur equilibrium shift driven by the concomitant release of the ^HO₂G residue⁵. The resultant thioester was then used to ligate two peptide fragments together. We envisioned that this elegant strategy could also be applied to peptide backbone

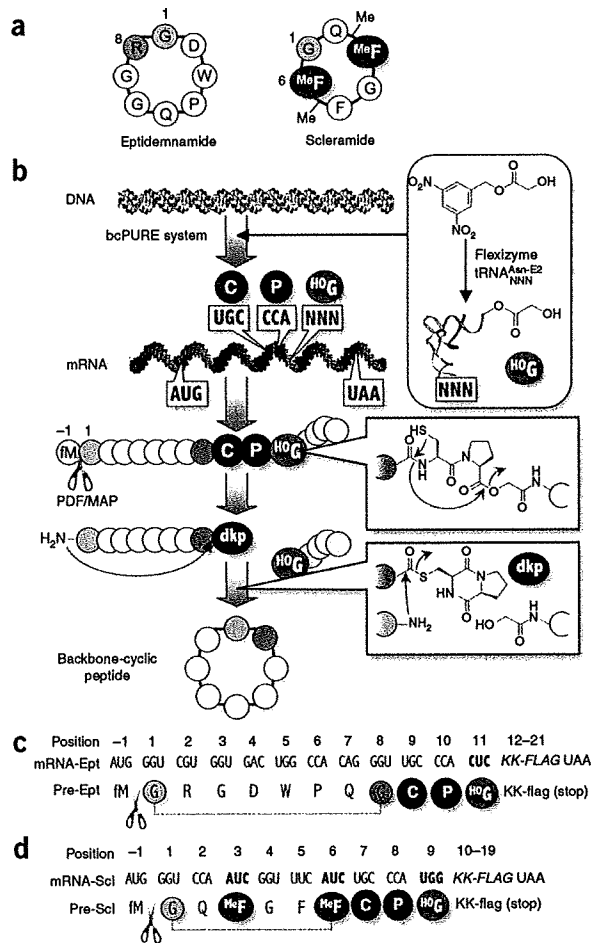


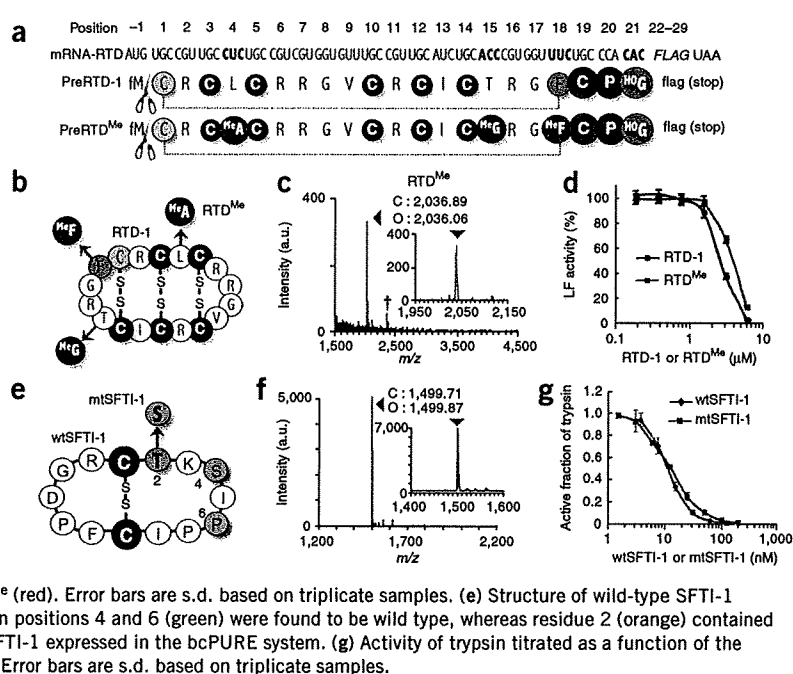
Figure 1 Ribosomal expression of backbone-cyclic peptides. (a) Structures of backbone-cyclic peptides expressed in this study. (b) mRNA-encoded expression of backbone-cyclic peptides in a withdrawn PURE system including PDF and MAP, referred to as the bcPURE system. Glycolic acid (^HO₂G) was charged onto a suppressor tRNA^{Asn-E2}_{NNN} (ref. 6) using dinitro-flexizyme⁶ and assigned to a vacant codon (NNN) created by withdrawing the corresponding proteinogenic amino acid. A DNA template, encoding a linear precursor peptide with a C-P-HOG sequence, was transcribed and translated in the bcPURE system. This segment spontaneously rearranges to form a diketopiperazine-thioester (dkp, dark green), subsequently reacting with the N-terminal amino group at residue 1 (yellow), which is liberated by *in situ* removal of fM(-1) by PDF and MAP. These processes result in closing the backbone of residue 1 and the C-terminal residue (green). (c) Expression of eptidamnamide. Sequences of mRNA template (mRNA-Ept) and linear precursor peptide (pre-Ept). Number denotes the position of residue in the linear precursor peptide. HOG11 is reprogrammed to the CUC codon by the addition of ^HO₂G-tRNA^{Asn-E2}_{GAG} to a leucine-withdrawn bcPURE system. KK-FLAG indicates an RNA sequence encoding the KK-flag peptide (KKDYKDDDDK). (d) Expression of scleramide. Sequences of the mRNA template (mRNA-Scl) and pre-scleramide (pre-Scl). HOG and N-methylphenylalanine (MeF) are reprogrammed to the UGG and AUC codons, respectively, by the addition of ^HO₂G-tRNA^{Asn-E2}_{CCA} and MeF-tRNA^{Asn-E2}_{GAU} to a tryptophan/leucine-withdrawn bcPURE system.

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Received 15 April; accepted 11 September; published online 25 October 2009; doi:10.1038/nchembio.259

Figure 2 One-pot expression of RTD-1, its N-methylated analog RTD^{Me}, and SFTI-1.

(a) Sequences of mRNA template (mRNA-RTD) expressing linear pre-peptides (preRTD-1 and preRTD^{Me}). For the expression of RTD-1, ^{HO}G is reprogrammed to the CAC codon by adding ^{HO}G-tRNA^{Asn-E2}_{GUG} to a histidine-withdrawn bcPURE system. For RTD^{Me} expression, ^{HO}G, ^{Me}A (10), ^{Me}G (11) and ^{Me}F are reprogrammed by adding ^{HO}G-tRNA^{Asn-E2}_{GUG}, ^{Me}A-tRNA^{Asn-E2}_{GAG}, ^{Me}G-tRNA^{Asn-E2}_{GGU} and ^{Me}F-tRNA^{Asn-E2}_{GAA} to a bcPURE system lacking histidine, leucine, threonine and phenylalanine. RTD-1 and RTD^{Me} were expressed in one pot from the DNA templates via preRTD-1 and preRTD^{Me}, respectively (Supplementary Fig. 4a,b). (b) Structure of RTD-1 and RTD^{Me}. (c) MALDI-TOF spectrum of RTD^{Me}. Arrowhead indicates a peak of a singly charged species [M+H]⁺ with the observed mass (O) along with the calculated mass (C). † denotes an unknown background peak obtained in the absence of the template DNA for mRNA-RTD. (d) Activity of anthrax lethal toxin protease titrated as a function of the concentration of RTD-1 (blue) or RTD^{Me} (red). Error bars are s.d. based on triplicate samples. (e) Structure of wild-type SFTI-1 (wtSFTI-1) and mutant SFTI-1 (mtSFTI-1). Residues in positions 4 and 6 (green) were found to be wild type, whereas residue 2 (orange) contained a T→S mutation. (f) MALDI-TOF spectrum of the mtSFTI-1 expressed in the bcPURE system. (g) Activity of trypsin titrated as a function of the concentration of wtSFTI-1 (purple) or mtSFTI-1 (red). Error bars are s.d. based on triplicate samples.



cyclization. We have previously demonstrated the incorporation of ^{HO}G (1) into a peptide chain using a reprogrammed genetic code⁶, creating a technical foundation for the ribosomal expression of C-P-^{HO}G segment-containing peptides. Unlike synthetic peptides, ribosomally expressed peptides typically begin with a formylated methionine (fM, 2). We anticipated that recombinant peptide deformylase (PDF) could remove the N-terminal formyl group and methionine aminopeptidase (MAP) could cleave the resultant methionine residue to generate a free N terminus of any desired amino acid. Our combined translation system is referred to as the bcPURE (backbone-cyclic peptide synthesis using recombinant elements) system, where certain proteinogenic amino acids are withdrawn from the ordinary PURE system⁷ to make vacant codons, while PDF and MAP facilitate *in situ* generation of N-terminal amino-peptides and thus cyclization (Fig. 1b). The system makes use of flexizymes—highly flexible tRNA acylation ribozymes—to charge ^{HO}G and nonproteinogenic amino acids onto any desired tRNAs^{8,9} and assign these residues to vacant codons. The integration of the bcPURE system and flexizymes provides a general platform for the production of cyclized, highly diverse peptides.

To build the foundation of this methodology, we first investigated whether dkp-thioester formation and PDF/MAP could function in the bcPURE system (Supplementary Results and Supplementary Methods). Initial studies using model peptides established several aspects of the method: (i) expression of a Cys-Pro-^{HO}G segment successfully resulted in a non-enzymatic self-arrangement to the corresponding dkp-thioester (Supplementary Fig. 1), (ii) the combination of PDF and MAP served to remove the N-terminal fM residue from peptides containing 7 different amino acids in the neighboring site (position 1) (Supplementary Fig. 2a,b) and (iii) initiation codon reprogramming¹⁰ in combination with PDF allowed the use of 7 additional residues as initiating amino acids, yielding further diversity at position 1 and bypassing the requirement for methionine (Supplementary Fig. 2c,d). These aspects, which can all be achieved *in situ* with the bcPURE system, will enable the design of any desired peptide by varying the connection site to assign the first residue as one of the 14 validated options (Fig. 1b).

To verify whether our methodology works as planned, two monocyclic backbone-cyclic peptides, eptidemnamide (3)¹¹ and scleramide (4)¹² (Fig. 1a), were chosen as the first set of synthetic targets. Eptidemnamide was retro-connected at the site between two glycine residues (G1 and G8; the number denotes the residue position), and the corresponding mRNA was designed to express the hexameric peptide followed by the C9-P10-^{HO}G11-KK-flag segment, where ^{HO}G11 was assigned by the CUC leucine codon (Fig. 1c, mRNA-Ept). This mRNA was then translated using the bcPURE system in the presence of 2,2-dithiodiethanol (DTDE) to trap the pre-cyclized intermediate as a 2-mercaptoethanol adduct (see Supplementary Fig. 1 for more information). The resulting product was subsequently treated with DTT during the flag purification to remove the 2-mercaptoethanol group. MALDI-TOF analysis of the isolated product gave a single major peak consistent with the expected molecular mass of the linear pre-peptide (Supplementary Fig. 3a,c, pre-Ept). The pre-Ept was then incubated in a pH 8.0 buffer with thiophenol. A MALDI-TOF analysis of the resulting product showed a single major peak with a mass that agreed with the mass value of eptidemnamide (Supplementary Fig. 3b,d).

Unlike eptidemnamide, which consists of only proteinogenic amino acids, scleramide contains two N-methylphenylalanine (^{Me}F, 5) residues. To incorporate the ^{Me}F residues into the specific positions¹³, the AUC isoleucine codon was reprogrammed to ^{Me}F and the UGG tryptophan codon was reprogrammed to ^{HO}G to express the C-P-^{HO}G segment. Accordingly, we prepared the corresponding mRNA (Fig. 1d, mRNA-Scl) where the G1 and ^{Me}F6 sites were retro-connected, and performed ribosomal expression of the linear peptide (Fig. 1d, pre-Scl) in a bcPURE system containing ^{Me}F-tRNA^{Asn-E2}_{GAU} and ^{HO}G-tRNA^{Asn-E2}_{CCA}. Following the same procedure as in the eptidemnamide synthesis, MALDI-TOF analysis of the intermediate and cyclic peptides confirmed the peaks for pre-Scl and scleramide (Supplementary Fig. 3e-h). These two demonstrations show the applicability of the bcPURE system integrated with flexizymes to the ribosomal expression of distinct backbone-cyclic peptides.

Rhesus-θ defensin-1 (RTD-1, 6), which belongs to a family of tetracyclic θ-defensins¹⁴, consists of three internal dithio bridges on

the ring structure (Fig. 2a,b). We envisioned that we could further promote cyclization by placing a cysteine residue at the N terminus, allowing an exchange from the dkp-thioester to the C1-thioester, and leading to spontaneous rearrangement from the C1-thioester to the free amine to create the cyclic backbone¹⁵ (Supplementary Fig. 4a). We therefore planned the ribosomal synthesis of RTD-1 via the backbone ligation between C1 and F18 (Fig. 2b and Supplementary Fig. 4a); at the C terminus, the segment of C19-P20-^{HO}G21-flag peptide was expressed, where ^{HO}G21 was assigned to the CAC histidine codon (Fig. 2a, mRNA-RTD and pre-RTD-1). In addition to wild-type RTD-1, we designed an RTD-1 analog containing three *N*-methyl amino acids, ^{Me}A4, ^{Me}G15 and ^{Me}F18, which were reassigned to the CUC leucine, ACC threonine and UUC phenylalanine codons¹³, respectively (Fig. 2a,b and Supplementary Fig. 4b, RTD^{Me}, 7). Expression of these peptides was executed in the one-pot bcPURE system with DTT instead of DTDE to avoid disulfide trapping, and the MALDI-TOF analysis of the respective peptides revealed the expected molecular mass with the reduced form (Supplementary Fig. 4c). The *in situ* formation of disulfide bridges was achieved by prolonging the translation reaction time via air oxidation, confirmed by a loss of 6 mass units from the reduced form of RTD-1 (Supplementary Fig. 4d and Fig. 2c for RTD-1 and RTD^{Me}, respectively) and no change in their mass after treatment of the product with 2-iodoacetoamide (data not shown). We also verified the inhibitory activity of RTD-1 and RTD^{Me} against anthrax lethal toxin protease (LF) using a fluorogenic substrate (Supplementary Fig. 4e)¹⁶. Their apparent half-maximal inhibitory concentration (IC₅₀) values were determined to be approximately 2.7 and 4.0 μM, respectively (Fig. 2d), which were nearly identical to the previously reported value, 2.4 μM, determined for authentic RTD-1 (ref. 16). These results suggest that the correct disulfide bridges in RTD-1 and RTD^{Me} are very likely formed. Importantly, the *N*-methyl backbone modification in RTD^{Me} did not perturb the intrinsic inhibitory activity of RTD-1. This demonstrates the usefulness of our methodology for rapid explorations of backbone and side chain modifications of natural peptides in order to alter or improve their properties.

Sunflower trypsin inhibitor-1 (SFTI-1, 8) is a serine protease inhibitor consisting of a 14-mer backbone-cyclic peptide with a single disulfide bridge (Fig. 2e)¹⁷. We chose this bicyclic peptide scaffold to build the technical foundation for the library synthesis and rapid screening of bioactive sequences integrated with limiting-dilution PCR deconvolution^{18,19}. First, the wild-type SFTI-1 (wtSFTI-1) was expressed in the bcPURE system under the assignment of the CUC leucine codon to ^{HO}G (Supplementary Fig. 5a–c), and its trypsin inhibitory activity was confirmed using a fluorogenic substrate (Supplementary Fig. 5d, and also see below). We then constructed a DNA library encoding SFTI-1 mutants in which three amino acid residues at positions 2, 4 and 6 embedded in the trypsin-binding loop were randomized by inserting NNK triplets into the corresponding positions in the parental DNA template (N = G, A, C or T and K = T or G) (Supplementary Figs. 6a and 7a). Based on the verified protocol (Supplementary Fig. 6b–f), we performed a 96-well parallel one-pot expression from PCR-amplified DNA libraries originating from approximately 60 unique sequences, and directly transferred the translation mixture in each well to the trypsin inhibition assay (Supplementary Fig. 7b). We performed a total of three rounds of screening and deconvolution of active sequences (Supplementary Fig. 7c,d; see details in Supplementary Results), identifying one mutant SFTI-1, referred to as mtSFTI-1 (9), with a T2S mutation (Fig. 2e,f). A titration of the activity as a function of mtSFTI-1 concentration resulted in an IC₅₀ value of 13.4 ± 0.7 nM ($K_i = 13 \pm 1$ nM, determined by fitting to the Morrison's slow-tight binding equation; Fig. 2g), which was comparable to that of wtSFTI-1 (IC₅₀ = 12.1 ± 0.3 nM

and $K_i = 8 \pm 1$ nM)¹⁷. Thus, integration of the ribosomal expression of a library SFTI-1 with activity screening and limiting-dilution PCR deconvolution enables rapid determination of active sequences.

Our proof-of-concept studies reported here have established a unique enabling methodology for the synthesis and discovery of backbone-cyclic peptides for potential therapeutic uses. Importantly, all processes—including transcription of the DNA template, translation of the peptide, generation of the N-terminal amino group, formation of the dkp-thioester and N-C termini cyclization—take place in one pot using the bcPURE system. This feature has enabled us to readily prepare and analyze a library of backbone-cyclic peptides based on a bicyclic scaffold. Although the screening/deconvolution methodology reported here is limited in that it can only be applied to rather small libraries (<10⁵ molecules)^{18,19}, it is inexpensive, easy and widely applicable to various *in vitro* assay methods. We envision that the combination of an *in vitro* display^{20,21} with the screening/deconvolution methodology will offer a rapid and reliable means for identification of functional peptides. Another important feature of our method is the ability to incorporate nonproteinogenic amino acids into peptide chains at multiple sites^{6,8,13,22–24}. This is in sharp contrast to other mRNA-directed backbone-cyclic peptide expression methods, such as those using inteins^{15,25}, and as such should accelerate the discovery of new classes of peptides.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

ACKNOWLEDGMENTS

This work was supported by grants from the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (16101007) and a research and development project of the Japanese Industrial Science and Technology Program in the New Energy and Industrial Technology Development Organization (NEDO) to H.S. T.K. and A.O. are supported by JSPS Research Fellowships for young scientists (20-664 and 19-1722, respectively).

AUTHOR CONTRIBUTIONS

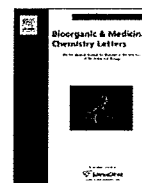
T.K., A.O. and H.S. designed the project. T.K., A.O., M.O., H.A. and H.M. performed experiments. The manuscript was written by T.K., A.O. and H.S.

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A flexizyme that selectively charges amino acids activated by a water-friendly leaving group

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ARTICLE INFO

Article history:

Received 11 February 2009

Revised 20 March 2009

Accepted 25 March 2009

Available online 28 March 2009

Keywords:

Ribozyme

In vitro selection

Non-proteinogenic amino acid

Aminoacylation

ABSTRACT

We have developed a new flexizyme (a flexible de novo tRNA acylation ribozyme) system, a pair of amino-derivatized benzyl thioester (ABT) and amino flexizyme (aF_x). ABT bearing the ammonium ion was designed to render the acyl-donor substrates better water solubility. Although the previously reported flexizymes (eF_x and dF_x) did not show acylation activity for the ABT derivatives, a new flexizyme variant aF_x, generated by in vitro selection against an amino acid activated ABT, exhibits high selectivity toward those activated ABT. The flexizymes system including aF_x, eF_x, and dF_x enables us to prepare a wide variety of acyl-tRNAs charged with non-proteinogenic amino acids.

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Engineering of the universal genetic code has given us a new opportunity to express peptides or proteins bearing non-proteinogenic amino acids.^{1–4} Recent developments in methodologies of the genetic code reprogramming have enabled us to reassign multiple codons to non-proteinogenic amino acids.^{5–21} By means of such methodologies, libraries of non-standard peptides can be prepared in the mRNA-encoding manner.¹⁴ We have engaged the development of such a methodology integrated with flexizymes.^{10,22–24} Flexizymes are flexible tRNA acylation ribozymes and facilitate the preparation of various non-proteinogenic aminoacyl-tRNAs, which are otherwise complex and technically difficult processes. Two flexizymes, referred to as eF_x and dF_x, have been devised (Fig. 1); the eF_x acylates tRNA with amino or hydroxy acids upon activation with cyanomethyl ester (CME) group or *p*-chlorobenzyl thioester (CBT), where its activity relies on the recognition of aromatic sidechain or the CBT group in the substrate; on the other hand, dF_x acylates tRNA with those activated with 3,5-dinitrobenzyl ester (DBE) group, where its activity fully relied on the recognition of the DBE group. Since both flexizymes bind the 3'-end sequence of tRNA via the 3-base pairing interaction (RCCA-3', R = G, A and U are the preferable discriminator base at position 73, but even C is acceptable under prolonged incubation), any tRNAs can be the acyl-acceptor for the flexizymes. Thus, by the combination of these two flexizymes, virtually any desired acyl-tRNAs can be readily prepared.

However, a shortcoming of the current flexizyme system is that since the CBT or DBE-derived the acyl-donor substrates make them more hydrophobic, some derivatives, particularly those with hydrophobic sidechains, are occasionally difficult to dissolve in the aqueous reaction buffer. Notably, eF_x and dF_x are able to retain their catalytic activity in the buffer containing up to 20% and 40% DMSO, respectively, but the addition of DMSO to the reaction buffer

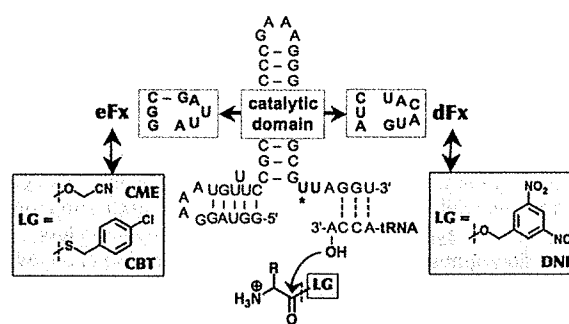


Figure 1. Flexizymes and their cognate leaving groups in substrates. Chemical structures of a benzyl ester and thioester leaving group. R represents amino acid sidechains including non-proteinogenic ones. Each flexizyme recognizes the specific leaving group and charges the acyl group onto the 3'-hydroxyl group at the tRNA 3'-end. Abbreviations: LG, leaving group; CME, cyanomethyl ester; CBT, *p*-chlorobenzyl thioester; eF_x, enhanced flexizyme; DNB, 3,5-dinitrobenzyl ester; dF_x, dinitro flexizyme. U with asterisk denotes the absolutely conserved U-turn base. Bold bases orchestrate to constitute the catalytic domain of each flexizyme.

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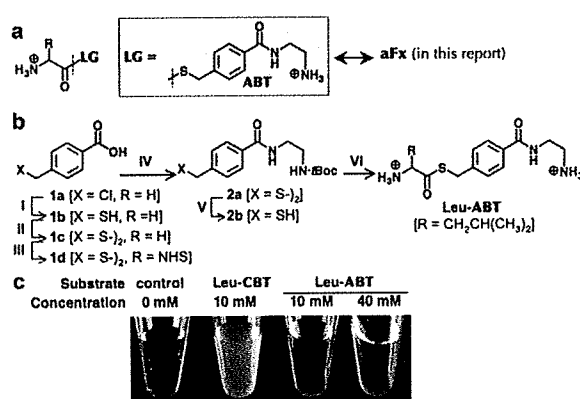


Figure 2. A new substrate design, synthesis, and solubility in the reaction buffer. (a) Chemical structures of acyl-donor substrates. R represents amino acid sidechains including non-proteinogenic ones. Abbreviations are as follows: LG, leaving group; ABT, amino-modified benzyl thioester; aFx, amino flexizyme. (b) Synthesis of aminoacyl-ABT. An example of Leu derivative is shown. Reagents and conditions: (I) thiourea, H_2O , 1 h, reflux, then 10% NaOH, 1 h, reflux, quant.; (II) I_2 , EtOH, 0°C , 1.5 h, quant.; (III) *N*-hydroxysuccinimide (NHS), EDC-HCl, 1,4-dioxane, CH_2Cl_2 , rt, 1 h, quant.; (IV) mono-*t*-Boc-ethylendiamine, H_2O , 1,4-dioxane, 0°C , 2 h, 91%; (V) DTT, DMF, 95°C , 1 h, 50%; (VI) *t*-Boc-L-leucine, EDC-HCl, DMAP, CH_2Cl_2 , rt, 1 h, then HCl, AcOEt, CH_2Cl_2 , rt, 0.5 h, 30%. (c) Solubility of Leu-CBT and Leu-ABT. Each compound was dissolved in 0.1 M HEPES-K (pH 7.5), 20% DMSO at the concentration as shown.

hampers the intrinsic ability of the flexizymes. Therefore we have a dilemma between poor solubility of the substrates and loss of flexizyme activity by the addition of DMSO. Here we report a new pair of flexizyme and substrate activated by a benzyl thioester in which *p*-position is derived to an amino group that is protonated in the reaction buffer to give the ammonium salt. By using this pair, we have demonstrated aminoacylation of tRNA with amino acids bearing hydrophobic sidechains or the α -*N*-octanoyl modification on the sidechain.

In order to make the leaving group more water-soluble, we derived a commercially available 4-chlorobenzoic acid to have a primary amino group and a thiol group for activation of the acyl group (Fig. 2a). Synthesis of this leaving group and its derivatization of amino acid was straightforward as shown in Figure 2b. We referred this amino-derivatized benzyl thioester aminoacyl-donor as aminoacyl-ABT (Fig. 2a).

First, we compared the solubility of 10 mM Leu-CBT and Leu-ABT in the flexizyme reaction buffer containing 20% (v/v) of DMSO. Despite the solution of Leu-CBT with a cloudy suspension, Leu-ABT gave a completely clear solution. The solution remained clear even with 40 mM Leu-ABT (Fig. 2c). Thus, the ABT leaving group significantly improved the water solubility of the Leu substrate. However, the alteration of the leaving group turned out to be detrimental for the acylation activity of the available flexizymes, dFx and eFx, yielding less than 1% leucylation onto a microhelix RNA (Fig. S1; regarding this analytical method see below). This observation imposed us to perform *in vitro* selection of a new flexizyme capable of reacting the ABT-derived substrate.

The method of *in vitro* selection and the flexizyme pool containing three random regions (Fig. S2a) were the same as our previous report¹⁰ except that the active sequences for self-aminoacylation were enriched by the reaction with Leu-ABT followed by the selective biotinylation on the α -amino group of the charged Leu. After six rounds of selection of active sequences by capturing on a streptavidin-agarose resin (Fig. S2b), we observed an enrichment of the pool. The 3'-terminal specific activity of the pool round 6 was confirmed by the activity loss upon the periodate oxidation (Fig. S2c). 23 clones isolated from the pool were sequenced, and their alignment revealed that 19 clones fell in a single class (Fig. S2d). We

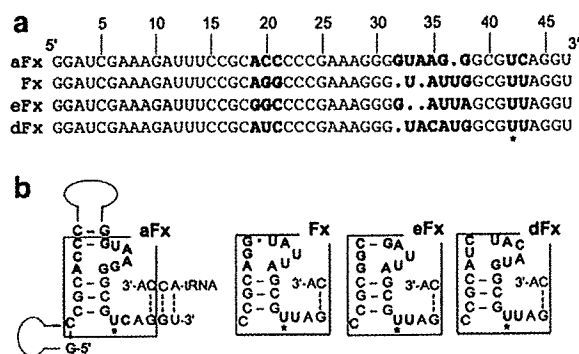


Figure 3. Sequence alignment and secondary structures of aFx and other flexizymes. (a) Sequence alignment. Bases originating from the random regions were shown in bold, and the conserved base (U42) was shown with asterisk. (b) The secondary structure of aFx compared with the catalytic domain in other flexizymes.

picked 5 representative clones and tested for self-aminoacylation; and found that all are active toward Leu-ABT. Among them, clone 11 exhibited the desired 3'-terminal specific activity in the highest efficiency (Fig. S3). Therefore, we focused on this clone for further studies. We referred this new flexizyme to as aFx as oppose to eFx and dFx.

The sequence alignment and drawing the individual secondary structure of aFx, eFx, dFx and the parental Fx revealed the difference in the composition of catalytic domain (Fig. 3a and b). The only conserved base is U42; this base is known to form a unique U-turn structure and plays a critical role in presenting the catalytic domain to the 3'-hydroxyl group at the tRNA 3'-end.²⁴ Because of lacking our knowledge of the 3-dimensional structure of the catalytic domain in the individual flexizyme except for Fx, it is yet unclear how the sequence variation is able to adapt to the respective leaving group (Fig. 3b). However, even for Fx we have shown that the catalytic domain is susceptible to mutagenesis,²⁴ it seems that the catalytic domain can be readily adapted to various benzyl leaving groups by the sequence alterations. Nonetheless, the present result indicates that the selection from the doped pool of flexizyme readily generates new flexizyme variants.

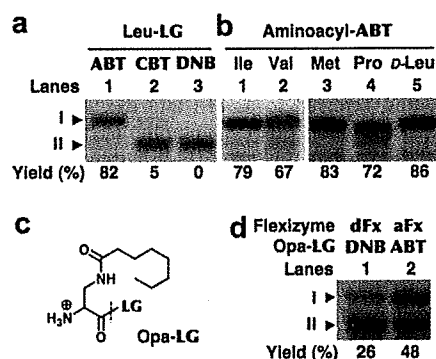


Figure 4. Aminoacylation ability of aFx. (a) Selectivity of aFx to cognate Leu-ABT against non-cognate substrates. Bands of I and II indicate aminoacyl-microhelix RNA and microhelix RNA, respectively. Conditions: 0.1 M HEPES-K (pH 7.5), 50 mM MgCl_2 , 20% DMSO, 20 μM microhelix RNA, 20 μM aFx, and 5 mM substrate on ice for 2 h; (b) Flexibility of aFx toward sidechain in the aminoacyl-ABT structures. Conditions for Ile-ABT (lane 1): 0.1 M HEPES-K (pH 7.5) and 10 mM substrate on ice for 24 h. Conditions for Val-ABT (lane 2): 0.1 M Bicine-K (pH 9.0) and 10 mM Val-ABT on ice for 6 h. Reactions in lanes 3–5 were carried out by the same conditions described in Figure 4a. (c) Chemical structure of Opa-LG. Opa, 2-amino-3-(octanamido)propanoic acid; LG, leaving group. (d) Aminoacylation of Opa onto a microhelix RNA using aFx or dFx against the cognate Opa-LG. The reaction conditions were the same as those described in Figure 4a, lane 1.

To verify the ability of aF_x for trans-activity, we prepared the aF_x domain alone by in vitro transcription from the corresponding DNA template, and examined the aminoacylation onto a microhelix RNA using denaturing acid PAGE (poly-acrylamide gel electrophoresis), a previously reported method for the conventional and reliable activity assay for trans-acting flexizymes.¹⁰ The trans-acting aF_x was able to charge Leu using Leu-ABT onto the microhelix RNA in 82% yield (Fig. 4a, lane 1). Interestingly, it was virtually inactive toward Leu-CBT and Leu-DNB (Fig. 4a, lanes 2 and 3), indicating that aF_x exhibited high specificity to the ABT leaving group.

To investigate the tolerance of aF_x toward amino acid side-chains and α -chirality, we synthesized five additional amino acids, Ile, Val, Met, Pro, and *D*-Leu, activated by ABT (Fig. 4b). The CBT or DNB derivatives of these amino acids were known to have modest or poor solubility at 5 or 10 mM in the reaction buffer unless including above 20% (v/v) DMSO. The ABT derivatives of these amino acids were, however, efficiently charged onto the microhelix RNA in high yields (Fig. 4b). This result shows that aF_x is able to charge the ABT-derived amino acids independent from their side-chain structure and α -chirality.

Lastly, we performed aminoacylation using a non-proteinogenic amino acid bearing a fatty acid, 2-amino-3-(octanamido)propanoic acid (Opa, Fig. 4c). We found that eF_x was able to charge Opa-CBT onto the microhelix RNA in 26% yield (Fig. 4d, lane 1), whereas aF_x was able to charge Opa-ABT in 48% yield (Fig. 4d, lane 2). Clearly, improving the water-solubility of the acyl-donor substrate offered a better yield.

In conclusion, we have devised a new pair of flexizyme and substrate activating group for tRNA aminoacylation, referred to as aF_x and ABT. aF_x selectively charges ABT-activated amino acids onto tRNA independent from the sidechain kinds. Importantly, the ABT leaving group makes hydrophobic amino acid substrates more water-friendly than CBT and DBE. We now have three pairs of flexizyme and acyl-donor, allowing us to choose the most appropriate pair of flexizyme and acyl-donor depending upon the substrate properties for the genetic code reprogramming.

Acknowledgments

This paper is dedicated to the recognition of the 2009 Tetrahedron Young Investigator Award in Bioorganic and Medicinal Chem-

istry for Professor Carlos F. Barbas. This work was supported by grants of Japan Society for the promotion of Science Grants-in-Aid for Scientific Research (S) (16101007) to H.S., Grants-in-Aid for JSPS Fellows (7734) to Y.Y., and a research and development projects of the Industrial Science and Technology Program in the New Energy and Industrial Technology Development Organization (NEDO).

Supplementary data

Supplementary data (synthetic procedures, a method of in vitro selection, and analysis for aminoacylation were reported in supplementary data) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.114.

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Ribosomal synthesis of dehydrobutyrine- and methyllanthionine-containing peptides†

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Received (in Cambridge, UK) 3rd March 2009, Accepted 31st March 2009

First published as an Advance Article on the web 30th April 2009

DOI: 10.1039/b904314d

We report here the ribosomal synthesis of methyllanthionine-containing cyclic peptides involving a site-specific incorporation of vinylglycine under the reprogrammed genetic code, followed by the isomerization of the vinylglycine to dehydrobutyrine, and the subsequent intramolecular Michael addition of a cysteine residue placed at a downstream position of the vinylglycine.

Dehydrobutyrine (Dhb), one of the family of dehydroamino acids, is frequently found in naturally occurring peptides.¹ For instance, lantibiotics, a class of bacteriocin, have Dhb residues that are generated by enzymatic dehydration of a threonine residue in the precursor peptides expressed by the translation machinery.² In addition, lantibiotics also contain thioether-bridged cyclic residues, referred to as methyllanthionine (MeLn). These unique residues are generated from Dhb residues by nucleophilic attack of the sulfhydryl group in cysteine (Cys) residues *via* enzymatic intramolecular Michael addition, forming cyclic structures.² The thioether bonds formed at MeLn residues are non-reducible, thereby providing physiological stability of their active structures. This suggests that MeLn-bridged cyclic structures provide excellent peptidic frameworks for the discovery of new drug candidates.³ However, since the formation of Dhb and MeLn residues from the precursor peptides relies on the actions of specific enzymes,² the biosynthetic method would not be synthetically versatile enough to generate diverse kinds of such peptides. On the other hand, direct incorporation of Dhb into the nascent peptide chain is very likely intractable due to the hydrolytic instability of the 2-amino group on Dhb itself (decomposing to the 2-oxoacyl group) and its inefficient elongation by the translation machinery. Hence, a generic and simple method that facilitates the synthesis of Dhb- and MeLn-containing peptides, particularly which enables the construction of a library is needed.

We report here a novel methodology for the ribosomal synthesis of Dhb-containing peptides and MeLn-containing cyclic peptides without enzymatic assistance by expressing the

precursor peptides bearing vinylglycine (Vgl) residue. We conceived that Vgl could be useful as a key non-proteinogenic amino acid based on the following considerations: (1) Vgl could be site-specifically incorporated into the desired peptide sequence by means of the genetic code reprogramming,⁴ and Cys could also be incorporated into a downstream position of the Vgl residue in the peptide chain. (2) Heating of the Vgl-containing peptide at nearly neutral pH could induce isomerization from Vgl to Dhb, and simultaneously, the resulting Dhb residue could accept the intramolecular Michael addition by Cys side-chains. Our report herein includes the ribosomal synthesis of MeLn-containing cyclic peptides that mimic the B- and C-ring segments of nisin,^{2c} one of the members of lantibiotics.

Although it was known that treatment of *N*-benzoyl-Vgl esters under acidic or basic conditions in non-aqueous media, resulted in isomerization from Vgl to Dhb residue,⁵ the chemical behaviour of Vgl at neutral or near-neutral pH in aqueous media was unknown in the literature. Therefore, at the first stage of this series of experiments, we synthesized two esters, *N*-acetyl-Vgl methyl ester and Vgl 3,5-dinitrobenzyl ester (**1** and **2** in Fig. 1A and B, respectively), and their chemical behaviours in D₂O buffered at pH 7.4 were monitored by ¹H-NMR. It should be noted that **1** was a mimic of Vgl in a peptidic structure⁶ and **2** was a substrate for our acylation RNA catalyst (flexible tRNA acylation ribozyme; flexizyme)^{4c} that would be used in the subsequent experiment. The summary of the observations was as follows (see more detailed observations and discussions in the ESI†): (1) The α -proton of **1** and **2** was deuterated at 37 °C over 2.5 h to yield **3a** and **4**, respectively, but no isomerization to Dhb was observed. (2) Upon heating **1** at 95 °C for 0.5 h, the isomerization of **1** occurred to yield **5** with a single deuteration on the γ -methyl group, along with a byproduct **3b** generated through the ester hydrolysis. The characteristic triplet at δ 6.83 ppm assigned to the β -olefinic proton of Dhb has a similar chemical shift to that of (*Z*)-Dhb found in lantibiotics.⁷ Based on the above results, we predicted that **2** would

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† Electronic supplementary information (ESI) available: Discussion about the model reactions for isomerization of Vgl and the configuration of MeLn, NMR spectra of the model reactions, and the experimental methods. See DOI: 10.1039/b904314d

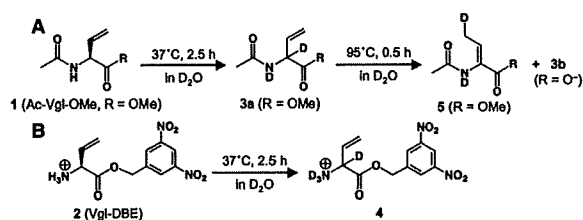


Fig. 1 Model reactions for the isomerization of Vgl to Dhb. Each reaction was monitored by ¹H-NMR. See ESI for more details.†

remain intact under mild conditions, *i.e.* Vgl would be likely intact during flexizyme-catalyzing aminoacylation without isomerization to Dhb, while at elevated temperatures Vgl in peptide chain would isomerize to (Z)-Dhb.

At the second stage, the ribosomal synthesis of a model peptide containing Vgl at a specific site was performed (Fig. 2A, P-vgl). We used the genetic code reprogramming by means of a withdrawn PURE (protein translation using recombinant elements)⁸ (*w*PURE) system integrated with the flexizyme.^{4c-e} In this particular experiment, a *w*PURE system lacking tryptophan (Trp) was prepared to make the cognate codon (UGG) vacant; Vgl-tRNA^{AsnE-2}_{CCA}^{4d} was prepared by the flexizyme and added to the *w*PURE system for the reassignment of UGG to Vgl. Tricine-SDS-PAGE analysis of the peptide expressed under three different conditions (Fig. 2B) revealed that P-vgl was expressed only in the presence of Vgl-tRNA^{AsnE-2}_{CCA} in the *w*PURE system (lanes 2 vs. 3) and its expression level was 44% relative to the wildtype peptide (P-wt) expressed in the *w*PURE system plus Trp (lanes 1 vs. 3). MALDI-TOF mass spectrometry of P-vgl showed a single major peak consistent with the desired molecular mass (Fig. 2C). Upon heating P-vgl at 95 °C, we expected that the Vgl would isomerize to Dhb residue. To confirm this Vgl → Dhb isomerization, P-vgl was incubated with an excess amount of 2-mercaptoethanol (ME), by which the generated Dhb was trapped to yield the corresponding Michael-adduct (P-vgl-ME). In fact, the molecular weight of the resulting peptide increased by 78 Da that was consistent with that of ME (Fig. 2D). This result clearly demonstrates that the isomerization of Vgl to Dhb occurs under heating and the resulting Dhb residue is readily reactive with a thiol *via* intermolecular Michael addition to afford thioethers.

At the final stage of our experiments, we synthesized MeLn-containing cyclic peptides. We chose two cyclic

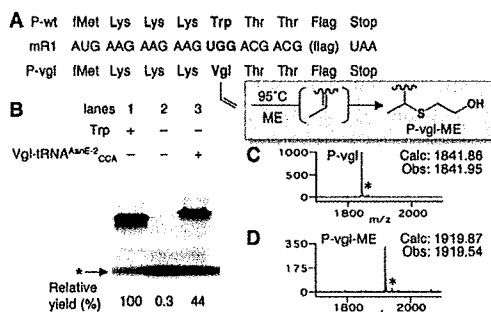


Fig. 2 Ribosomal synthesis of a Dhb-containing peptide. (A) mRNA and the expressed peptide sequences used in this study. Conversions from Vgl to Dhb, and to the ME adduct was illustrated in the grey box. Flag in parentheses indicates the RNA sequence encoding a Flag peptide (DYKDDDDK). (B) Tricine-SDS PAGE analysis of the translation products. Lane 1, wild type expression in *w*PURE system plus Trp; lane 2, *w*PURE system with tRNA^{AsnE-2}_{CCA}, lanes 3, *w*PURE system with Vgl-tRNA^{AsnE-2}_{CCA}. The band indicated by the asterisk corresponds to the remaining [¹⁴C]-Asp that was not incorporated into the peptide. (C) MALDI-TOF mass spectrum of the expressed peptide in *w*PURE system before ME treatment, and (D) that after. The calculated (Calc) and observed (Obs) mass values are shown in each spectrum. Each peak labelled with asterisk denotes a sodium adduct of the major product.

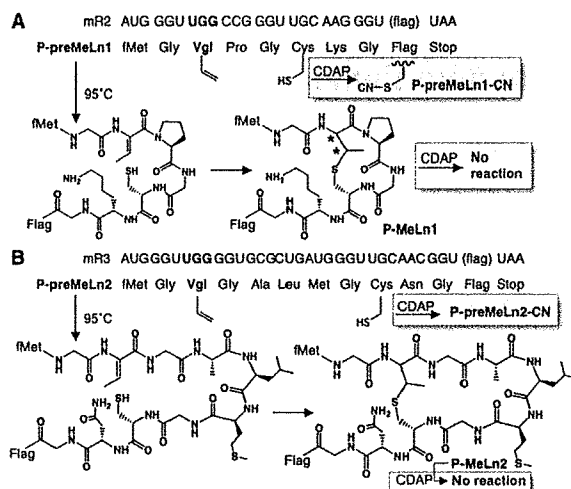


Fig. 3 Synthesis of B-ring (A) and C-ring (B) segments of nisin. CDAP reaction of each peptide is shown in the grey box. Asterisks indicate stereogenic centers newly produced by the intramolecular Michael addition. See ESI for the MALDI-TOF mass spectra of the respective peptides and the stereochemistry.†

peptides as synthetic targets based on the sequences of B- and C-ring segments of nisin (Fig. 3A and B, P-MeLn1 and P-MeLn2). We designed two mRNAs encoding the MeLn-containing cyclic peptide sequences along with the C-terminal Flag-tag appendix, in which the MeLn residue was replaced with Vgl and Cys assigned by UGG and UGC codons (mR2 and mR3). MALDI-TOF mass spectrometry of the expressed peptides showed the consistent mass values with those of Vgl-containing peptides (P-preMeLn1 and P-preMeLn2, respectively; ESI Fig. S1A and E).† We then performed thermal isomerization of the Vgl to Dhb residue and simultaneous intramolecular Michael addition of the Cys thiol. Unlike the intermolecular reaction demonstrated in Fig. 2, the molecular weight of the peptide before and after the tandem isomerization-cyclization would not change; therefore we verified if the Cys thiol in the products reacted with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP), which is known as a thiol-selective cyanylation agent⁹ suitable for monitoring a loss of the free thiol group. As expected, when both precursor peptides were treated with CDAP, their respective mass values increased by 25 Da corresponding to the cyano group (ESI Fig. S1B and F).† In contrast, upon the CDAP treatment of the heated peptides (MnLn1 and MnLn2), their mass values did not change (ESI Fig. S1D and H).† This concluded that the desired cyclization took place smoothly to afford the MeLn-containing cyclic peptides.^{10,11}

Here we have reported a novel methodology for the ribosomal synthesis of MeLn-containing cyclic peptides without assistance of modification enzymes. The key chemistry utilized in this method is the incorporation of Vgl into a specific site designated by a reprogrammed genetic code and the thermal isomerization of the Vgl residue to Dhb followed by simultaneous intramolecular Michael addition of the sulfhydryl group of a Cys residue present in the peptide chain. The virtue of this method requires no reagents that potentially damage the amino acid residues after the incorporation of Vgl.

Moreover, the peptide sequence is readily programmable by the mRNA template sequence. Seebeck *et al.* recently reported a method to generate dehydroalanine-containing peptides from the selenalysine-containing precursor peptides expressed by translation.¹² This elegant method relies on oxidative elimination of selenium by hydrogen peroxide to afford a dehydroalanine residue. However, strong oxidative reagents such as hydrogen peroxide could also oxidize the thiol in Cys (as well as the sulfide group in methionine) existing in the peptide chain, so that Cys may be no longer reactive with Dha as a Michael donor. In contrast, the method reported here resulted in a clean formation of peptides bearing intact Dhb and Cys residues, which can readily be transformed into MeLn residues by heating. Hence, a coupling of ribosomal peptide library synthesis with this methodology opens a new avenue for the discovery of novel MeLn-containing cyclic peptides with not only antimicrobial but also other biological activities when it is integrated with an appropriate screening technique.

This work was supported by grants of the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (S) (16101007) to H. S., Grants-in-Aid for JSPS Fellows (18-10526) to Y. G., and a research and development projects of the Industrial Science and Technology Program in the New Energy and Industrial Technology Development Organization (NEDO).

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DOI: 10.1002/cbic.200900021

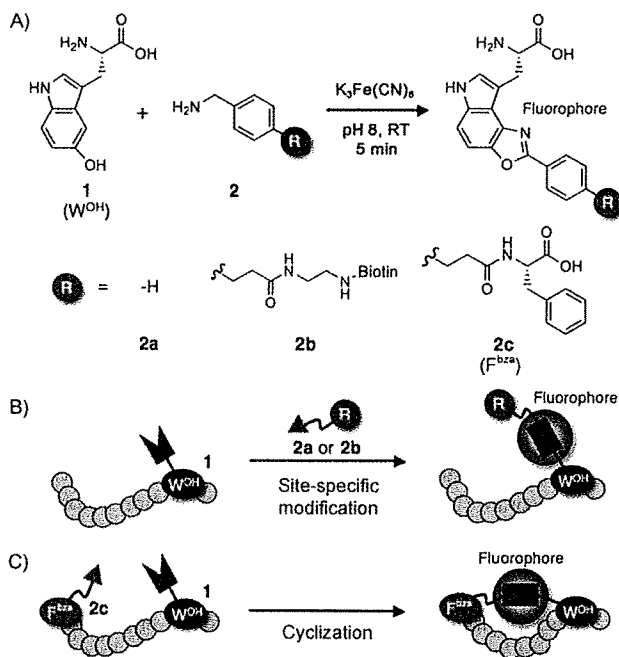
Ribosomal Synthesis of Cyclic Peptides with a Fluorogenic Oxidative Coupling Reaction

Yusuke Yamagishi,^[a] Hiroshi Ashigai,^[a] Yuki Goto,^[c] Hiroshi Murakami,^[b] and Hiroaki Suga^{*,[a, b, c]}

Cyclic peptides often have better physiological properties than linear peptides due to their increased structural rigidity and peptidase resistance.^[1–3] Despite the fact that translation provides an attractive platform for the synthesis of cyclic peptides, those expressed by the translation apparatus have cysteine–cysteine (Cys–Cys) disulfide bridges, and this limits their use. However, because substitution of the disulfide bond with a nonreducible bond is expected to give better physiological stability, it has been of interest to develop novel methodologies for generating such nonstandard cyclic peptides expressed by translation. For instance, a non-natural amide linkage was introduced between the N-terminal α -amino and the lysine ϵ -amino groups by the treatment of peptides with a crosslinking reagent that had been activated by *N*-hydroxysuccinimide.^[4,5] Likewise, the sulfhydryl groups of two Cys residues existing in peptides were crosslinked with α, α' -dibromo-*m*-xylene to generate a unique cyclic peptide.^[6] In contrast to these approaches, we have devised unique methods that use nonproteinogenic amino acids with sidechains that selectively form linkages to the cognate pair.^[7–9] Here, we extend such methodology to incorporate a pair of nonproteinogenic amino acids containing benzylamine and 5-hydroxyindole functionalities into designated sites assigned by reprogrammed codons. Under oxidative conditions, these groups instantly react with each other and form a fluorescent heterocyclic moiety. Thus, this method enables the conversion of a nonfluorescent linear peptide to a fluorescent cyclic peptide.

5-Hydroxytryptamine (serotonin) is known to react rapidly and under mild conditions with benzylamine (**2a**) in the presence of potassium ferricyanide, $K_3Fe(CN)_6$, to yield a benzoxazole derivative (**14**, Figures S1 and S5 in the Supporting Information).^[10–12] It is noteworthy that the resulting adduct is fluorescent with an excitation maximum at 345 nm and an emission maximum at 460 nm. We have envisioned that this oxidative reaction would allow us to develop a new class of

orthogonal pairs for the intermolecular and intramolecular reactions of polypeptides involving 5-hydroxytryptophan (W^{OH} , **1**) and three benzylamine derivatives (**2a–c**, Scheme 1A). As a



Scheme 1. A) Fluorogenic oxidative coupling reactions between 5-hydroxytryptophan **1** (W^{OH}) and benzylamine derivatives **2**. Reaction between **1** and **2** is achieved by treatment with $K_3Fe(CN)_6$ in aqueous media to form the fluorophore structure. B) Schematic presentation of the site-specific modification of peptides bearing W^{OH} by intermolecular reaction with benzylamine derivatives (**2a** or **2b**). Blue symbol and red triangle represent 5-hydroxyindole and benzylamine groups, respectively. C) Schematic presentation of the cyclization of peptides by the intramolecular reaction between **1** and **2c** (F^{Bza}).

preliminary study, W^{OH} was incorporated into a model peptide at a specific site, and the resulting peptide could be site-selectively modified with a benzylamine (**2a**) or its biotin-bearing derivative (**2b**) (Scheme 1B). Upon successful demonstration of the above intermolecular coupling reaction, we attempted cyclization of peptides between the benzylamine attached to the α -amino group of phenylalanine (F^{Bza} , **2c**) and W^{OH} in the nascent chain to give fluorescent cyclic peptides (Scheme 1C). In both cases, incorporation of the above nonproteinogenic amino acids into peptides was achieved by genetic code reprogramming by using the flexizyme system,^[13,14] and a modified peptide-translation by using the recombinant elements (PURE) system.^[15–17] The former system consists of flexible tRNA acylation ribozymes, and the latter system is a reconstituted

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Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbic.200900021>; further details of all experiments.

cell-free translation system in which isoleucine (Ile) and/or methionine (Met) were withdrawn, referred to as wPURE system.

To reassign W^{OH} to AUC (one of the vacant Ile codons), W^{OH} -tRNA^{Asn-E1}_{GAU} prepared by the flexizyme was added to a wPURE system lacking Ile. An mRNA template expressing a dodecamer model peptide containing the Ile codon at position 11 (**3**, Figure S5) was subjected to translation. The expressed peptide was desalted by using a conventional C₁₈-resin filter, and the isolated peptide was analyzed by using a MALDI-TOF mass spectrometer. The observed molecular mass (ms) was consistent with the expected ms of peptide **3** containing W^{OH} 11 (Figure S5 B). The expression level of **3** was approximately 51% relative to that of the wild-type peptide containing Ile11 expressed in the ordinary PURE system (Figures S3 and S4); this value translated to approximately 0.46 pmol μ L⁻¹ of **3** compared with 0.91 pmol μ L⁻¹ of the wild-type as determined by the quantification method reported elsewhere.^[18]

For the conjugation, the translation solution containing crude peptide **3** was treated with the benzylamine derivatives **2a** or **2b** in the presence of K₃Fe(CN)₆ at room temperature for 5 min (Figure S5A) and then desalted. MALDI-TOF analysis revealed that **3** was modified by the respective reagents to yield **3a** and **3b** without undesirable side-reactions (Figure S5C and S5D). To further confirm the formation of the conjugation through a heterocyclic structure, we next examined the fluorescent properties of peptides **3**, **3a**, and **3b** (Figure S5E). The unmodified peptide **3** showed nearly the same emission profile as a blank sample; this indicates that it does not exhibit any fluorescent properties under such conditions (Figure S5E, **3**). In contrast, both modified peptides **3a** and **3b** exhibited similar fluorescent profiles with an excitation maximum at 345 nm and an emission maximum at 460 nm (Figure S5E, **3a** and **3b**). The above fluorescent profiles were nearly identical to that of fluorophore **14** (Figures S1 and S5E), which is derived from 5-hydroxyindole and **2a**; this implies that the same fluorophore was generated. Because the molecular absorption coefficient and fluorescent quantum yield were not available in the literature, we determined these parameters for **14** under similar conditions to which the peptides were monitored. These values were determined to be 1.54×10^4 cm⁻¹ m⁻¹ and 0.54 at pH 8.0 in 250 mM borate buffer. The above data encouraged us to perform the same chemistry in intramolecular fashion and apply it to the directed cyclization of peptides.

We synthesized a phenylalanine derivative bearing a benzylamine group on the α -amino group (Scheme 1A, **2c**) and charged it onto tRNA^{Met}_{CAU} by means of flexizyme.^[7,8,19,20] A wPURE system lacking Met and Ile was prepared, to which F^{bza}-tRNA^{Met}_{CAU} and W^{OH} -tRNA^{Asn-E1}_{GAU} were added in order to initiate the translation with F^{bza} instead of *N*-formylmethionine (fMet); this was followed by elongation with the corresponding amino acid sequence including W^{OH} 11 (Figure 1A, **4**). Oxidation of dodecamer **4** in the presence of K₃Fe(CN)₆ resulted in a decrease

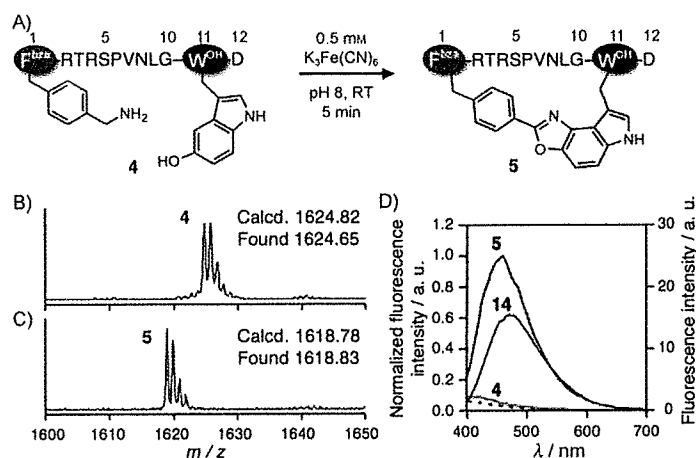


Figure 1. A) Intramolecular cyclization of peptide **4** through an oxidative coupling reaction. B) MALDI-TOF spectra of linear peptide **4** and C) cyclic peptide **5**. The calculated molecular mass (Calcd.) and found molecular mass (Found) for singly charged species, $[M+H]^+$ are shown in the spectra. D) Emission spectra of **4**, **5**, and **14**. The spectra were recorded in 250 mM borate buffer and DMSO 10% (v/v) adjusted pH to 8.0. The green line indicates the fluorescent profile of 300 nm **14** at an excitation wavelength of 326 nm (right axis), and other lines show the emission profiles at an excitation wavelength of 345 nm (left axis). The dashed line shows a blank profile obtained in the above buffer, while red and blue lines indicate the profiles of **4** and **5**, respectively.

in molecular weight that corresponded to a loss of six protons observed in MALDI-TOF analysis of the product; this suggested that **5** was being produced (Figure 1B and C). The excitation and emission profiles of **5** showed the respective maxima at 345 and 460 nm, whereas such a profile was not observed for **4** (Figure 1D). The fluorescent profile of **5** was similar to that of **14** as well as the modified peptides **3a** and **3b**. This result solidified the idea that the linear peptide **4** was cyclized to afford **5** with the expected eleven-membered ring structure closed by the fluorescent linker (Figure 1A).

To survey the orthogonality of the oxidative coupling reaction with various sidechains of proteinogenic amino acids, we designed peptide sequences containing F^{bza}, W^{OH} and those amino acids that are potentially reactive under oxidative conditions, such as Lys, Cys, Tyr and Trp (as opposed to an unreactive Leu) at position 13 (Figure 2A, **6–10**). Expression of peptides **7–10** took place smoothly with efficiencies of the range of 51–75% relative to the control Leu13-containing peptide **6** (Figure 2B, lanes 1–5). MALDI-TOF analysis of the resulting peptides indicated that all were converted to the desired cyclic peptides based on the observation of the same mass change corresponding to the loss of six protons (Figure S6A–E) with one exception; peptide **8**, which contains Cys13, seemed to have an extra set of peaks with an average of -8.07 mass shift along with the set of peaks with a -6.04 shift (Figure S6C, **8**). To the best of our knowledge, the sulfhydryl group of glutathione intermolecularly reacts with a dione derived from the oxidation of W^{OH} ; this results in a 7-S-glutathionyl-tryptophan-4,5-dione adduct.^[21] Thus, we attributed the above observation to a similar side-reaction between Cys13 and W^{OH} 7 that competed with the reaction between F^{bza} and W^{OH} 7. To further confirm the oxidative coupling between F^{bza} and W^{OH} 7, we mea-

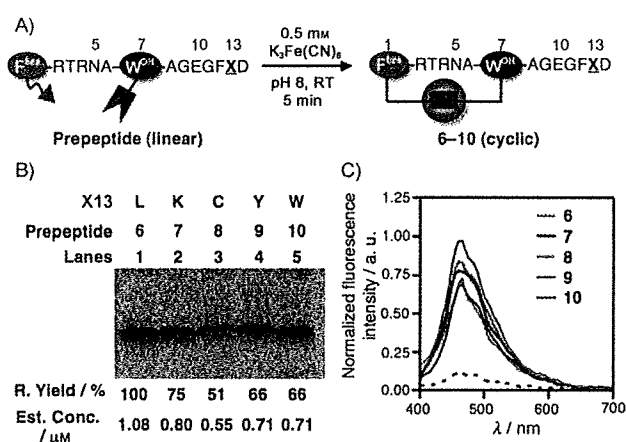


Figure 2. Competitive reaction between W^{OH} and potentially reactive proteinogenic amino acids against F^{bza} and F^{bza} . A) Peptide sequences used for the verification. The proteinogenic residue X13 was altered to L, K, C, Y, and W. B) Tricine SDS-PAGE analysis of the linear peptide labeled with [^{14}C]-Asp. The band of peptide was quantified by radio-autoradiography. Lane 1, Pre-6 (X13=L); lane 2, Pre-7 (K); lane 3, Pre-8 (C); lane 4, Pre-9 (Y); lane 5, Pre-10 (W). The peptide concentration was estimated by our standard quantification method in which the radioisotope (RI) counts of [^{14}C]-Asp were plotted against its known concentration to determine the calibration line. Then, the RI counts of the expressed peptides were fitted to the calibration line to estimate their respective concentration. R. Yield and Est. Conc. denote relative yield and estimated concentration. C) Emission spectra of cyclic 6–10. The spectra were recorded in 250 mM borate buffer and DMSO 10% (v/v) adjusted pH at 8.0. Solid lines show the emission profiles at an excitation wavelength of 345 nm. The dashed line shows a blank profile obtained in translation without DNA template, while red, blue, orange, green and cyan lines indicate those of 6, 7, 8, 9, and 10, respectively.

sured the fluorescence spectra of the product (Figure 2C). Fluorescence intensities were normalized according to the concentration of the respective linear peptides 7–10 against the control peptide 6 and quantified by tricine SDS-PAGE analysis (Figure 2B). All peptides exhibited similar fluorescent properties; this is consistent with the idea that they were cyclized. It should be noted that even though production of peptide 8 was accompanied by side-product formation, its fluorescent intensity was similar to that of 6 (Figure 2C, spectrum 8). Because we expected that the side-product would not show a similar fluorescent property, we concluded that the side-reaction between Cys13 and W^{OH} was fairly minor; this provided evidence that the intramolecu-

lar coupling reaction between F^{bza} and W^{OH} took place in a fairly selective manner.

Lastly, we verified that this cyclization method would be applicable to the synthesis of peptides with various ring sizes (Figure 3). We prepared three different mRNAs capable of expressing decamer, octamer, and hexamer peptides in the presence of F^{bza} -tRNA $^{Met}_{CAU}$ and W^{OH} -tRNA $^{Asn-E1}_{GAU}$ in the Met/Ile-lacking wPURE system. Treatment of the expressed peptides with $K_3\text{Fe}(\text{CN})_6$ was expected to produce the corresponding cyclic peptides rings containing W^{OH} at positions 9, 7, and 5 in peptides 11–13, respectively (Figure 3A–C). In all cases, the linear peptides were cleanly converted to the cyclic peptides with the expected loss of six protons detected by MALDI-TOF analysis (Figure 3A–C, ms data in the middle panel). Moreover, all of the cyclic peptides 11–13 exhibited nearly identical fluorescent profiles to 14, whereas the linear ones showed no fluorescence (Figure 3A–C, the bottom panel). These data clearly demonstrate that this method is applicable to the cyclization of peptides with a wide range of ring sizes (from five- to eleven-membered rings).

In conclusion, we have developed a new method for the cyclization of peptides that involves oxidative coupling of W^{OH} and F^{bza} attached to their N terminus by means of reprogramming the genetic code. The selective coupling of the above pairs takes place rapidly upon addition of $K_3\text{Fe}(\text{CN})_6$ and generates the desired fluorescent heterocyclic linkage. This chemistry enables us to convert nonfluorescent linear peptides to fluorescent cyclic peptides. Notably, the reaction takes place independently from the forming ring sizes, at least in the range of five- to eleven-membered rings as demonstrated in the current study. The chemistry demonstrated here by using the wPURE system is readily applicable to the platform of solid-phase or

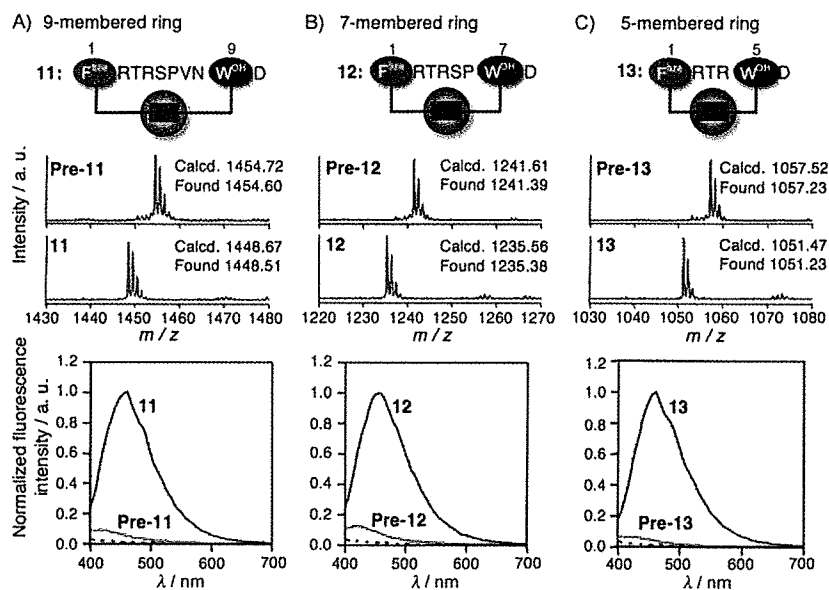


Figure 3. Synthesis of cyclic peptides with various ring sizes. Peptides 11–13 consist of A) 9, B) 7, and C) 5-membered rings. The upper panel of each Figure shows the structure of cyclic peptide, the middle panel exhibits MALDI-TOF spectra of precursor (Pre-11–13) and cyclic peptides (11–13), and bottom panel shows the excitation and emission profiles of Pre-11–13 and 11–13. The dashed line shows a blank profile. The conditions and designations of lines are the same as Figure 1.

solution-phase chemical synthesis, and thereby allows us to access to the large-scale production of the fluorescent cyclic peptide. On the other hand, the ribosomal expression of the peptides in the mRNA-encoding manner enables us to couple with an appropriate in vitro display technique that facilitates the screening of a large sequence space of cyclic peptides. Thus, the synthetic methodology reported herein offers us a new means for the discovery of cyclic peptide probes containing a unique fluorescent heterocyclic structure against various biological targets.

Experimental Section

Chemical synthesis of the amino acid derivatives, biotiny benzylamine, and the benzoxazole fluorophore is described in the Supporting Information. Flexizyme, tRNA^{Asn-E1}_{GAU}, tRNA^{Met}_{CAU}, and DNA templates coding peptides were prepared as previously reported.^[13] Aminoacyl-tRNAs (W^{OH}-tRNA^{Asn-E1}_{GAU} and F^{bza}-tRNA^{Met}_{CAU}) were prepared by using the flexizyme system. The wPURE system was reconstituted with the same components as the PURE system except that the necessary proteinogenic amino acids were added. Peptide **3**, which contained W^{OH}, was synthesized by using W^{OH}-tRNA^{Asn-E1}_{GAU} and the wPURE system without Ile. Linear peptides **4**, and **6–13** containing W^{OH} and F^{bza} were synthesized by using W^{OH}-tRNA^{Asn-E1}_{GAU}, F^{bza}-tRNA^{Met}_{CAU} and the wPURE system without Ile and Met. Translation mixtures were incubated at 37 °C for 30 min. For MALDI-TOF analysis, 5.0 µL of reaction mixture was acidified with TFA (1% (v/v)) in H₂O and purified by using a PerfectPure C-18 Tip (Eppendorf, Hamburg, Germany). Peptide product on the resin was eluted with an acetonitrile (50% (v/v))/TFA (0.1% (v/v)) solution (2.0 µL total) saturated with the matrix α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany).

For MALDI-TOF analysis of oxidation coupling reactions, modification was performed as follows: borate buffer (1.0 M, 2.5 µL, pH 8.0), **2a** or **2b** in DMSO (100 mM, 1.0 µL), and K₃Fe(CN)₆ in H₂O (13.3 mM, 1.5 µL) were added to the translation mixture (5.0 µL) containing **3** (0.46 µM). Components and concentrations of translation mixture were described in elsewhere.^[22] On the other hand, cyclization was carried out as follows: borate buffer (500 mM, 2.5 µL, pH 8.0), and K₃Fe(CN)₆ in H₂O (2.5 mM, 2.5 µL) were added to the translation mixture (5.0 µL). The resulting mixture was left for 5 min at room temperature. The translation mixture was acidified with TFA in H₂O (1% (v/v)). The product was purified by using a C-18 Tip and eluted with acetonitrile (50% (v/v))/TFA (0.1% (v/v)) solution (2.0 µL total) saturated with the matrix α-cyano-4-hydroxycinnamic acid.

For fluorescence analysis of oxidation coupling reactions, each peptide was purified by using a C-18 Tip from translation mixture (5.0 µL), and eluted with an acetonitrile (50% (v/v))/TFA (0.1% (v/v)) solution (10 µL total). The eluent was evaporated for 30 min at room temperature, and the resulting residue was dissolved in H₂O (1.25 µL). Borate buffer (1.0 M, 1.25 µL, pH 8.0), **2a** or **2b** in DMSO (100 mM, 0.5 µL) were added to modified peptide **3**, and then K₃Fe(CN)₆ in H₂O (5 mM, 2.0 µL) was added. To generate cyclic peptide **5** and **6–13**, the peptide residue was dissolved in H₂O (1.25 µL), borate buffer (1.0 M, 1.25 µL, pH 8.0), and DMSO (0.5 µL), and then added to K₃Fe(CN)₆ in H₂O (1.25 mM, 2.0 µL). The resulting mixture was incubated for 5 min at room temperature. The emission and excitation spectra were measured by using a 1536-well micro plate (BD Falcon, Franklin Lakes, USA) at 25 °C. Excitation and emission slit-widths were kept constant at 5.0 nm.

Acknowledgements

We thank Dr. M. Komiyama for the use of UV-Vis spectrophotometer and spectrofluorometer and Dr. P. C. Reid for critical proof-reading. We thank Drs. I. Hirao and T. Mitsui in the RIKEN Genomic Sciences Center for the use of a FAB mass spectrometer. This work was supported by grants of Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (S) (16101007) and a research and development projects of the Industrial Science and Technology Program in the New Energy and Industrial Technology Development Organization (NEDO) to H.S.

Keywords: cyclization · fluorescent probes · oxidation · peptides · protein modifications

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Received: January 12, 2009

Published online on May 26, 2009

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

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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 wPURE, 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

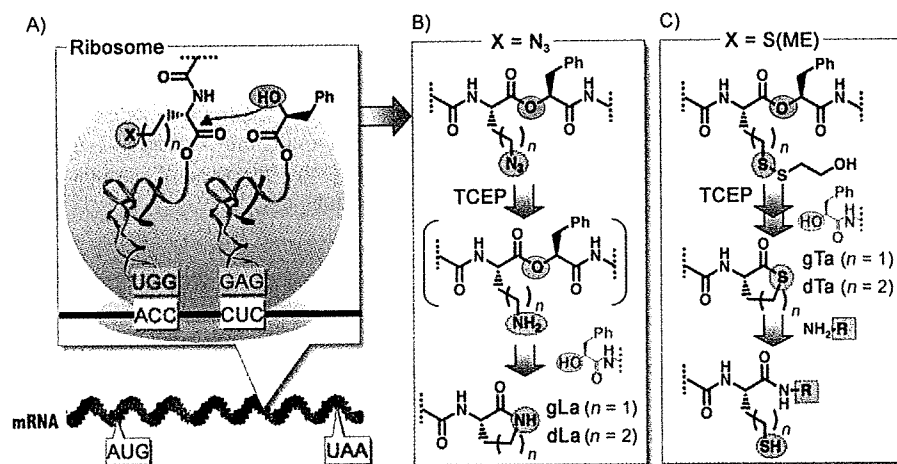
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Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbic.200900058>.

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 1A). The resulting peptides contain a peptide bond originating from the above nonproteinogenic amino acid, fol-



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.

lowed 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 1B, gLa) or into a γ -thiolactone (gTa) that can be converted into a variety of alkylamides (Scheme 1C). 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.

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 1A, Aha). In the case of Hcy, its sulfhydryl group was masked by a disulfide bond with 2-mercaptoethanol (ME; Figure 1A, (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 wPURE systems. Xaa and F^{lac} were esterified with DBE and CME groups, respectively, and thus were made suitable substrates for dFx and eFx (Figure 1A). To make the ACC and CUC codons vacant, we prepared a wPURE 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} 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)mercaptanorvaline ((ME)Mnv; Figure 1A, $n=2$).^[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 1B, 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

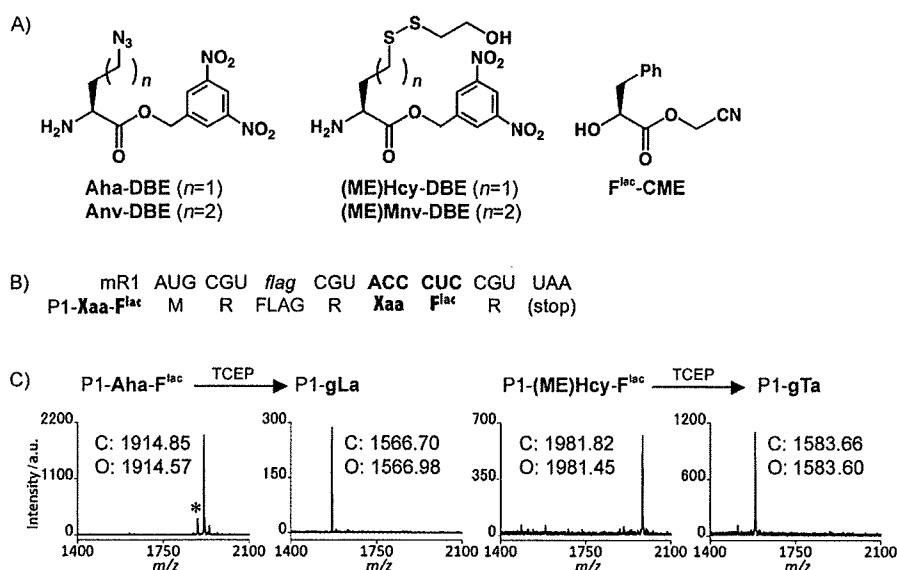


Figure 1. Ribosomal synthesis of the C-terminal γ/δ -lactam- and γ/δ -thiolactone-containing peptides, P1-gLa and P1-gTa, respectively. A) Structures of the nonproteinogenic amino acid DBEs and phenyllactic acid CME; Aha: azidothioalanine; Anv: azidonorvaline; (ME)Hcy: 2-mercaptoethanol-protected homocysteine; (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 wPURE 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 clearly 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 [¹⁴C]-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 μ 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

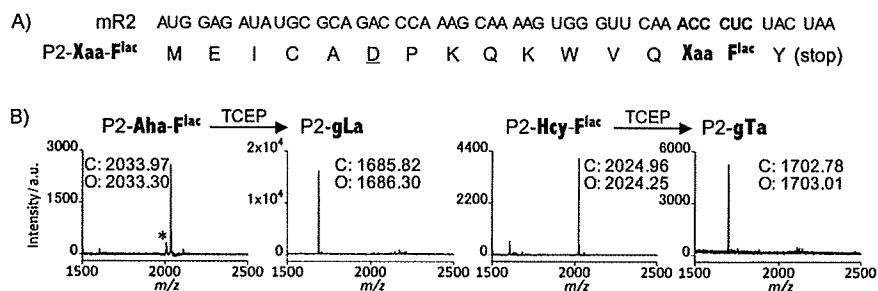


Figure 2. Ribosomal synthesis of the C-terminal γ/δ -lactam- and γ/δ -thiolactone-containing peptides, P2-gLa and P2-gTa, respectively. A) Sequences of mR2 and P2-Xaa-F^{lac}. Xaa and F^{lac} are assigned to codons ACC and CUC, respectively. B) MALDI-TOF analysis of the peptides. Calculated (C) and observed (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.

the precursor peptide (Figure 2B, P2-Aha-F^{lac} or P2-Hcy-F^{lac}) and of the peptide containing C-terminal γ -lactam or γ -thiolactone moieties (Figure 2B, 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 3A). P1-gTa was accordingly treated with thirteen different amines, from simple ammonium hydroxide to a fluorescent-labeled alkylamine (Figure 3B, 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 3C). It should be noted that ME was one of the standard reagents included both in the ordinary PURE and in the wPURE 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 4A). 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 4B). MALDI-TOF analyses both of the intermediate peptide (P3-gTa) and of the macrocyclic peptide (mcP3-(ME)Hcy) confirmed the desired conversion (Figure 4C). 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 treat-

ment 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-E1} (40 μ M) in Tris-HCl (0.2 M, pH 8.0, 6.25 μ L) was heated at 95 °C for 1 min, and the system was then allowed to cool to room temperature over 5 min. MgCl₂ (3 M, 2.5 μ L) and dFx (200 μ M, 1.25 μ L) were then added and the solution was incubated at room temperature for 5 min. Substrate in DMSO (25 mM, 2.5 μ L) 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 M, pH 5.0, 40 μ L), and the RNA was recovered by ethanol precipitation. The pellet was rinsed twice with ethanol (70%) containing sodium acetate (0.1 M, 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 mM each); for tricine SDS-PAGE analysis, [¹⁴C]-Asp (50 μ M) 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 nM) at 37 °C for 1 h, in a total volume of 5 μ L. The wPURE system was reconstituted with the same components as the PURE system, except that only the necessary proteinogenic amino acids (0.2 mM each) and acyl-tRNAs (50 μ M 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 wPURE system in the presence of DNA template D1 (40 nM), Met, Arg, Tyr, Lys, and Asp (0.2 mM each), and Aha-tRNA^{Asn-E1}_{GGU}, Anv-tRNA^{Asn-E1}_{GGU}, (ME)Hcy-tRNA^{Asn-E1}_{GGU}, or (ME)Mnv-tRNA^{Asn-E1}_{GGU} (50 μ M each), and F^{lac}-tRNA^{Asn-E1}_{GAG} (50 μ M) 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 wPURE system in the presence of DNA template D2 (40 nM), Met, Glu, Ile, Cys, Ala, Pro, Lys, Gln, Trp, Val, Arg, Tyr, Asp (0.2 mM each); Asp was replaced with 50 μ M [¹⁴C]-Asp for tricine SDS-PAGE analysis), and Aha-tRNA^{Asn-E1}_{GGU} or Anv-tRNA^{Asn-E1}_{GGU}, (ME)Hcy-tRNA^{Asn-E1}_{GGU}, or (ME)Mnv-tRNA^{Asn-E1}_{GGU} (50 μ M each), and F^{lac}-tRNA^{Asn-E1}_{GAG} (50 μ M) were used. For tricine SDS-PAGE analysis the reaction mixture (2.5 μ L) was mixed with loading buffer (2.5 μ L; 0.9 M 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 μ L) of the reaction mixture was mixed with 2 \times TBS (5 μ L; 100 mM Tris-HCl, pH 8.0, 300 mM NaCl) and incubated in prewashed FLAG-M2 agarose (Sigma) for 1 h. The resin was then washed once with TBS (50 mM Tris-HCl, pH 8.0, 150 mM NaCl; 10 μ L), and

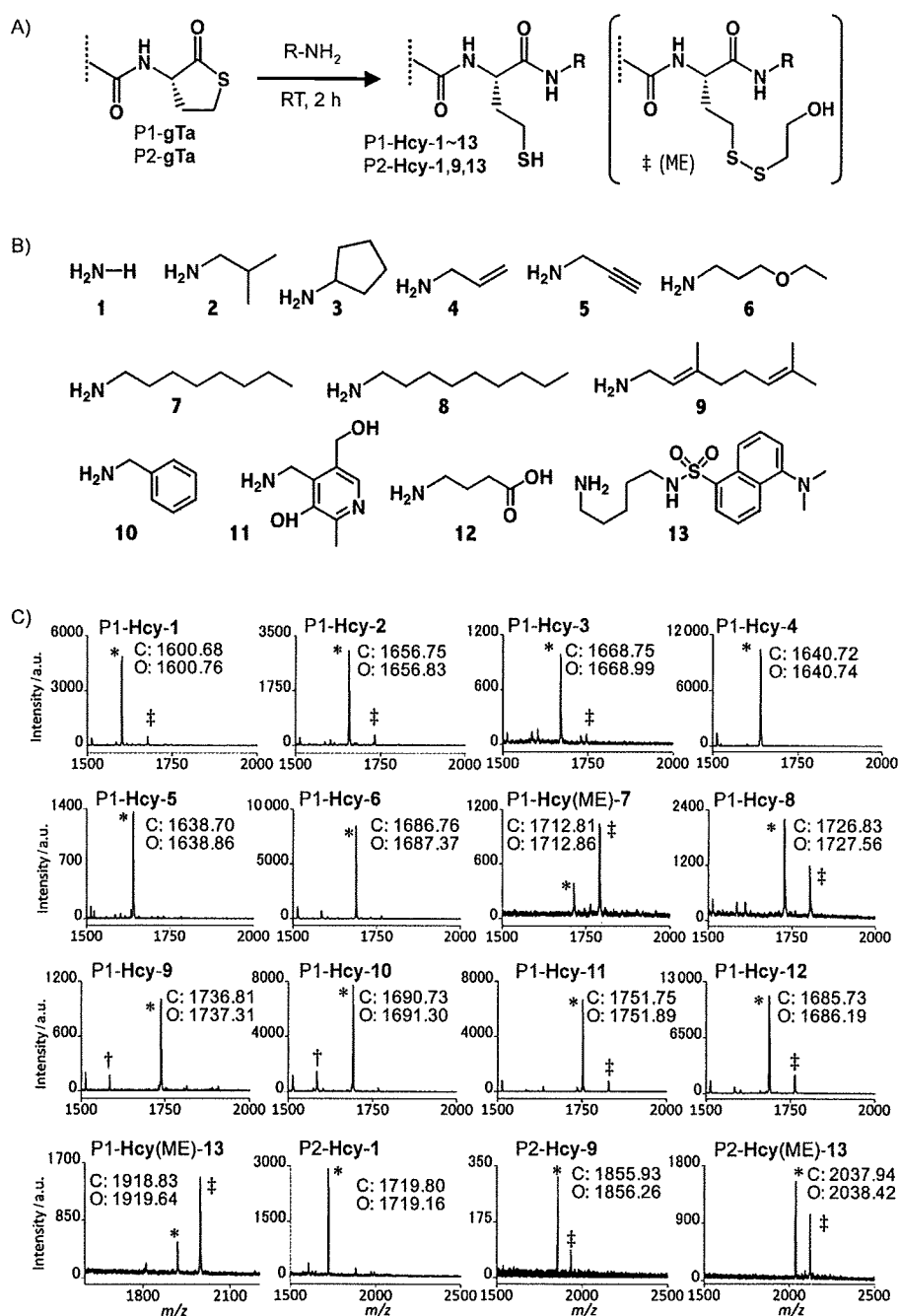


Figure 3. Amidation of P1-gTa and P2-gTa with a variety of amines. A) Schematic representation of C-terminal amidation with γ -thiolactones. P1-gTa and P2-gTa were incubated with amines that opened their γ -thiolactone moieties to yield the alkylamides. The products in parentheses represent the oxidation 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.