Chemistry & Biology
Article

Messenger RNA-Programmed Incorporation of Multiple N-Methyl-Amino Acids into Linear and Cyclic Peptides

Takashi Kawakami,[1] Hiroshi Murakami,[2] and Hiroaki Suga[1,2,*]
[1]Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, Tokyo 113-8656, Japan
[2]Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-0894, Japan
*Correspondence: hsuga@rcast.u-tokyo.ac.jp
DOI 10.1016/j.chembiol.2007.12.008

SUMMARY

Natural peptide products often contain N-methylated backbones, and such a modification plays a crucial role in making natural peptides peptidase resistant and membrane permeable. Here, we demonstrate the ribosomal synthesis of N-methyl-peptides by means of genetic code reprogramming. Two key technologies, a ribozyme-based de novo tRNA acylation (flexizyme) system and an *E. coli* reconstituted cell-free translation (PURE) system, were used in order to reassign arbitrarily chosen codons to N^{α}-methylated amino acids (^{Me}aa). Using this combination, we determined the general structural requirement of "accessible" ^{Me}aa and demonstrated their multiple incorporations into the nascent peptide chain according to the assignments made on mRNA, giving linear and cyclic N-methyl-peptides in high purities. This platform technology offers a convenient tool for the construction of N-methyl-peptide libraries, potentially leading to the discovery of therapeutic peptides.

INTRODUCTION

Natural peptide products isolated from various organisms often contain N-methylated backbones (Billich and Zocher, 1990; Hornbogen and Zocher, 2005). Such a modification of peptide backbone alters the properties of the peptide bond, which confers their conformational rigidity (Sagan et al., 2004). This modification contributes to improvements in the biological properties of natural peptides, such as target affinity, proteolytic stability, and/or membrane permeability. Thus, N^{α}-methylated amino acids (^{Me}aa) are invaluable components for the synthesis of peptide libraries in screening for peptides with suitable drug-like properties for potential therapeutic use. The backbone N-methylation of these peptides are generally executed by one or more of enzymes in the multienzyme clusters, called nonribosomal peptide synthetases (NRPSs) (Billich and Zocher, 1990; Hornbogen and Zocher, 2005; Sieber and Marahiel, 2005; Walsh et al., 2001). This type of peptide synthesis machinery is known to be template independent, in contrast to the mRNA template-dependent ribosomal machinery. Unfortunately, their complexity demands an enormous effort to manipulate the systems, thereby making it difficult to generate desired peptide libraries (Baltz, 2006; Fischbach and Walsh, 2006; Hahn and Stachelhaus, 2006).

On the other hand, the translation machinery expresses peptides in an mRNA template-dependent manner, which makes this system exceptionally versatile and useful for the synthesis of peptides or proteins. Unlike NRPSs, the ordinary translation system strictly incorporates 20 proteinogenic amino acids into the nascent peptide chain. However, an appropriate manipulation of the translation apparatus enables us to incorporate nonproteinogenic amino acids into peptides (Hendrickson et al., 2004; Hohsaka and Sisido, 2002; Link et al., 2003; Wang and Schultz, 2004). A classical example is that when a nonproteinogenic amino acid is charged onto an orthogonal $tRNA_{CUA}$ (the subscript base sequence indicates the anticodon), this aminoacyl-$tRNA_{CUA}$ (aa-$tRNA_{CUA}$) is able to suppress UAG amber stop codon on mRNA; thereby, the amino acid can be incorporated into the nascent peptide at the designated site (Bain et al., 1989; Noren et al., 1989). Despite encouraging results from a number of successful examples for the incorporation of nonproteinogenic amino acids with various nonnatural side chains, it has been known that some ^{Me}aa are incorporated into a peptide chain with good or modest efficiencies, and some are not at all (Bain et al., 1991; Chung et al., 1993; Ellman et al., 1992; Gilmore et al., 1999; Karginov et al., 1997; Mendel et al., 1995; Murakami et al., 2006; Short et al., 2000). To the best of our knowledge in the literature, only three ^{Me}aa, ^{Me}Gly, ^{Me}Ala, and ^{Me}Phe, have been successfully incorporated into the nascent peptide chain by means of amber suppression. Moreover, neither incorporation of multiple ^{Me}aa nor a single ^{Me}aa with amino acids bearing noncanonical side chains has been thus far reported.

More recently, a new concept of genetic code reprogramming was introduced by Forster et al. and applied to the incorporation of nonproteinogenic amino acids into peptides (Forster et al., 2003). Genetic code reprogramming involves the reassignment of codons from proteinogenic amino acids to nonproteinogenic ones via multiple sense suppressions. Thus, this methodology enables us to simultaneously incorporate multiple nonproteinogenic amino acids into peptides, which represents a major advantage over the aforementioned amber suppression method (Forster et al., 2003; Josephson et al., 2005; Murakami et al., 2006; Ohta et al., 2007; Ohuchi et al., 2007; Tan et al., 2005).

In the context of ^{Me}aa using the sense suppressions, there were three examples in the literature (Frankel et al., 2003; Merryman and Green, 2004; Tan et al., 2004). Merryman and Green have reported that aa-tRNAs prepared by cognate aminoacyl-tRNA

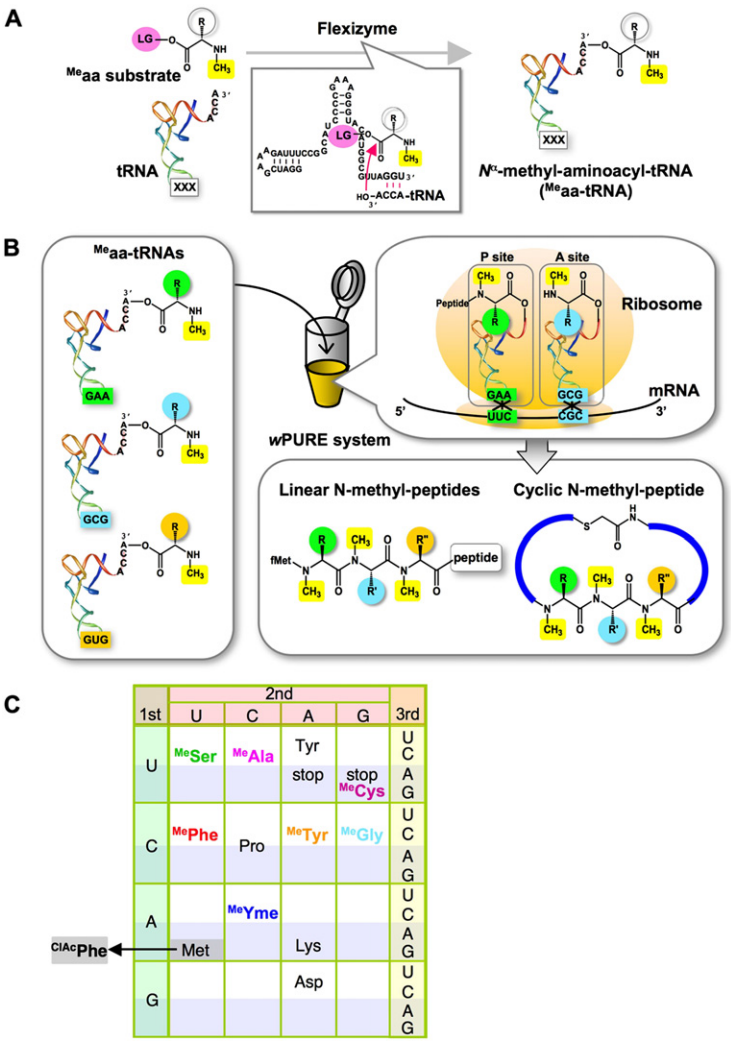

Figure 1. Messenger RNA-Programmed Synthesis of N-Methyl-Peptides by Genetic Code Reprogramming

(A) Synthesis of N^{α}-methyl-aminoacyl-tRNA (^{Me}aa-tRNA) by flexizyme system. Flexizyme recognizes leaving group (LG: highlighted in pink) on the ester bond of N^{α}-methyl-amino acid (^{Me}aa) substrate and conserved three bases on the 3′-terminus of tRNA, which allows for the aminoacylation of desired tRNA with any ^{Me}aa. N^{α}-methyl groups are highlighted in yellow.

(B) Ribosomal synthesis of N-methyl-peptide. ^{Me}aa-tRNAs bearing various combinations of ^{Me}aa and anticodon are added to the *w*PURE (*withdrawn* protein synthesis using recombinant elements) system for mRNA-programmed incorporation of ^{Me}aa into peptides. The *w*PURE system is an *E. coli* reconstituted cell-free translation system in which some components (amino acids and aminoacyl-tRNA synthases) are withdrawn from the ordinary translation system to reassign multiple sense codons to various ^{Me}aa.

(C) Reprogrammed genetic code table for the mRNA-programmed synthesis of N-methyl-peptides. Codons that are reassigned to various ^{Me}aa are shown in color letters. Initiation codon (AUG) highlighted in gray is reassigned from Met to N^{α}-(α-chloroacetyl)-Phe (^{ClAc}Phe) for the ribosomal synthesis of cyclic N-methyl-peptides.

synthetases (aaRSs) were converted to ^{Me}aa-tRNAs by the three-step procedure, where the α-amino group was alkylated by consecutive reductive amination with 2-nitrobenzaldehyde and formaldehyde, and then the 2-nitrobenzyl group was deprotected by UV irradiation (Merryman and Green, 2004). These ^{Me}aa-tRNAs derived from 20 proteinogenic amino acids were surveyed for the synthesis of a dipeptide, fMet-^{Me}aa, where thin-layer chromatographic electrophoresis was mainly used to discern the product of fMet-^{Me}aa from fMet (also possibly fMet-aa) and to determine the incorporation efficiency of each ^{Me}aa in a semiquantitative manner. In the second example, Tan and Cornish et al. prepared ^{Me}Ala-tRNA$^{AsnB}_{GAC}$ and ^{Me}Phe-tRNA$^{AsnB}_{GAC}$ by the chemoenzymatic aminoacylation procedure (Hecht et al., 1978; Robertson et al., 1991) and performed their single incorporation into a tripeptide (fMet-Xaa-Glu, where Xaa represents ^{Me}aa) upon the sense suppression of the Val codon (GUU) (Tan et al., 2004). Significantly, this work determined the incorporation efficiencies of these two ^{Me}aa into the peptide chain in a quantitative manner and also confirmed the product peptide by liquid chromatography based on its retention time by comparison with that of the corresponding synthetic authentic sample. Both examples above showed only a single incorporation of ^{Me}aa into a di- or tripeptide backbone, and therefore the concept of genetic code reprogramming has not yet been fully explored for the synthesis of N-methyl-peptides.

In the third example, Frankel and Roberts et al. have shown polymerization of ^{Me}Phe assigned to in repetition of two, five, or ten residues in an in vitro display system (Frankel et al., 2003). This work represents, to our knowledge, the first demonstration of consecutive incorporations of a single type of ^{Me}aa into a peptide stretch by using sense suppression. However, because the polymerization of ^{Me}Phe was only evidenced by observing the protease resistance of the respective peptide, it still remains unknown to what degree of the contamination of natural amino acids in the poly-^{Me}Phe chain occurred. In fact, the full-length peptide bearing multiple ^{Me}Phe was susceptible to protease to some extent, suggesting that competing incorporations of likely Val or possibly other proteinogenic amino acids occurred as Frankel et al. discussed in their report (Frankel et al., 2003). Taken together, although the above three examples clearly documented that the sense-suppression method could be used for the incorporation of ^{Me}aa, the proof-of-concept study on the genetic code reprogramming, i.e., performing multiple incorporations of two or more different ^{Me}aa with high fidelity control, remains to be demonstrated.

We report here incorporation of multiple ^{Me}aa into the peptide backbone with a nearly perfect control of sequences and lengths by using the concept of genetic code reprogramming. To reprogram the genetic code, we used two technologies, flexizyme and PURE (protein synthesis using recombinant elements) systems. Flexizyme system is a ribozyme-based de novo tRNA acylation system that is able to charge virtually any amino acids onto desired tRNAs with any body and anticodon sequences (Figure 1A) (Kourouklis et al., 2005; Murakami et al., 2003a, 2003b, 2006; Ohuchi et al., 2007; Saito et al., 2001). PURE system is a

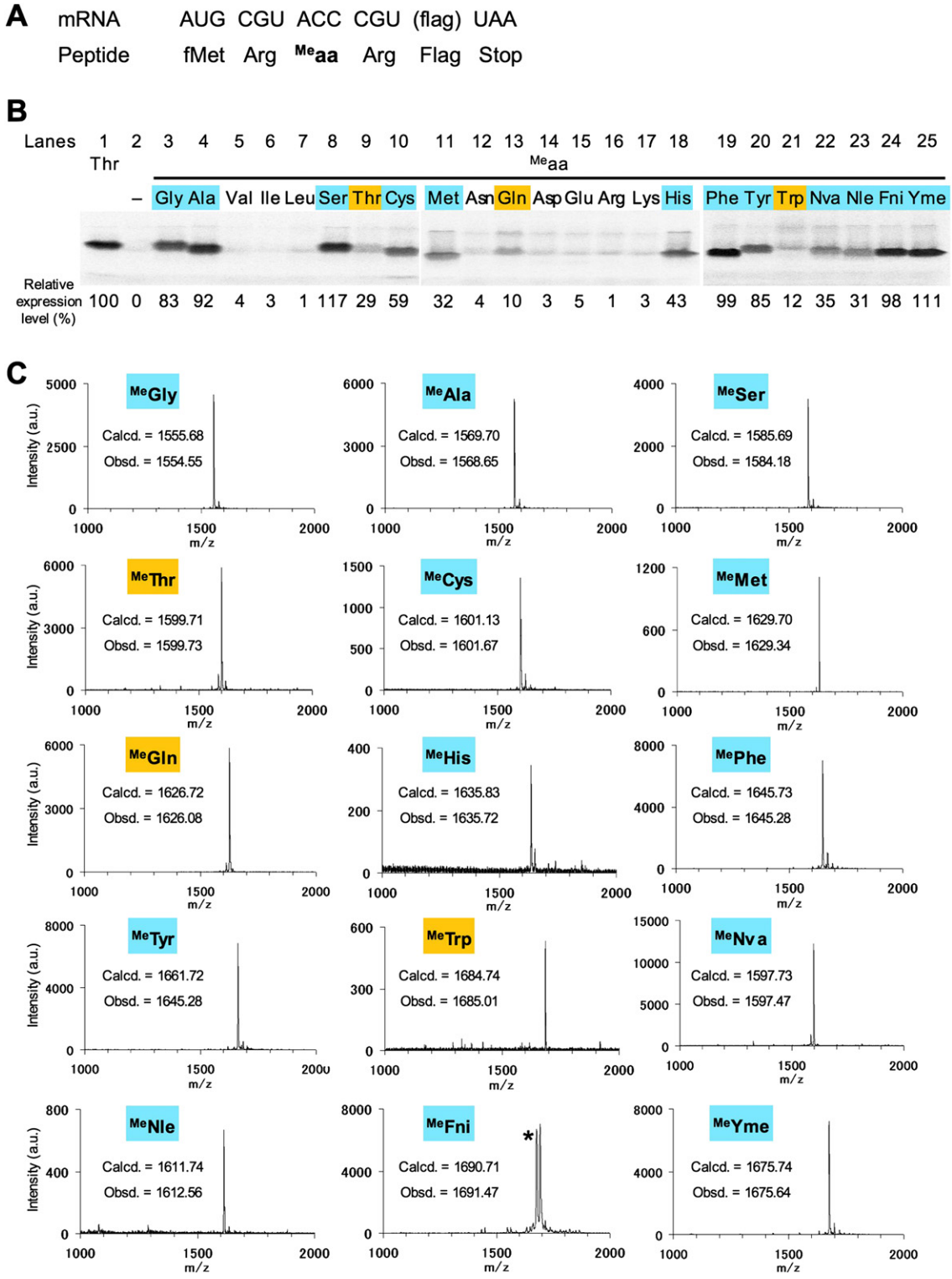

Figure 2. Single Incorporations of N^{α}-Methylated Amino Acids

(A) Sequences of mRNA and peptide. Flag in parentheses indicates the RNA sequence coding the Flag peptide (DYKDDDDK).

(B) Tricine-SDS-PAGE analysis of the expressed peptides labeled with [^{14}C]-Asp detected by autoradiography. Lane 1, the wild-type peptide expressed in the ordinary PURE system where ACC assigns Thr; lane 2, a negative control using *w*PURE system in the presence of uncharged $tRNA^{Asn\text{-}E1}_{GGU}$; lanes 3–25, expression of the peptide containing a single ^{Me}aa in the presence of designated ^{Me}aa-$tRNA^{Asn\text{-}E1}_{GGU}$ prepared by the flexizyme system. Each expression level relative to wild-type was determined by a mean score of triplicates. The amino acids giving over 30% and 10% expression levels relative to the wild-type are highlighted in cyan and orange, respectively.

reconstituted *Escherichia coli* cell-free translation system in which transcription and translation are coupled (Shimizu et al., 2001). The most important feature of this translation apparatus is that some components, such as amino acids and aminoacyl-tRNA synthases (aaRSs), can be withdrawn from the translation elements (Figure 1B). By means of such a *withdrawn* PURE system, named *w*PURE, we are able to vacant certain codons at our will. Combining these two systems enables us to readily reassign the vacant codons to ^{Me}aa and express peptides as designed on mRNA sequences according to the newly designated genetic table (Figure 1C).

In this report, we initially screened N^{α}-methylated proteinogenic and nonproteinogenic amino acids to evaluate the efficiency of their single incorporation into a peptide backbone. Then, we investigated the mRNA-programmed synthesis of peptides containing multiple ^{Me}aa, achieving the compositions of continuous stretches of up to ten ^{Me}aa residues. Finally, we investigated the synthesis of cyclic N-methyl-peptides closed by a physiologically stable thioether bond, showing its potential for the generation of in vivo compatible peptide libraries therapeutically targeting various proteins.

RESULTS

Single Incorporations of N^{α}-Methylated Amino Acids

To investigate the incorporation of multiple ^{Me}aa into a peptide backbone, various N^{α}-methylated proteinogenic and nonproteinogenic amino acids charged onto a tRNA by the flexizyme system were comprehensively evaluated for the efficiency of their single incorporation into a peptide backbone. As was discussed earlier, Merryman and Green performed a similar experiment with a dipeptide synthesis format (Merryman and Green, 2004). In contrast, we here incorporated ^{Me}aa into a 12-mer model peptide, thus involving the incorporation of ^{Me}aa into a specific site followed by elongation and termination of peptide synthesis as a format of normal peptide synthesis (Figure 2A, more details are discussed below).

For this study, we chose 23 ^{Me}aa substrates, 19 of which were derived from proteinogenic amino acids, and 4 of which were derived from nonproteinogenic amino acids (Figure S1, see the Supplemental Data available with this article online). The flexizyme-catalyzed aminoacylation efficiency for each ^{Me}aa was verified by our standard protocol (Figure S2), showing that the majority of ^{Me}aa could be charged onto tRNA with over 30% yields. Three amino acids, ^{Me}Val, ^{Me}Ile, and ^{Me}Asn, showed less than 30% yields. In the former two amino acids, their steric hindrance resulted from the combination of the β-branched side chain and the N^{α}-methyl-amino group presumably made these particular substrates much less reactive to flexizyme compared with other ^{Me}aa. In the case of ^{Me}Asn, based on our past experience working on Asn-tRNA, its tRNA-adduct might be labile due to the intramolecular attack of the amide side chain (Lee and Suga, 2001; Murakami et al., 2006); thereby the yield might appear to be low in PAGE analysis. However, we have previously observed that such aa-tRNAs prepared in similar ranges of quality (Val-, Ile-, and Asn-tRNAs in 13%–30% yield) could still be used for the incorporation into the nascent peptide chain with satisfactory yields (30%–70%) (Murakami et al., 2006). Thus, we pursued to survey all ^{Me}aa-tRNAs for the single incorporation into the model peptide.

The *w*PURE system used in our survey was composed of a limited member of amino acids and aaRSs. We designed an open-reading frame (ORF) in mRNA (Figure 2A) that expresses a short peptide consisting of fMet-Arg-^{Me}aa-Arg followed by a Flag peptide (DYKDDDDK; D, Asp; Y, Tyr; K, Lys). We selected a Thr codon (ACC) to assign ^{Me}aa and an orthogonal tRNA$^{Asn\text{-}E1}_{GGU}$ (Ohta et al., 2007) for its suppression. When the model peptide was expressed in the presence of uncharged tRNA$^{Asn\text{-}E1}_{GGU}$ in *w*PURE system, no product band appeared on tricine-SDS-PAGE (Figure 2B, lane 2), suggesting that tRNA$^{Asn\text{-}E1}_{GGU}$ could act as an orthogonal tRNA inert against the endogenous aaRSs added to the *w*PURE system.

We next tested the single incorporation of ^{Me}aa charged onto tRNA$^{Asn\text{-}E1}_{GGU}$ into the model peptide sequence. Eight out of 19 ^{Me}aa derived from proteinogenic amino acids were incorporated into the peptide with more than 30% efficiencies relative to wild-type expression (Figure 2B, lanes 1 versus those highlighted in cyan). The incorporation of the designated ^{Me}aa was also confirmed by MALDI-TOF analysis, giving the expected molecular mass as a sole peak in all cases (Figure 2C). Moreover, ^{Me}Thr, ^{Me}Gln, and ^{Me}Trp were modestly incorporated yet gave the single desired product (Figures 2B and 2C, those highlighted in orange). We called these ^{Me}aa molecules that could be incorporated into the peptide chain with more than 10% efficiencies as "accessible" ^{Me}aa. Interestingly, all of these "accessible" ^{Me}aa shared similar structural features of their side chains; either aromatic side chains (^{Me}Phe, ^{Me}Tyr, ^{Me}His, and ^{Me}Trp) or noncharged and nonbulky side chains (^{Me}Gly, ^{Me}Ala, ^{Me}Ser, ^{Me}Cys, ^{Me}Met, ^{Me}Thr, and ^{Me}Gln).

It should be noted that our results were mostly consistent with Merryman's observations, with some contradictions (Merryman and Green, 2004). In their experiments, ^{Me}Val, ^{Me}Ile, and ^{Me}Leu were ranked in the "efficient," or what we referred to as the "accessible," ^{Me}aa group. However, our experiments showed that ^{Me}Val, ^{Me}Ile, and ^{Me}Leu were grouped as rather "inaccessible" ^{Me}aa for incorporation (Figure 2B, lanes 5–7). The difference between the in vitro-transcribed tRNA$^{Asn\text{-}E1}$ and the native cognate tRNAs that Merryman et al. used as ^{Me}aa-carriers may have an effect on their incorporation efficiencies (Dale and Uhlenbeck, 2005; LaRiviere et al., 2001). However, it should be noted that in the Merryman's TLC electrophoresis assay, the mobility of these branched aliphatic ^{Me}aa were indistinguishable from that of the cognate natural ones. Therefore, it was not clearly defined if the observed product spot originated from fMet-^{Me}aa or fMet-aa. Because the reductive alkylation of some aa-tRNAs to generate ^{Me}aa-tRNAs possibly left a small amount of unreacted aa-tRNAs as Merryman et al. discussed (Merryman and Green, 2004), it could not be ruled out that such aa-tRNA contamitants competed out ^{Me}aa-tRNAs for incorporation into the nascent

(C) MALDI-TOF-MS spectra of the Flag-purified N-methyl-peptides. The calculated molecular mass (Calcd.) and observed molecular mass (Obsd.) for singly charged species, $[M+H]^{+1}$ are shown in each spectrum. In the spectrum of peptide with ^{Me}Fni, an additional peak (asterisk) was detected since the additional peak corresponds to N^{α}-methyl-*p*-nitroso-Phe (Calcd. = 1674.72, Obsd. = 1675.45) generated by photodecomposition of NO_2 to NO during the MALDI-TOF-MS analysis (Ho and Chow, 1996).

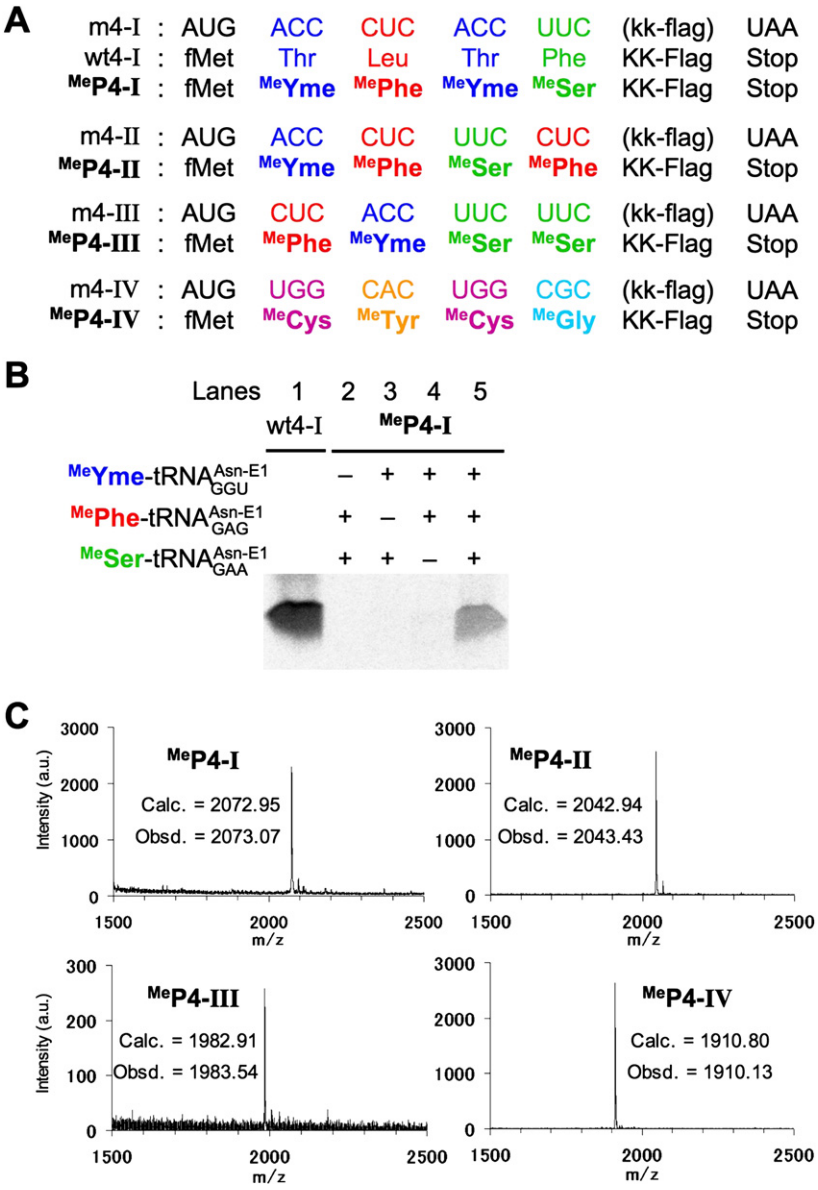

Figure 3. Four Successive Incorporations of Multiple N^{α}-Methylated Amino Acids

(A) Sequences of mRNA templates (m4-I–IV), tetra-N-methyl-peptides (^{Me}P4-I–IV), and the control wild-type peptide (wt4-I). The kk-flag in parentheses indicates the RNA sequence coding a KK-Flag peptide (KKDYKDDDDK). Arabic number denotes the number of N-methylated peptide bonds. The codons and amino acids are colored according to the reprogrammed genetic code table in Figure 1C.

(B) Tricine-SDS-PAGE analysis of the peptides expressed from m4-I labeled with [^{14}C]-Asp detected by autoradiography. Lane 1, the wild-type peptide expressed in the ordinary PURE system; lanes 2–4, negative controls in the presence of two of ^{Me}aa-tRNA^{Asn-E1} as shown; lane 5, in the presence of all three ^{Me}aa-tRNA^{Asn-E1}.

(C) MALDI-TOF-MS spectra of ^{Me}P4-I–IV. The calculated mass (Calcd.) and observed mass (Obsd.) are shown in each spectrum.

peptide chain. In contrast, in our studies, because the flexizyme system ensures the purity of each ^{Me}aa-tRNA, i.e., no contamination of aa-tRNA as confirmed by MALDI-TOF analysis (Figure 2C), we are able to avoid such issues and judge its intrinsic incorporation efficiency more reliably and quantitatively. To this end, we propose that ^{Me}Val, ^{Me}Ile, and ^{Me}Leu are inaccessible substrates due to the combination of bulkiness in the branched side chain and the N^{α}-methylated α-amino group.

Four ^{Me}aa derived from nonproteinogenic amino acids (Figure S1) were also incorporated into the corresponding peptide chain (Figure 2B, lanes 21–25), each of which the MALDI-TOF data was consistent with the expected molecular mass (Figure 2C). Again, the same trend for the incorporation efficiency seemed to apply to these nonproteinogenic ^{Me}aa, where the aromatic ones were incorporated more efficiently than the aliphatic ones. All data taken together, we concluded that ^{Me}aa having "aromatic" or "nonbulky and noncharged" side chains were preferable for the incorporation into the peptide backbone. This information is invaluable for us to select appropriate ^{Me}aa and design specific compositions of N-methylated peptides or peptide libraries while avoiding the risk of potential failure in the synthesis.

Four Successive Incorporations of Multiple N^{α}-Methylated Amino Acids

We next attempted the consecutive incorporations of multiple ^{Me}aa into the peptide backbone via genetic code reprogramming. Five ^{Me}aa derived from proteinogenic amino acids (^{Me}Ser, ^{Me}Phe, ^{Me}Tyr, ^{Me}Cys, ^{Me}Gly) and one derived from a nonproteinogenic amino acid (^{Me}Yme) were selected and assigned arbitrarily to six codons as shown in Figure 1C. The flexizyme system was utilized to charge the respective ^{Me}aa on tRNA^{Asn-E1} bearing the anticodon that reads the assigned codon. We also designed four mRNA templates containing ORFs (Figure 3A, m4-I–IV) that express the corresponding peptides comprised of three different ^{Me}aa in four successive residues (Figure 3A, ^{Me}P4-I–IV). The C terminus of the respective peptides was also designed to contain a modified Flag sequence (KK-Flag = KKDYKDDDDK; D, Asp; Y, Tyr; K, Lys) to facilitate its isolation as well as ionization upon MALDI-TOF analysis.

We first used m4-I to monitor the expression level of ^{Me}P4-I by tricine-SDS-PAGE with a series of appropriate controls (Figure 3B). ^{Me}P4-I was expressed only when all designated ^{Me}aa-tRNA^{Asn-E1}s were present in the *w*PURE system (lanes 2–5), suggesting that no competing background expression of undesignated peptides occurred. The expression level of ^{Me}P4-I was an approximately 20% relative to that of wild-type (wt4-I) expressed in the ordinary PURE system (lanes 1 versus 5). Despite the modest expression level of ^{Me}P4-I, MALDI-TOF analysis of the Flag-purified ^{Me}P4-I gave a single peak with the expected molecular mass, indicating that the assigned codons on mRNA were

correctly read by these ^{Me}aa-tRNA$^{Asn\text{-}E1}$s and the designated tetra-N-methyl-peptide was expressed.

Likewise, ^{Me}P4-II–IV peptides were expressed at levels approximately 15%–20% of the corresponding wild-type peptides (data not shown). Again, MALDI-TOF data of ^{Me}P4-II–IV peptides were consistent with the expected mass (Figure 3C). Thus, all mRNA ORFs tested in this study could be correctly translated to the tetra-N-methyl-peptides according to the assigned codons with high fidelities. This study represents, to our knowledge, the first proof-of-concept experiment for the ribosomal synthesis of N-methyl-peptides containing multiple kinds of ^{Me}aa by the genetic code reprogramming.

Expression of Longer N-Methyl-Peptides

The achieved high fidelity control in the ribosomal synthesis of the tetra-N-methyl-peptides encouraged us to attempt the expression of longer N-methyl-peptides. We designed four mRNA templates that express N-methyl-peptides containing five, six, eight, or ten consecutive N-methyl-peptide bonds (Figure 4A) (m5/^{Me}P5, m6/^{Me}P6, m8/^{Me}P8, m10/^{Me}P10, respectively). These peptides consisted of three ^{Me}aa in a repetition of the sequence ^{Me}Yme, ^{Me}Phe, and ^{Me}Ser for the corresponding length. To compare the expression level of these N-methyl-peptides, the respective wild-type peptides (wt5–10) were also expressed by using the ordinary PURE system.

All mRNA templates successfully expressed the peptides in the presence of the corresponding ^{Me}aa-tRNA$^{Asn\text{-}E1}$s in *w*PURE system (Figure 4B, gel figure). The absolute expression level for each N-methyl-peptide, determined by its radioisotope intensity originating from the incorporation of five [^{14}C]-Asp residues in the Flag peptide, gradually decreased with increasing peptide length (Figure 4B, upper graph). However, since wild-type also exhibited an inverse-correlation between expression level and peptide length, the relative expression levels of N-methyl-peptides to the corresponding wild-types remained in a similar range (10%–20%), suggesting that the relative expression level was independent of length (Figure 4B, lower graph). Despite their modest yields, the respective N-methyl-peptide had a single peak with the expected molecular mass in MALDI-TOF analysis, indicating that all peptides were expressed with high fidelities according to the corresponding mRNA templates assigned with the reprogrammed genetic code (Figure 4C).

In conclusion, our work has clearly demonstrated up to ten successive incorporations of ^{Me}aa into the peptide backbone via genetic code reprogramming with high fidelity control of both the peptide sequence and length. This technology offers a new means for the sequence-controlled synthesis of peptides containing multiple N-methyl-modifications on their backbone.

Synthesis of Cyclic N-Methylated Peptides Closed by a Thioether Bond

With the above method in our hands, we next applied it to the synthesis of cyclic peptides closed by a physiologically stable thioether bond. The foundation of this peptide ring-closing method has been established during the course of our studies on initiation reprogramming (Goto et al., 2008). Briefly, we prepared a *w*PURE system where Met was depleted so that the initiation codon became vacant. This vacant initiation codon was then filled with initiator tRNA$^{fMet}_{CUA}$ charged with an arbitrarily chosen proteinogenic or nonproteinogenic amino acid by the flexizyme system, so that the translation starts from this newly assigned initiator amino acid. When N^{α}-(α-chloroacetyl)-amino acid (N^{α}-ClAc-aa) and Cys were assigned to the initiation and elongation codons, respectively, the expressed full-length linear peptide spontaneously cyclized via an intramolecular nucleophilic attack of the sulfhydryl group of the Cys side chain to the N-terminal α-carbon of ClAc group, resulting in the formation of a thioether bond. This technology has enabled us to synthesize various cyclic peptides with any sequences and ring sizes. We here attempted to combine initiation reprogramming with elongation reprogramming using ^{Me}aa, to enable us to devise a flexible and reliable methodology for the mRNA-programmed synthesis of cyclic N-methyl-peptides.

We designed three mRNA templates that express peptides containing multiple N-methylated peptide bonds (Figure 5A, mc-I–III). Six ^{Me}aa (^{Me}Ala, ^{Me}Yme, ^{Me}Tyr, ^{Me}Gly, ^{Me}Ser, ^{Me}Phe) and six proteinogenic amino acids (Phe, Lys, Tyr, Pro, Asp, Cys) were chosen as the components of the cyclic N-methyl-peptides in this study (Figures 5A and 5B, ^{Me}cP-I–III). The respective peptides ^{Me}cP-I–III have two, three, and four N-methylated peptide bonds, respectively, implanted by the newly assigned ^{Me}aa in the codon table shown in Figure 1C. It should also be noted that ^{Me}cP-I and ^{Me}cP-II contain Pro as a proteinogenic component that also gives an additional secondary amide bond. These peptides were expressed in the presence of initiator tRNA$^{fMet}_{CAU}$ charged with N^{α}-ClAc-Phe and the corresponding ^{Me}aa-tRNAs in the above *w*PURE system. The MALDI-TOF analysis of the Flag-purified ^{Me}cP-I–III showed that all peptides were spontaneously cyclized via a thioether bond upon completion of translation, giving only the programmed cyclic N-methyl-peptides (Figure 5C). This study indicates that this technology is applicable to a wide array of cyclic peptides with multiple N-methyl-peptide bonds.

DISCUSSION

In the first stage of our proof-of-concept studies, we evaluated 19 ^{Me}aa derived from proteinogenic amino acids and 4 ^{Me}aa derived from nonproteinogenic amino acids, to determine the accessible ^{Me}aa. This study has revealed that the ribosome is accessible to ^{Me}aa with aromatic or nonbulky and noncharged side chains for the incorporation into the growing peptide. Most likely the N^{α}-methyl group added a steric bulkiness to the already bulky structure of certain amino acids, thereby reducing their incorporation levels. It is not yet clear what is the most important filter for the selection of accessible or inaccessible ^{Me}aa, but either EF-Tu or ribosome (or possibly both) is very likely the determinant(s) for their selection (Dale and Uhlenbeck, 2005). Therefore, it is of importance in future experiments to define the selection filter and engineer it to increase the tolerance toward inaccessible ^{Me}aa (Dedkova et al., 2003, 2006).

In this study, we depleted both aaRSs and cognate amino acids from the translation components in order to create vacant codon boxes for ^{Me}aa assignments. In previous studies, the genetic code reprogramming for nonproteinogenic amino acids bearing nonstandard side chains could be achieved by using a PURE system in which only proteinogenic amino acids, not aaRSs, were withdrawn (Murakami et al., 2006). We found,

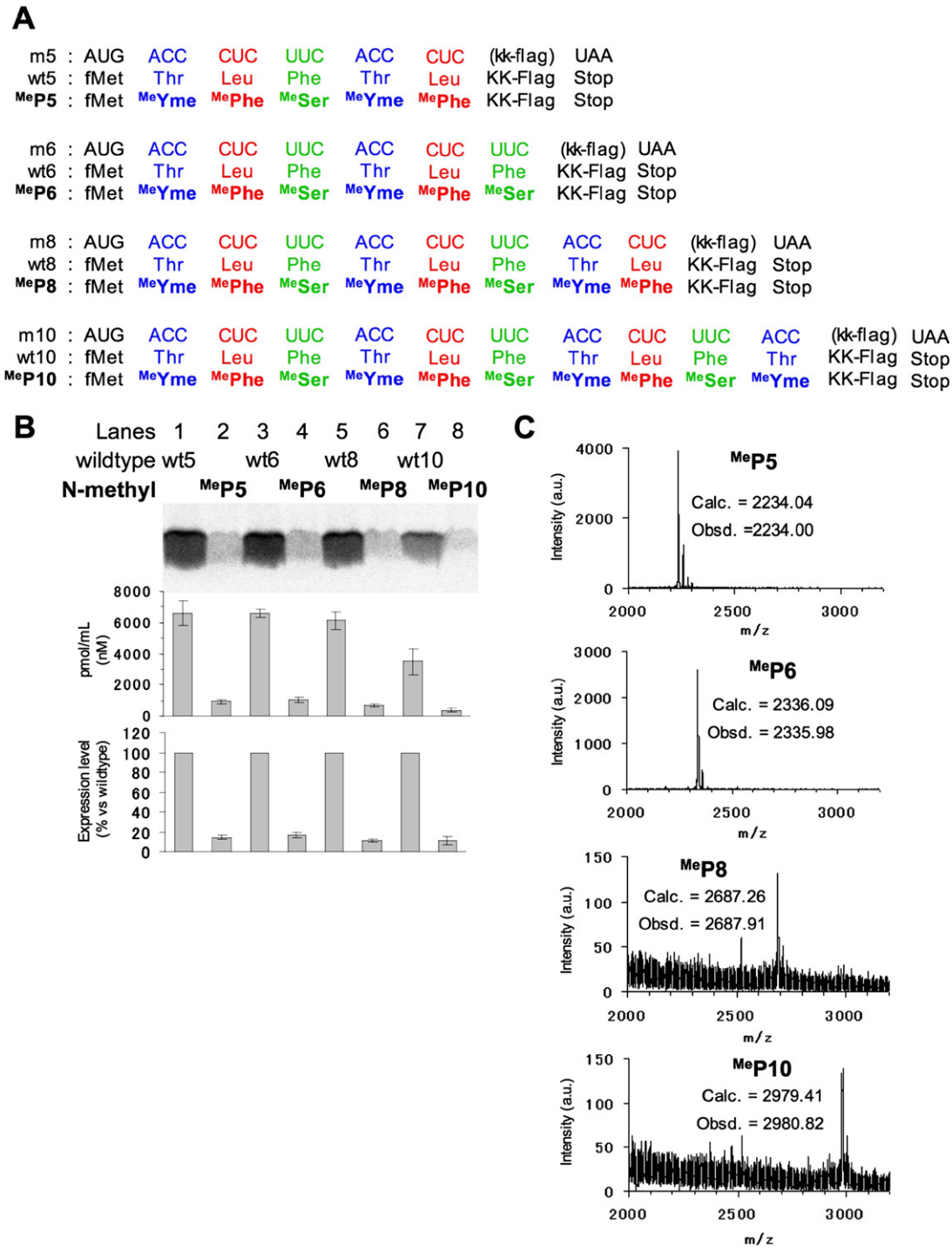

Figure 4. Expression of Longer N-Methyl-Peptides

(A) Sequences of mRNA templates (m5–10), control wild-type peptides (wt5–10), and peptides containing five, six, eight, or ten consecutive N-methyl-peptide bonds (^{Me}P5–10).

(B) Tricine-SDS-PAGE analysis of control wild-type peptides and N-methyl-peptides expressed from the respective mRNA. The peptides were labeled with [^{14}C]-Asp and detected by autoradiography. The wild-type peptides (lanes with odd numbers) were expressed in the ordinary PURE system, while N-methyl-peptides (lanes with even numbers) were expressed in the *w*PURE system containing ^{Me}Yme-tRNA$^{Asn\text{-}E1}_{GGU}$, ^{Me}Phe-tRNA$^{Asn\text{-}E1}_{GAG}$, and ^{Me}Ser-tRNA$^{Asn\text{-}E1}_{GAA}$. Absolute expression level of each peptide based on its observed radioisotope counts is shown in the upper graph, and relative expression level of each N-methyl-peptide against the corresponding wild-type peptide is shown in the lower graph. Each expression level was determined by a mean score of triplicates.

(C) MALDI-TOF-MS spectra of ^{Me}P5–10. The calculated mass (Calcd.) and observed mass (Obsd.) are shown in each spectrum.

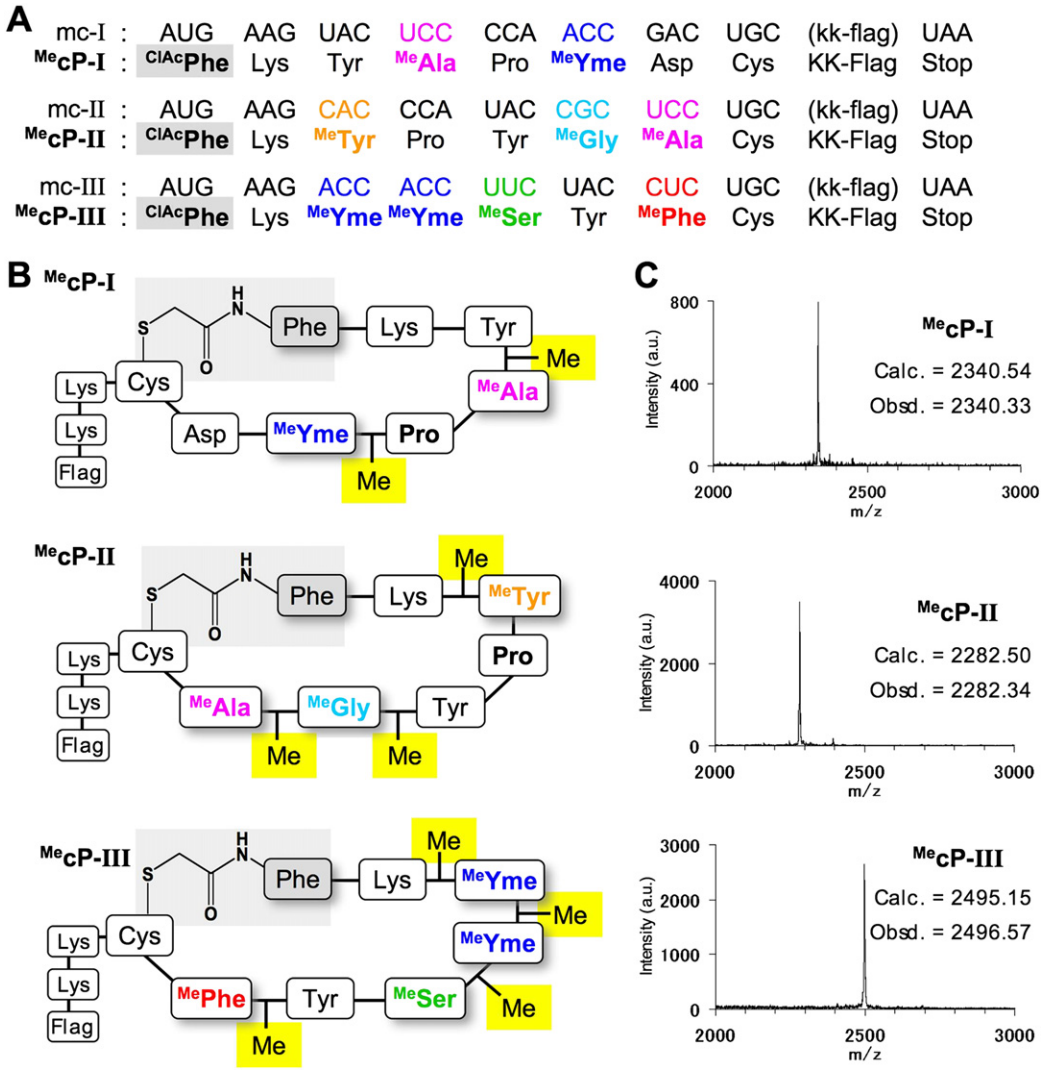

Figure 5. Synthesis of Cyclic N-Methyl-Peptides Closed by a Thioether Bond

(A) Sequences of mRNA templates (mc-I–III) and the precursor N-methyl-peptides (the linear version of ^{Me}cP-I–III). The codons and amino acids are colored according to reprogrammed genetic code table in Figure 1C.

(B) Structures of cyclic N-methyl-peptides. The locations of N-methyl groups on the peptide bond are shown in yellow.

(C) MALDI-TOF-MS spectra of cyclized ^{Me}cP-I–III. The calculated mass (Calcd.) and observed mass (Obsd.) are shown in each spectrum.

however, that in such a *w*PURE system, the incorporations of ^{Me}aa often suffered from contaminations caused by the competing incorporations of the proteinogenic amino acids to the assigned sites (data not shown). This was because that even though proteinogenic amino acids were supposedly depleted from the translation components, the recombinant translation factors and ribosome were often contaminated with small amounts of proteinogenic amino acids; therefore, they were charged onto the endogenous cognate tRNAs and readily competed out ^{Me}aa for the incorporation at the reprogrammed codon sites. Thus, it was critical to remove both aaRSs and proteinogenic amino acids from the translation components in order to repress the background incorporations of proteinogenic amino acids into the peptide. Moreover, we used orthogonal tRNAs as ^{Me}aa carriers aiming at eliminating potential mischarging events with proteinogenic amino acids catalyzed by aaRSs left in the *w*PURE system. Combining all of these controlled elements in the *w*PURE system, we were able to wipe out undesirable backgrounds and thus succeeded in synthesizing N-methyl-peptides as programmed by the mRNA.

Another key technology used in our proof-of-concept study is the flexizyme system. This system enables us to readily prepare a wide variety of ^{Me}aa-tRNAs with high purities, i.e., no contamination of the competing aa-tRNAs, so that we can ensure ^{Me}aa reassignments to the reprogrammed codons and thus determine the accessible ^{Me}aa in a quantitative manner. This technology has given us advantage over Merryman's method involving reductive alkylations on the precharged aa-tRNAs, where certain amounts of competing aa-tRNAs could remain unreacted and therefore contaminate the ^{Me}aa-tRNAs (Merryman and Green, 2004). As was discussed earlier, this might have led them to a different conclusion from ours on the accessible ^{Me}aa.

In a similar context, it would be difficult to perform ^{Me}aa assignments by the Szostak's method, where endogenous aaRSs or their mutants are used to mischarge nonproteinogenic amino acids onto endogenous tRNAs (Hartman et al., 2006; Josephson et al., 2005; Seebeck and Szostak, 2006). The major advantage of the Szostak's method is to in situ generate the mischarged aa-tRNAs and thus express the nonnatural peptides by simply adding nonproteinogenic amino acids to the *w*PURE system that lacks the corresponding proteinogenic amino acids. This system has been proven to be well suited for the incorporation of ''highly efficient'' nonproteinogenic amino acids into the target peptide, generally those structurally similar to proteinogenic amino acids that can readily compete out the background incorporations of amino acids originated by their contaminations (Seebeck and Szostak, 2006). Szostak et al. have also surveyed the aminoacylation ability of aaRSs toward ^{Me}aa, suggesting that ^{Me}Leu, ^{Me}Asp, ^{Me}Lys, ^{Me}His, and ^{Me}Trp could be charged onto the cognate tRNAs (Hartman et al., 2006). Unfortunately, among them only ^{Me}His and ^{Me}Trp were the ''accessible'' ^{Me}aa according to our experimental results shown in Figure 2C. As was discussed earlier, because of the intrinsic poor incorporation efficiency of ^{Me}aa, such background incorporations would give impure products containing certain or occasionally significant amounts of partially non-N-methylated peptides. In fact, by means of in situ aaRS's aminoacylation we found that ^{Me}His could be incorporated to the designated site of the same model peptide with competing incorporation of a smaller amount of His, whereas no incorporation of ^{Me}Trp, i.e., only incorporation of the competing Trp, was observed (T.K. and H.S., unpublished data).

Under the optimal conditions for the *w*PURE and flexizyme systems, we performed the mRNA-dependent synthesis of N-methyl-peptides containing more than four successive ^{Me}aa utilizing three different kinds of ^{Me}aa (Figures 3A and 4A). We monitored the expression level of each N-methyl-peptide by tricine-SDS-PAGE compared with the wild-type expression level based on their radioisotope intensities originating from the incorporation of [^{14}C]-Asp in the Flag peptide (Figures 3B and 4B). Using this strategy, we were able to directly compare the expression levels of the N-methyl-peptides in a quantitative manner.

Because we chose only accessible ^{Me}aa for the polymerization, we expected that the expression level would not be drastically reduced compared to the single incorporation. However, the expression of a tetra-N-methyl-peptide (^{Me}P4-I) gave only 20% of the wild-type expression level (Figure 3B, lanes 1 versus 5). Moreover, when longer N-methyl-peptides (from 5-mer to 10-mer) were expressed, their relative expression levels did not significantly change (Figure 4B). We wondered why the observed relative expression level of the tetra-N-methyl-peptides drastically dropped from that observed for the mono-N-methyl-peptide. To investigate this, we expressed mono-, di-, tri-, and tetra-N-methyl-peptides (Figure S3) (^{Me}P1-II, ^{Me}P2-II, ^{Me}P3-II, and ^{Me}P4-II) and compared their expression levels with those of the corresponding wild-type peptides (wt1-II, wt2-II, wt3-II, and wt4-II). Interestingly, the absolute expression levels of wt3-II and wt4-II were approximately three and four times higher, respectively, than that of wt1-II. On the other hand, the absolute expression levels of all N-methyl-peptides remained nearly the same. Thus, it became apparent that the relative expression levels of ^{Me}P3-II and ^{Me}P4-II to wt3-II and wt4-II appeared lower because the expression levels of wt3-II and wt4-II increased from wt1-II, whereas the absolute expression level of all N-methyl-peptides including 5- to 10-mers did not change significantly. Most importantly, MALDI-TOF analysis of the respective N-methyl-peptide gave the corresponding single peak with the correct molecular mass regardless of the length of the N-methyl-peptide. These findings led us to attempt the synthesis of cyclic-N-methyl-peptides.

In this application, we combined our technology for the ^{Me}aa incorporation into peptides with the method previously developed for the initiation reprogramming with N^{α}-ClAc-aa where a linear peptide containing Cys is expressed to afford the corresponding cyclic-peptide closed by a thioether bond between the N-terminus and the sulfhydryl group of the Cys. Because the thioether bond is nonreducible and thereby physiologically stable, it has been implanted into some therapeutic peptides in order to make them compatible for in vivo examination. Although such cyclic peptides were previously generated by only solid-phase chemistry, this method has enabled us to synthesize such cyclic peptides by means of the translation apparatus. Thus, the combination of the above two methods has offered us a platform technology for the synthesis of cyclic N-methyl-peptides by designing the corresponding mRNA templates. Remarkably, the expressed full-length cyclic N-methyl-peptides were highly pure in all three examples presented in this work. Thus, this technology is well suited for the preparation of libraries of cyclic N-methyl-peptides. Particularly, when this is coupled with in vitro display systems, such as ribosome (Hanes and Pluckthun, 1997) or mRNA displays (Nemoto et al., 1997; Roberts and Szostak, 1997), it becomes a very powerful means of screening large libraries and selecting in vivo-compatible cyclic N-methyl-peptides against therapeutic targets. Such experimental examples are shortly up coming from our laboratory.

SIGNIFICANCE

Reprogramming the genetic code allows for the simultaneous incorporation of multiple amino acid monomers with a wide variety of side chains into peptides through the ribosomal translation reaction. In this study, we achieved the mRNA-directed synthesis of various sequences and lengths of peptides containing multiple N-methylated amino acid residues upon reprogramming the genetic code by using the *w*PURE and flexizyme systems. Our demonstration shows the potential of this technology that leads us to a new strategy to construct libraries of linear and cyclic N-methyl-peptides simply by designing mRNA sequence libraries. Upon combining with appropriate screening strategies, this technology will yield a novel class of therapeutic peptides with protease resistance and cell permeability.

EXPERIMENTAL PROCEDURES

Materials and Synthesis of N^{α}-Methyl-Amino Acid Substrates

All N^{α}-methylated amino acids except N^{α}-methyl-L-cysteine and N^{α}-methyl-L-methionine were purchased from Watanabe Chemical, Japan. N^{α}-methyl-L-cysteine was synthesized by the procedure reported before from L-cysteine as a starting material (Park and Kim, 2002). N^{α}-tBoc-N^{α}-methyl-L-methionine was synthesized by the procedure reported before from N^{α}-tBoc-L-methionine as a starting material (Lee et al., 1990). N^{α}-methylated amino acids with the

aromatic side-chain (^{Me}Phe, ^{Me}Tyr, ^{Me}Trp, ^{Me}Fni, and ^{Me}Yme) were converted to cyanomethyl esters (CMEs), and the others were converted to 3,5-dinitrobenzyl esters (DBEs) by the procedure reported elsewhere (Murakami et al., 2006).

Synthesis of N^{α}-Methyl-Aminoacyl-tRNA

Aminoacylation reactions were generally performed as follows: 12.5 μl of 20 μM tRNA in 0.2 M HEPES-KOH (pH 7.5) was heated at 95°C for 1 min and cooled to room temperature over 5 min. Five microliters of 0.1 M $MgCl_2$ and 2.5 μl of 0.1 mM dFx or 5 μl of 3 M $MgCl_2$ and 2.5 μl of 0.1 mM eFx were added to the solution. Five microliters of 25 mM N^{α}-methylated amino acid substrate (DBE or CME) in DMSO was then added to the mixture and incubated on ice for 2–24 hr. For ^{Me}Thr, ^{Me}Tyr, ^{Me}Fni, and ^{Me}Yme, 5 μl of 200 mM substrate was used. The acylation reaction was quenched by addition of 150 μl of 0.6 M sodium acetate (pH 5.0), and the tRNA was recovered by ethanol precipitation. The pellet was rinsed twice with 70% ethanol with 0.1 M sodium acetate (pH 5.0) and once with 70% ethanol.

Ribosomal Synthesis of Linear N-Methylated Peptides and Wild-Type Peptides

The following conditions were used for the peptide synthesis without [^{14}C]-labeling. For the synthesis of N-methylated peptides (^{Me}P4-I, ^{Me}P4-II, ^{Me}P4-III, ^{Me}P4-IV, ^{Me}P5, ^{Me}P6, ^{Me}P8, ^{Me}P10, ^{Me}P1-II, ^{Me}P2-II, and ^{Me}P3-II), translation reaction was carried out by using the *w*PURE system (see the Supplemental Data) in the presence of 40 nM DNA template (one of the following templates: m4-I, m4-II, m4-III, m4-IV, m5, m6, m8, m10, m1-II, m2-II, or m3-II), 0.1 mM of the respective ^{Me}aa-tRNA(s), and 0.2 mM each of Met, Asp, Tyr, and Lys. For the synthesis of wild-type peptides (wt4-I, wt5, wt6, wt8, wt10, wt1-II, wt2-II, wt3-II, and wt4-II), translation reaction was carried out by using the ordinary PURE system in the presence of 40 nM DNA template (one of the following templates: m4-I, m5, m6, m8, m10, m1-II, m2-II, m3-II, or m4-II) and 0.2 mM each of Met, Thr, Leu, Phe, Asp, Tyr, and Lys. The translation reactions were assembled on ice and started by incubating at 37°C for 3 hr. For the detection of the peptides using autoradiography, see the section of tricine-SDS-PAGE analysis.

Tricine-SDS-PAGE Analysis

For tricine-SDS-PAGE analysis, peptides were synthesized by using 50 μM [^{14}C]-Asp in the place of 0.2 mM Asp. The translation reaction (2.5 μl) was stopped by the addition of 2.5 μl loading buffer consisting of 0.9 M Tris-HCl (pH 8.5), 30% glycerol, and 8% SDS for loading to 15% tricine-SDS-PAGE. Separation gel contained 15% acrylamide (acrylamide: bisacrylamide = 19:1), 1 M Tris•HCl (pH 8.5), 0.1% SDS, and 13% Glycerol, while the stacking gel contained 4% acrylamide (acrylamide: bisacrylamide = 29:1), 0.75 M Tris•HCl (pH 8.5), 0.075% SDS. The anode running buffer was 200 mM Tris•HCl (pH 8.9), while the cathode running buffer contains 100 mM Tris, 100 mM tricine, 0.1% SDS (the resulting buffer was approximately pH 8.3). The products, labeled with [^{14}C]-Asp in the C terminus FLAG peptide, were quantified by autoradiography with an image analyzer, FLA-5100 (Fuji, Japan), and the specific activity of [^{14}C]-Asp.

Ribosomal Synthesis of Cyclic N-Methylated Peptides

For the synthesis of cyclic N-methylated peptide (^{Me}Pc1, ^{Me}Pc2, or ^{Me}Pc3), translation reaction was carried out by using the *w*PURE system with 40 nM DNA template (mc1, mc2, or mc3) in the presence of 0.1 mM N^{α}-ClAc-Phe-tRNA$^{fMet}_{CAU}$, 0.1 mM of the respective ^{Me}aa-tRNAs, and 0.2 mM each of Met, Lys, Tyr, Pro, Asp, and Cys. The translation reactions were assembled on ice and started by incubation at 37°C for 3 hr.

MALDI-TOF-MS Analysis

The respective peptide was incubated in prewashed Flag-M2 agarose (Sigma) for an hour. After the resin was washed with TBS (50 mM Tris-HCl [pH 8.0], 150 mM NaCl), the peptide was eluted with 0.2% TFA. Eluted peptide was desalted with C18 zip tips (Millipore) and eluted with 50% acetonitrile, 0.1% TFA solution saturated with the matrix (*R*)-cyano-4-hydroxycinnamic acid. MALDI-TOF-MS analysis was performed by using Autoflex TOF/TOF (Bruker Daltonics) operated in the linear positive mode and externally calibrated with Substance P (1348.66 Da), Bombesin (1620.88 Da), ACTH clip 1–17 (2094.46 Da), and Somatostatin 28 (3149.61 Da) standards.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures for RNA synthesis, tRNA aminoacylation, and preparation of the translation systems and are available at http://www.chembiol.com/cgi/content/full/15/1/32/DC1/.

ACKNOWLEDGMENTS

We thank Y. Goto for providing N^{α}-ClAc-Phe-CME. We thank T.J. Kang, A. Ohta, and P.C. Reid for proofreading of this manuscript. 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. and a research and development projects of the Industrial Science and Technology Program in the New Energy and Industrial Technology Development Organization (NEDO).

Received: September 19, 2006
Revised: November 25, 2007
Accepted: December 6, 2007
Published: January 25, 2008

REFERENCES

Bain, J.D., Diala, E.S., Glabe, C.G., Dix, T.A., and Chamberlin, A.R. (1989). Biosynthetic site-specific incorporation of a non-natural amino acid into a polypeptide. J. Am. Chem. Soc. *111*, 8013–8014.

Bain, J.D., Wacker, D.A., Kuo, E.E., and Chamberlin, A.R. (1991). Site-specific incorporation of non-natural residues into peptides: effect of residue structure on suppression and translation efficiencies. Tetrahedron *47*, 2389–2400.

Baltz, R.H. (2006). Molecular engineering approaches to peptide, polyketide and other antibiotics. Nat. Biotechnol. *24*, 1533–1540.

Billich, A., and Zocher, R. (1990). Formation of N-methylated peptide bonds in peptides and peptidols. In Biochemistry of Peptide Antibiotics, H. Kleinkauf and H. Von Doehren, eds. (Berlin: Walter de Gruyter & Co.), pp. 57–79.

Chung, H.H., Benson, D.R., and Schultz, P.G. (1993). Probing the structure and mechanism of Ras protein with an expanded genetic code. Science *259*, 806–809.

Dale, T., and Uhlenbeck, O.C. (2005). Amino acid specificity in translation. Trends Biochem. Sci. *30*, 659–665.

Dedkova, L.M., Fahmi, N.E., Golovine, S.Y., and Hecht, S.M. (2003). Enhanced D-amino acid incorporation into protein by modified ribosomes. J. Am. Chem. Soc. *125*, 6616–6617.

Dedkova, L.M., Fahmi, N.E., Golovine, S.Y., and Hecht, S.M. (2006). Construction of modified ribosomes for incorporation of d-amino acids into proteins. Biochemistry *45*, 15541–15551.

Ellman, J.A., Mendel, D., and Schultz, P.G. (1992). Site-specific incorporation of novel backbone structures into proteins. Science *255*, 197–200.

Fischbach, M.A., and Walsh, C.T. (2006). Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. Chem. Rev. *106*, 3468–3496.

Forster, A.C., Tan, Z., Nalam, M.N., Lin, H., Qu, H., Cornish, V.W., and Blacklow, S.C. (2003). Programming peptidomimetic syntheses by translating genetic codes designed de novo. Proc. Natl. Acad. Sci. USA *100*, 6353–6357.

Frankel, A., Millward, S.W., and Roberts, R.W. (2003). Encodamers: unnatural peptide oligomers encoded in RNA. Chem. Biol. *10*, 1043–1050.

Gilmore, M.A., Steward, L.E., and Chamberlin, A.R. (1999). Incorporation of noncoded amino acids by in vitro protein biosynthesis. Top. Curr. Chem. *202*, 77–99.

Goto, Y., Ohta, A., Sako, Y., Yamagishi, Y., Murakami, H., and Suga, H. (2008). Reprogramming the translation initiation for the synthesis of physiologically stable cyclic peptides. ACS Chem. Biol., in press.

Hahn, M., and Stachelhaus, T. (2006). Harnessing the potential of communication-mediating domains for the biocombinatorial synthesis of nonribosomal peptides. Proc. Natl. Acad. Sci. USA *103*, 275–280.

Hanes, J., and Pluckthun, A. (1997). In vitro selection and evolution of functional proteins by using ribosome display. Proc. Natl. Acad. Sci. USA *94*, 4937–4942.

Hartman, M.C., Josephson, K., and Szostak, J.W. (2006). Enzymatic aminoacylation of tRNA with unnatural amino acids. Proc. Natl. Acad. Sci. USA *103*, 4356–4361.

Hecht, S.M., Alford, B.L., Kuroda, Y., and Kitano, S. (1978). "Chemical aminoacylation" of tRNA's. J. Biol. Chem. *253*, 4517–4520.

Hendrickson, T.L., de Crecy-Lagard, V., and Schimmel, P. (2004). Incorporation of nonnatural amino acids into proteins. Annu. Rev. Biochem. *73*, 147–176.

Ho, T.-I., and Chow, Y.L. (1996). Photochemistry of nitro and nitroso compounds. In The Chemistry of Amino, Nitroso, Nitro and Related Groups. Supplement F2, S. Patai, ed. (New York: Wiley), pp. 757–821.

Hohsaka, T., and Sisido, M. (2002). Incorporation of non-natural amino acids into proteins. Curr. Opin. Chem. Biol. *6*, 809–815.

Hornbogen, T., and Zocher, R. (2005). Biosynthesis of N-methylated peptides in fungi. In Handbook of Industrial Mycology, Z. An, ed. (New York: Marcel Dekker), pp. 449–477.

Josephson, K., Hartman, M.C., and Szostak, J.W. (2005). Ribosomal synthesis of unnatural peptides. J. Am. Chem. Soc. *127*, 11727–11735.

Karginov, V.A., Mamaev, S.V., An, H., Van Cleve, M.D., Hecht, S.M., Komatsoulis, G.A., and Abelson, J.N. (1997). Probing the role of an active site aspartic acid in dihydrofolate reductase. J. Am. Chem. Soc. *119*, 8166–8176.

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

LaRiviere, F.J., Wolfson, A.D., and Uhlenbeck, O.C. (2001). Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. Science *294*, 165–168.

Lee, N., and Suga, H. (2001). A minihelix-loop RNA acts as a trans-aminoacylation catalyst. RNA *7*, 1043–1051.

Lee, J.P., Dunlap, B., and Rich, D.H. (1990). Synthesis and immunosuppressive activities of conformationally restricted cyclosporin lactam analogues. Int. J. Pept. Protein Res. *35*, 481–494.

Link, A.J., Mock, M.L., and Tirrell, D.A. (2003). Non-canonical amino acids in protein engineering. Curr. Opin. Biotechnol. *14*, 603–609.

Mendel, D., Cornish, V.W., and Schultz, P.G. (1995). Site-directed mutagenesis with an expanded genetic code. Annu. Rev. Biophys. Biomol. Struct. *24*, 435–462.

Merryman, C., and Green, R. (2004). Transformation of aminoacyl tRNAs for the in vitro selection of "drug-like" molecules. Chem. Biol. *11*, 575–582.

Murakami, H., Kourouklis, D., and Suga, H. (2003a). Using a solid-phase ribozyme aminoacylation system to reprogram the genetic code. Chem. Biol. *10*, 1077–1084.

Murakami, H., Saito, H., and Suga, H. (2003b). A versatile tRNA aminoacylation catalyst based on RNA. Chem. Biol. *10*, 655–662.

Murakami, H., Ohta, A., Ashigai, H., and Suga, H. (2006). A highly flexible tRNA acylation method for non-natural polypeptide synthesis. Nat. Methods *3*, 357–359.

Nemoto, N., Miyamoto-Sato, E., Husimi, Y., and Yanagawa, H. (1997). In vitro virus: bonding of mRNA bearing puromycin at the 3′-terminal end to the C-terminal end of its encoded protein on the ribosome in vitro. FEBS Lett. *414*, 405–408.

Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C., and Schultz, P.G. (1989). A general method for site-specific incorporation of unnatural amino acids into proteins. Science *244*, 182–188.

Ohta, A., Murakami, H., Eri, H., and Suga, H. (2007). Synthesis of polyester using genetic code reprogramming. Chem. Biol. *14*, 1315–1322.

Ohuchi, M., Murakami, H., and Suga, H. (2007). The flexizyme system: a highly flexible tRNA aminoacylation tool for the translation apparatus. Curr. Opin. Chem. Biol. *11*, 537–542.

Park, J.D., and Kim, D.H. (2002). Cysteine derivatives as inhibitors for carboxypeptidase A: synthesis and structure-activity relationships. J. Med. Chem. *45*, 911–918.

Roberts, R.W., and Szostak, J.W. (1997). RNA-peptide fusions for the in vitro selection of peptides and proteins. Proc. Natl. Acad. Sci. USA *94*, 12297–12302.

Robertson, S.A., Ellman, J.A., and Schultz, P.G. (1991). A general and efficient route for chemical aminoacylation of transfer RNAs. J. Am. Chem. Soc. *113*, 2722–2729.

Sagan, S., Karoyan, P., Lequin, O., Chassaing, G., and Lavielle, S. (2004). N- and Calpha-methylation in biologically active peptides: synthesis, structural and functional aspects. Curr. Med. Chem. *11*, 2799–2822.

Saito, H., Kourouklis, D., and Suga, H. (2001). An in vitro evolved precursor tRNA with aminoacylation activity. EMBO J. *20*, 1797–1806.

Seebeck, F.P., and Szostak, J.W. (2006). Ribosomal synthesis of dehydroalanine-containing peptides. J. Am. Chem. Soc. *128*, 7150–7151.

Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., and Ueda, T. (2001). Cell-free translation reconstituted with purified components. Nat. Biotechnol. *19*, 751–755.

Short, G.F., 3rd, Laikhter, A.L., Lodder, M., Shayo, Y., Arslan, T., and Hecht, S.M. (2000). Probing the S1/S1′ substrate binding pocket geometry of HIV-1 protease with modified aspartic acid analogues. Biochemistry *39*, 8768–8781.

Sieber, S.A., and Marahiel, M.A. (2005). Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. Chem. Rev. *105*, 715–738.

Tan, Z., Forster, A.C., Blacklow, S.C., and Cornish, V.W. (2004). Amino acid backbone specificity of the *Escherichia coli* translation machinery. J. Am. Chem. Soc. *126*, 12752–12753.

Tan, Z., Blacklow, S.C., Cornish, V.W., and Forster, A.C. (2005). De novo genetic codes and pure translation display. Methods *36*, 279–290.

Walsh, C.T., Chen, H., Keating, T.A., Hubbard, B.K., Losey, H.C., Luo, L., Marshall, C.G., Miller, D.A., and Patel, H.M. (2001). Tailoring enzymes that modify nonribosomal peptides during and after chain elongation on NRPS assembly lines. Curr. Opin. Chem. Biol. *5*, 525–534.

Wang, L., and Schultz, P.G. (2004). Expanding the genetic code. Angew. Chem. Int. Ed. Engl. *44*, 34–66.

