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# In Vitro Genetic Code Reprogramming for the Expansion of Usable Noncanonical Amino Acids

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## Keywords

genetic code reprogramming, aminoacylation, D-amino acid, β-amino acid, EF-P, foldamer

## Abstract

Genetic code reprogramming has enabled us to ribosomally incorporate various nonproteinogenic amino acids (npAAs) into peptides in vitro. The repertoire of usable npAAs has been expanded to include not only L-α-amino acids with noncanonical sidechains but also those with noncanonical backbones. Despite successful single incorporation of npAAs, multiple and consecutive incorporations often suffer from low efficiency or are even unsuccessful. To overcome this stumbling block, engineering approaches have been used to modify ribosomes, EF-Tu, and tRNAs. Here, we provide an overview of these in vitro methods that are aimed at optimal expansion of the npAA repertoire and their applications for the development of de novo bioactive peptides containing various npAAs.

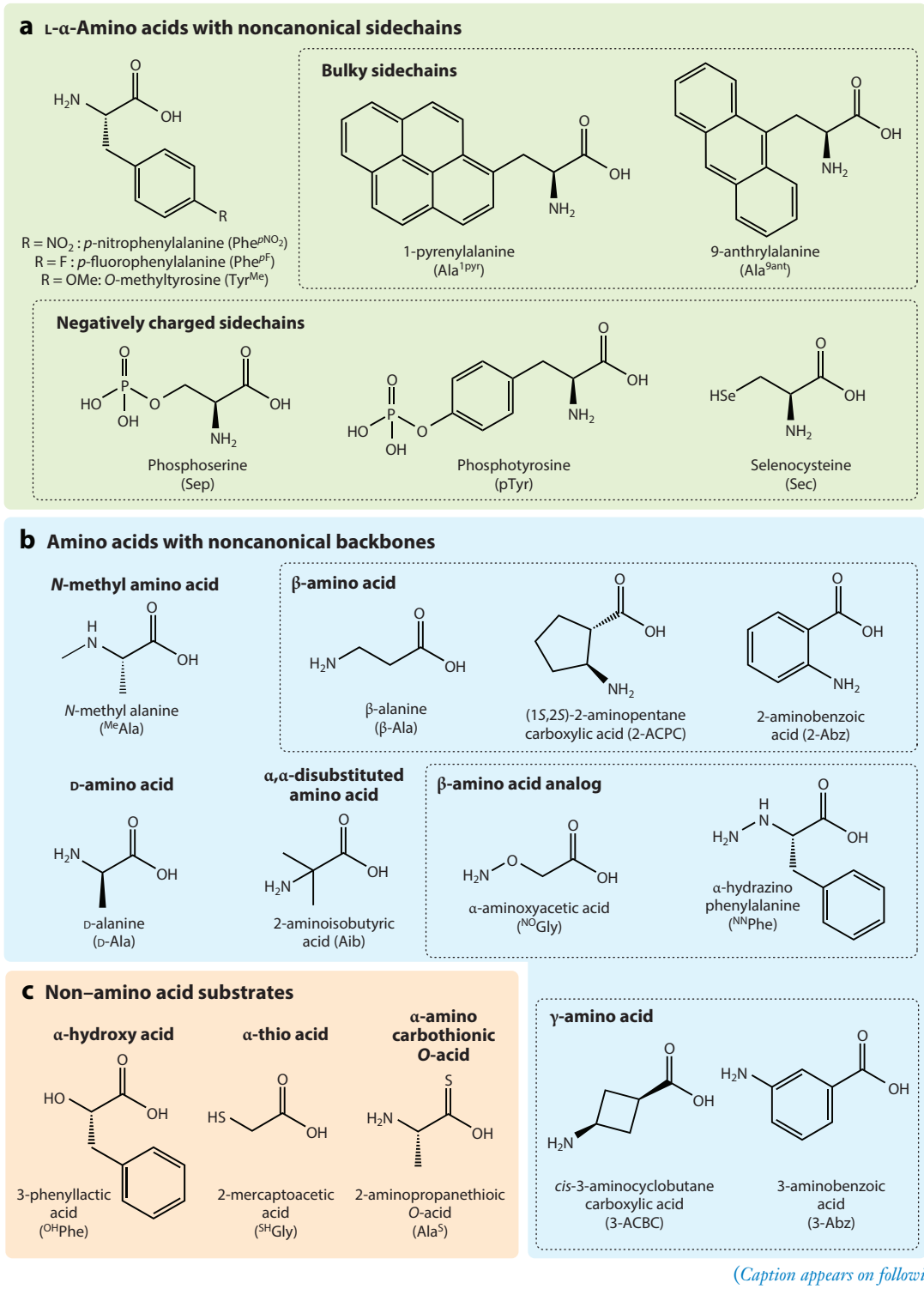
## Contents



## 1. INTRODUCTION

In nature, ribosomes use the 20 canonical proteinogenic L-α-amino acids (pAAs) as building blocks for peptide (protein) biosynthesis. However, beyond the 20 pAAs, various nonproteinogenic amino acids (npAAs) have also been found in diverse bioactive peptides and proteins (**Figure 1**). Such npAAs are generally introduced by posttranslational modification (PTM) or nonribosomal peptide synthesis (NRPS) within cells (1–4), i.e., they are not directly introduced by ribosomes. Since such npAAs play structurally and functionally important roles in these peptides, novel methods for preparing peptides and proteins containing npAAs should greatly benefit the study of their functions and structures. However, their synthesis via both PTM and NRPS generally occurs in a sequence-specific manner and therefore cannot be easily adapted for the synthesis of diverse peptide sequences.

The translation system is much more flexible in its ability to synthesize diverse peptide sequences, since sequence changes can be readily achieved by simply altering the mRNA template. In translation, a triplet of nucleotides constitutes a codon and encodes one amino acid. There are four mRNA nucleotides: A, U, G, and C. Out of the 64 possible triplet combinations ($4^3$), 61 are used as sense codons that encode the 20 pAAs in the canonical genetic code, whereas the remaining 3 codons, UAG (amber), UAA (ochre), and UGA (opal), are used as stop codons that terminate translation (**Figure 2*a***). However, an npAA of interest can be introduced at a stop codon (generally the UAG codon), and this is often referred to as genetic code expansion or nonsense codon suppression. Since aminoacyl-tRNA is the key molecule for decoding the genetic code,




*(Caption appears on following page)*

**Figure 1** (*Figure appears on preceding page*)

Examples of nonproteinogenic amino acids that are ribosomally incorporated into peptides and proteins by genetic code manipulation. (*a*) L-α-Amino acids with noncanonical sidechains. (*b*) Amino acids with noncanonical backbones. (*c*) Non–amino acid substrates.

a specific mischarged nonproteinogenic aminoacyl-tRNA (npAA-tRNA) that can recognize the stop codon can be prepared to insert the desired npAA. Despite the fact that this pioneering approach worked well for npAAs with noncanonical sidechains, it often suffered from an inability to expand to multiple npAAs or impaired incorporation efficiency for npAAs with noncanonical backbone structures, such as D-α-amino acids, *N*-methylamino acids, and β- and γ-amino acids. Two major issues must be considered to solve these shortcomings. First, even though two other stop codons, UAA and UGA, are available, npAA-tRNA suppression using these codons turns out to be much poorer than with UAG. Second, such suppressor npAA-tRNAs compete with release factors (RFs) (see Section 2.2). To avoid these problems, a method to exclusively introduce npAAs at particular codons of choice has been developed, referred to as genetic code reprogramming. This strategy has expanded the usable building blocks to include npAAs ranging from L-α-amino acids with noncanonical sidechains to amino acids with noncanonical backbones (**Figure 1*a*,*b***). This has also made it possible for atypical substrates, e.g., α-hydroxy acids and α-thio acids, to be incorporated, allowing the expression of peptides containing atypical peptide bonds (**Figure 1*c***).

In this review, we provide an overview of in vitro approaches involving genetic code reprogramming, nonsense codon suppression, quadruplet codon suppression, and genetic alphabet expansion. We next discuss the most advanced in vitro method of genetic code reprogramming, which involves the engineered ribosomal translation system and enhances npAA incorporation efficiency, and its applications for the development of de novo bioactive peptides containing various npAAs.

## 2. OVERVIEW OF GENETIC CODE MANIPULATION METHODS

### 2.1. Genetic Code Reprogramming

Genetic code reprogramming is a method that enables npAA incorporation at any of the 61 sense codons in place of pAAs (**Figure 2*b***); it requires that the corresponding pAA-tRNAs are depleted from the translation system, so that no competition between npAA and pAA occurs. The first work demonstrating this concept was reported in 1962 (5); in this study, Cys codons (UGU and UGC) were reprogrammed to Ala by means of chemical desulfurization of Cys-tRNA$^{Cys}$ into Ala-tRNA$^{Cys}$ using Raney nickel. Consequently, Cys-tRNA$^{Cys}$ was depleted to generate Ala-tRNA$^{Cys}$ in a cell-free translation system. Since no additional npAA was introduced in this case, the number of available amino acids in this reprogrammed genetic code simply decreased to 19. Nearly a decade later, another demonstration of genetic code reprogramming was reported in 1971, in which Phe codons (UUU and UUC) were reprogrammed to 3-phenyllactic acid ($^{OH}$Phe) by deamination of Phe-tRNA$^{Phe}$ into $^{OH}$Phe-tRNA$^{Phe}$ (**Figure 1*c***) (6). Since $^{OH}$Phe is not an amino acid but an α-hydroxy acid, ester bonds were formed instead of peptide bonds, leading to polyester formation. Unfortunately, these early works suffered from severe misincorporation of Cys and Phe due to their incomplete chemical conversion into Ala and $^{OH}$Phe, respectively, and the use of crude *Escherichia coli* translation systems. These results tell us that complete depletion of the competing pAA-tRNA is essential for clean expression of the desired products bearing npAAs.

To overcome this issue, Forster et al. (7) constructed an *E. coli* cell-free translation system made of individually purified components: ribosomes, translation factors, amino acids, tRNAs, aminoacyl-tRNA synthetases (ARSs), and other essential factors. In such a reconstituted translation system, unnecessary pAAs and their corresponding ARSs can be arbitrarily omitted to prepare vacant codons; npAAs can then be assigned to the vacant codons by adding npAA-tRNAs

**a** The canonical genetic code

| 1st | 2nd U | 2nd C | 2nd A | 2nd G | 3rd |
|---|---|---|---|---|---|
| U | Phe | Ser | Tyr | Cys | U |
| | | | | | C |
| | Leu | | Ochre | Opal | A |
| | | | Amber | Trp | G |
| C | Leu | Pro | His | Arg | U |
| | | | | | C |
| | | | Gln | | A |
| | | | | | G |
| A | Ile | Thr | Asn | Ser | U |
| | | | | | C |
| | | | Lys | Arg | A |
| | fMet/Met | | | | G |
| G | Val | Ala | Asp | Gly | U |
| | | | | | C |
| | | | Glu | | A |
| | | | | | G |

**b** Genetic code reprogramming

| 1st | 2nd U | 2nd C | 2nd A | 2nd G | 3rd |
|---|---|---|---|---|---|
| U | npAA | Ser | Tyr | Cys | U |
| | | | | | C |
| | Leu | | Ochre | Opal | A |
| | | | Amber | Trp | G |
| C | Leu | Pro | His | Arg | U |
| | | | | | C |
| | | | Gln | | A |
| | | | | | G |
| A | Ile | Thr | Asn | Ser | U |
| | | | | | C |
| | | | Lys | Arg | A |
| | fMet/Met | | | | G |
| G | Val | Ala | Asp | Gly | U |
| | | | | | C |
| | | | Glu | | A |
| | | | | | G |

**c** Nonsense codon suppression

| 1st | 2nd U | 2nd C | 2nd A | 2nd G | 3rd |
|---|---|---|---|---|---|
| U | Phe | Ser | Tyr | Cys | U |
| | | | | | C |
| | Leu | | Ochre | Opal | A |
| | | | npAA | Trp | G |
| C | Leu | Pro | His | Arg | U |
| | | | | | C |
| | | | Gln | | A |
| | | | | | G |
| A | Ile | Thr | Asn | Ser | U |
| | | | | | C |
| | | | Lys | Arg | A |
| | fMet/Met | | | | G |
| G | Val | Ala | Asp | Gly | U |
| | | | | | C |
| | | | Glu | | A |
| | | | | | G |

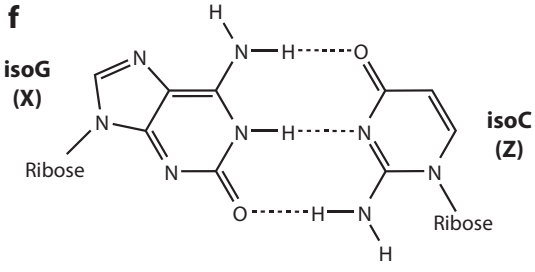
**d** Quadruplet codon suppression

| 1st | 2nd U | 2nd C | 2nd A | 2nd G | 3rd |
|---|---|---|---|---|---|
| U | Phe | Ser | Tyr | Cys | U |
| | | | | | C |
| | Leu | | Ochre | | A |
| | | | Amber | Trp | G |
| C | Leu | Pro | His | Arg | U |
| | | | | | C |
| | | | Gln | | A |
| | | | | | G |
| A | Ile | Thr | Asn | Ser | U |
| | | | | | C |
| | | | Lys | Arg | A |
| | fMet/Met | | | | G |
| | | | | npAA | GN |
| G | Val | Ala | Asp | Gly | U |
| | | | | | C |
| | | | Glu | | A |
| | | | | | G |

**e** Genetic alphabet expansion

| 1st | 2nd U | 2nd C | 2nd A | 2nd G | 2nd X | 2nd Z | 3rd |
|---|---|---|---|---|---|---|---|
| U | Phe | Ser | Tyr | Cys | | | U |
| | | | | | | | C |
| | Leu | | Ochre | Opal | | | A |
| | | | Amber | Trp | | | G |
| | | | | | | | X |
| | | | | | | | Z |
| C | Leu | Pro | His | Arg | | | U |
| | | | | | | | C |
| | | | Gln | | | | A |
| | | | | | | | G |
| | | | | | | | X |
| | | | | | | | Z |
| A | Ile | Thr | Asn | Ser | | | U |
| | | | | | | | C |
| | | | Lys | Arg | | | A |
| | fMet/Met | | | | | | G |
| | | | | | | | X |
| | | | | | | | Z |
| G | Val | Ala | Asp | Gly | | | U |
| | | | | | | | C |
| | | | Glu | | | | A |
| | | | | | | | G |
| | | | | | | | X |
| | | | | | | | Z |
| X | Available for npAA | | | | | | U |
| | | | | | | | C |
| | | | | | | | A |
| | | | | | | | G |
| | | | | | | | X |
| | | | | | | | Z |
| Z | | | | | | | U |
| | | | | | | | C |
| | | | | | | | A |
| | | | | | | | G |
| | | | | | | | X |
| | | | | | | | Z |

**f**




(Caption appears on following page)

**Figure 2** (*Figure appears on preceding page*)

The canonical and reprogrammed genetic codes. (*a*) The canonical genetic code. Artificial genetic codes reprogrammed by (*b*) genetic code reprogramming, (*c*) nonsense codon suppression, (*d*) quadruplet codon suppression, and (*e*) genetic alphabet expansion. (*f*) An example of artificial base pairs, isoG and isoC, used for genetic alphabet expansion. Abbreviations: npAA, nonproteinogenic amino acid; GN, a set of two nucleotides, G and N.

chemoenzymatically prepared by a classical method developed for nonsense codon suppression (see Section 3.2). Consequently, the authors reprogrammed AAC (Asn), ACC (Thr), and GUU (Val) codons to 2-amino-4-pentynoic acid, 2-amino-4-pentenoic acid, and *O*-methylserine, respectively. This work demonstrated the expression of short peptides, up to seven residues. For polyester synthesis, Ohta et al. (8) used a reconstituted *E. coli* translation system, referred to as the FIT (flexible in vitro translation) system, containing four amino acids (Met, Lys, Asp, and Tyr) and their cognate ARSs. Seven kinds of α-hydroxy acids were charged on tRNA by means of flexizyme (see Section 3.3) and assigned to the UUC (Phe), CUC (Leu), GUU (Val), ACC (Thr), CAG (Gln), AAC (Asn), and AGU (Ser) codons. Since serious competition between npAA-tRNA and pAA-tRNA was avoided in this system, clean expression of the desired polyester–polypeptide hybrid products was accomplished without significant misincorporation. Since the incorporation of α-hydroxy acids was efficient enough, 10 μL or less of the translation reaction was sufficient for detection of the polyester–polypeptide hybrid products by mass spectrometry and autoradiography. Although the establishment of such reconstituted translation systems requires the overexpression and purification of individual translation components, several commercial in vitro translation kits are available nowadays and used for npAA incorporation. However, the incorporation of inefficient substrates, such as β-, γ-, and D-α-amino acids, requires more intensive manipulation and optimization of translation components (see Section 4), which is not easily accomplished with commercial kits.

## 2.2. Nonsense Codon Suppression

Nonsense codon suppression is a method that utilizes stop codons to introduce npAAs (**Figure 2*c***). Since the amber codon (UAG) is most frequently used for suppression, this method is also referred to as amber suppression or amber codon suppression. By introducing npAA-$tRNA_{CUA}$ into a translation system, the npAA can be incorporated at the UAG codon. In 1989, Schultz and colleagues (9) developed a $tRNA^{Phe}$-based amber suppressor tRNA whose anticodon was substituted with CUA for the incorporation of Phe analogs, such as *p*-nitrophenylalanine ($Phe^{pNO2}$), *p*-fluorophenylalanine ($Phe^{pF}$), and homophenylalanine (hPhe), at the active center of β-lactamase by in vitro translation (**Figure 1*a***). The drawback of this method is the competition between RFs and the suppressor npAA-tRNA, which leads to premature translation termination and yields a truncated peptide/protein. Since RF1 recognizes UAG and UAA, whereas RF2 recognizes UGA and UAA in bacterial translation systems, competition between RF1 and npAA-$tRNA_{CUA}$ may take place during amber suppression (**Figure 3**, step 4). In a reconstituted in vitro translation system, this issue can be circumvented by removing the competing RF from the system. For instance, RF1 could be removed for amber codon suppression, whereas RF2 should be retained for translation termination at UGA and UAA codons.

## 2.3. Quadruplet Codon Suppression

Quadruplet codon suppression utilizes quadruplets of nucleotides as codons, with the quadruplets containing an additional fourth nucleotide following the canonical triplet codon (**Figure 2*d***). Some canonical triplet codons cannot be efficiently decoded due to the low abundance of cognate

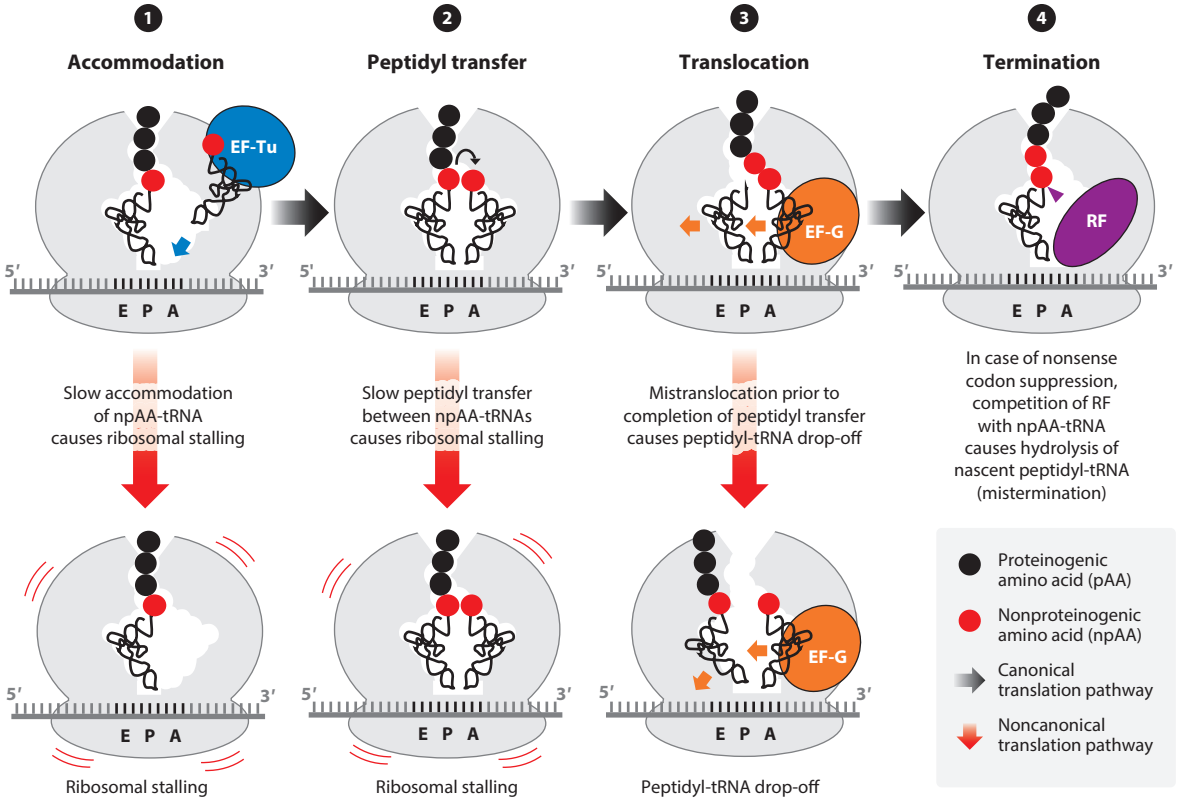



**Figure 3**

Schematic overview of npAA incorporation by ribosomal translation. In the canonical translation pathway (*black arrows*), elongation of the nascent peptide occurs by repeated ① accommodation of aminoacyl-tRNA onto the ribosomal A site, ② peptidyl transfer between the P-site peptidyl-tRNA and A-site aminoacyl-tRNA, and ③ translocation of the P-site deacylated tRNA and A-site peptidyl-tRNA to the E site and P site, respectively. ④ Translation is eventually terminated at a stop codon by an RF. In the case of npAA incorporation, slow accommodation and peptidyl transfer, mistranslation, and mistermination cause ribosomal stalling, peptidyl-tRNA drop-off, and hydrolysis of the nascent peptidyl-tRNA, leading to truncation of the peptides/proteins. Abbreviations: npAA, nonproteinogenic amino acid; pAA, proteinogenic amino acid; RF, release factor.

tRNAs in cells. They are not frequently used for decoding their corresponding amino acids, and therefore are generally called rare codons. At such rare codons, the competition between an npAA-tRNA bearing a quadruplet anticodon and a pAA-tRNA with a triplet anticodon is nearly negligible because of the low abundance of the pAA-tRNA. For example, the expression level of $tRNA^{Arg}_{CCU}$ that designates arginine with the AGG codon is low in *E. coli* cells, and therefore, an AGGN quadruplet can be utilized for suppression of npAAs with minimal competition from Arg incorporation. Moreover, the quadruplet npAA-tRNA acts as a programmed frameshifter, i.e., one nucleotide upstream of the triplet codon on mRNA is read to shift the frame of reading from the normal triplet pAA-tRNA. Hohsaka and colleagues (10, 11) utilized an artificially designed tRNA bearing an ACCU anticodon for the incorporation of $Phe^{pNO2}$, 2-naphthylalanine ($Ala^{2nap}$), 2-anthrylalanine ($Ala^{2ant}$), and *p*-phenylazophenylalanine ($Phe^{pa}$) into streptavidin at AGGN codons. Suppression of not only quadruplets but also quintuplets, consisting of five nucleotides, has been demonstrated by the same group using tRNAs with quintuplet anticodons (12).

## 2.4. Genetic Alphabet Expansion

In order to expand the genetic code, various artificial base pairs have been created in addition to the canonical base pairs, A–U and G–C. If a pair of artificial nucleotides, X–Z, are added to the genetic code, the number of available codons should theoretically increase from 64 ($4^3$) to 216 ($6^3$), providing 152 additional codons that can be used for npAA incorporation (**Figure 2*e***). For instance, in 1992, Benner and coworkers (13) devised the isoG–isoC pair to expand the codon table such that the (isoC)AG codon was used for incorporation of 3-iodotyrosine ($Tyr^{I}$) by means of an artificial tRNA bearing a CU(isoG) anticodon (**Figure 2*f***). They expressed a 16-mer peptide containing $Tyr^{I}$ using a rabbit reticulocyte lysate translation system. In 2002, the Hirao group (14) devised a pair of artificial nucleotides, 2-amino-6-(2-thienyl)purine (designated s) and pyridin-2-one (designated y), which they used to introduce 3-chlorotyrosine ($Tyr^{Cl}$) at a yAG codon using a tRNA bearing a CUs anticodon. They expressed Ras protein containing $Tyr^{Cl}$ at position 32 using *E. coli* extract as an in vitro translation system. In 2019, the Romesberg group (15) applied a pair of two artificial nucleotides, NaM and TPT3, to in-cell expression of superfolder green fluorescent protein in the *E. coli* YZ3 strain, introducing N6-[(2-propynyloxy)carbonyl]-lysine ($Lys^{pr}$) or *p*-azidophenylalanine ($Phe^{paz}$) at an A(NaM)C codon at position 151.

# 3. PREPARATION OF NONPROTEINOGENIC AMINOACYL TRANSFER RNA

To introduce npAAs by means of any of the genetic code manipulation methods described in Section 2, we need to prepare npAA-tRNA bearing a corresponding anticodon that recognizes the reprogrammed codon. The methods for preparing npAA-tRNA that have been developed to date can be classified into the following three types: (*a*) use of artificial ARSs, (*b*) chemical synthesis, and (*c*) use of aminoacylation ribozymes. These methods are summarized in this section.

## 3.1. Preparation of npAA-tRNA by Aminoacyl-tRNA Synthetases

In nature, synthesis of aminoacyl-tRNAs is catalyzed by specific ARSs. Naturally occurring ARSs strictly recognize the structures of both substrates, amino acids and tRNAs, so that mischarge of a noncognate amino acid on the wrong tRNA does not take place. The high substrate specificity of ARSs and their proofreading mechanism ensure the accuracy of aminoacylation. Therefore, we cannot generally use naturally occurring ARSs to prepare noncognate npAA-tRNAs. However, the development of artificial ARSs that can charge particular npAAs on specific tRNAs has been reported. Schultz (16) is the pioneer of this approach, and his group succeeded in charging $Tyr^{Me}$ on *Methanocaldococcus jannaschii* $tRNA^{Tyr}$ using an engineered *M. jannaschii* TyrRS bearing five point mutations around the active site (**Figure 1*a***). Since the mutant *M. jannaschii* TyrRS/$tRNA^{Tyr}$ pair and the *E. coli* TyrRS/$tRNA^{Tyr}$ pair are orthogonal to each other, $Tyr^{Me}$ and Tyr are specifically charged onto *M. jannaschii* $tRNA^{Tyr}$ and *E. coli* $tRNA^{Tyr}$, respectively, and can be independently introduced at different codons in an *E. coli* translation system. On the basis of this strategy, many TyrRS-based artificial ARSs have been established to date (17–19). The pyrrolysyl-tRNA synthetase (pylRS)/pyrrolysyl-tRNA (pylT) pairs from *Methanosarcinaceae* species are also orthogonal in both prokaryotic and eukaryotic translation systems. Therefore, the pylRS/pylT systems have often been engineered and utilized for the incorporation of various npAAs (20, 21).

## 3.2. Chemical Synthesis of npAA-tRNA

npAA-tRNA can also be prepared by chemical modification of pAA-tRNA after the aminoacylation of pAA by ARSs. For instance, deamination of the amino group of pAA-tRNA yields an α-hydroxyacyl-tRNA. In the ribosomal polyester synthesis mentioned in Section 2.1,

Phe-tRNA$^{Phe}$ was converted to $^{OH}$Phe-tRNA$^{Phe}$ by deamination in the presence of nitrous acid (**Figure 1*c***) (6). *N*-methylation of pAA-tRNA can be conducted by reductive alkylation using 2-nitrobenzaldehyde and sodium cyanoborohydride to give *N*-nitrophenyl aminoacyl-tRNA, followed by reaction with formaldehyde to give *N*-nitrophenyl-*N*-methyl aminoacyl-tRNA, and deprotection of the 2-nitrobenzaldehyde by ultraviolet light (22). The reaction of pAA-tRNA with succinimide ester is also useful for introducing a desired functional group at the amino group. For instance, a succinimide ester of BODIPY or biotin can be used for BODIPY and biotin labeling of an amino group (23–25).

Chemical synthesis of a 3′-end fragment of npAA-tRNA and its enzymatic ligation to the rest of the tRNA body (minus CA) also enable the preparation of full-length npAA-tRNAs. For instance, the Hecht group (26) used a combination of aminoacylated $P^1$,$P^2$-di(adenosine 5′-)diphosphate and a tRNA body lacking the 3′-end A, which were eventually conjugated by T4 RNA ligase. To improve its low ligation efficiency, they also established a method using aminoacyl-pCpA and a tRNA body lacking the 3′-end dinucleotide (27). Later, the Schultz group further modified this method to use aminoacyl-pdCpA, in which deoxycytidine is used instead of cytidine, and a tRNA lacking the 3′-end dinucleotide (28). They also introduced a photocleavable protective group to the α-amino group of the aminoacyl-pdCpA to make it more easily treated.

Use of amino acid *N*-carboxyanhydrides (NCAs) is a practical approach for the aminoacylation of 2-aminobenzoic acid (2-Abz) and its derivatives on tRNA (**Figure 1*b***) (29–31). The reaction occurs by simply mixing the NCA and tRNA under mild basic conditions (pH 8.8–10.5). However, application of this method is limited to amino acids with reduced nucleophilicity of the amino group, such as 2-Abz, because polymerization of amino acids occurs if the amino group is highly reactive.

### 3.3. Flexizyme-Catalyzed Aminoacylation

As discussed in Section 3.1, protein-based ARSs are highly specific to their substrates, amino acids, and tRNAs, and therefore, they are not a fully versatile tool for the preparation of multiple diverse npAA-tRNAs at once. Even though the development of artificial ARSs is possible, their substrates are limited to those that are structurally similar to the original substrate, making it unrealistic to develop specific ARSs for a wide variety of npAA-tRNAs. Chemoenzymatic synthesis of npAA-tRNAs can be an alternative method for gaining versatility, yet its labor intensity and the uncertainty of success with this method of preparation hinder the production of a variety of npAA-tRNAs in parallel.

To overcome this major stumbling block, Suga and others (32–36) conducted a series of experiments to develop a family of aminoacylation ribozymes, referred to as flexizymes, that have multiple turnover activities for charging npAA donors, which must be appropriately activated with leaving groups, onto tRNAs. Further optimization and in vitro evolution yielded a number of flexizyme variants, as reported in 2006 (37), that enable the acylation of diverse substrates on various tRNAs (38). There are three variants of flexizyme currently available. Enhanced flexizyme (eFx) utilizes cyanomethyl esters or *p*-chlorobenzyl thioesters of amino acids (37). Dinitro-flexizyme (dFx) (37) and amino flexizyme (aFx) (39) utilize amino acids activated as 3,5-dinitrobenzyl esters and 4-[(2-aminoethyl)carbomoyl]benzyl thioesters (ABTs), respectively (38). Due to the high water solubility of ABTs, aFx is favorable for the acylation of hydrophobic substrates with low water solubility. All of these flexizymes recognize only the 3′-CCA end of tRNA and the aromatic ring of an ester or thioester group or sidechain. Therefore, virtually any substrate can be acylated on any tRNA bearing the conserved 3′-CCA end. To date, various substrates, including L-α-amino acids with nonnatural sidechains, D-α-amino acids, *N*-alkyl-α-amino acids, *N*-acyl-α-amino acids,

β- and γ-amino acids, and even non–amino acid substrates like α-hydroxy acids, α-thio acid, and α-aminocarbothionic *O*-acid, have been successfully charged onto tRNAs by flexizymes (8, 31, 37, 40–50).

## 4. EXPANSION OF USABLE NONPROTEINOGENIC AMINO ACIDS IN THE RIBOSOMAL TRANSLATION SYSTEM

Although the methods for genetic code manipulation mentioned in Section 2 have enabled the incorporation of various npAAs, not all npAAs are able to be efficiently introduced into peptides or proteins. Therefore, the scope of npAAs that are usable in the ribosomal translation systems should be evaluated. In 2004, Tan et al. (51) examined the ribosomal incorporation of diverse npAAs, including *N*-methyl-α-amino, α,α-disubstituted amino, β-amino, D-α-amino, and α-hydroxy acid analogs of Ala and Phe, into a tripeptide fMet-npAA-Glu by means of genetic code reprogramming. For the incorporation of *N*-methyl-α-amino and α-hydroxy acids, an acceptable level of translation efficiency was achieved. However, some α,α-disubstituted amino acids were incorporated only at low efficiencies, and D-α-amino and β-amino acids were even worse. Several groups showed that incorporation of D-α-amino and β-amino acids is also possible, but their efficiency is at a far lower or barely detectable level compared with that of canonical L-α-amino acids (45, 46, 52–56). The most systematic study, reported by Fujino et al. (45), classified 19 D-amino acids, the counterparts of pAAs, into three groups based on their incorporation efficiency: Group I had a yield of over 40% relative to the incorporation of their L-amino acid counterparts (Ala, Ser, Cys, Met, Thr, His, Phe, and Tyr), Group II had a yield of 10%–40% (Asn, Gln, Val, and Leu), and Group III had a yield of less than 10% (Arg, Lys, Asp, Glu, Ile, Trp, and Pro). Compared to single incorporation, consecutive incorporation of multiple npAAs is generally more difficult. Achenbach et al. (53) determined the efficiency of consecutive D-amino acid incorporation to be generally less than 10% relative to L-amino acid incorporation.

The low incorporation efficiency of npAAs can be mainly attributed to the following three causes: (*a*) slow accommodation of npAA-tRNAs onto the ribosomal A site, (*b*) slow peptidyl transfer of npAAs, and (*c*) peptide truncation caused by mistranslocation (**Figure 3**). In this section, we discuss the mechanisms by which npAA incorporation is made inefficient and introduce methods to improve npAA incorporation efficiency.

### 4.1. Improving Accommodation of npAA-tRNAs onto the Ribosomal A Site

The slow accommodation of npAA-tRNAs onto the ribosome is attributed to the low binding affinity of npAA-tRNAs for EF-Tu, a translation factor responsible for accommodation (**Figure 3**, step 1). EF-Tu has been reported to recognize two parts of aminoacyl-tRNA, the amino acid moiety and the T-stem region (**Figure 4*a***) (57–60). EF-Tu binds to the amino acid moiety of pAA-tRNA with different affinities, depending on their amino acid structures. However, the affinity of the T-stem region compensates for this nonuniformity so that all pAA-tRNAs have uniform affinities for EF-Tu (61). For instance, Glu, whose affinity toward EF-Tu is low, is charged onto $tRNA^{Glu}$, which bears a high affinity T-stem. Therefore, the rate of accommodation of Glu-$tRNA^{Glu}$ is maintained within an appropriate range.

In the case of npAA-tRNAs, the binding affinity of the npAA is generally lower than those of the pAAs, and this difference is not appropriately compensated for unless we choose strong tRNAs with high EF-Tu affinities. Iwane et al. (62) surveyed the binding affinities of 13 kinds of *N*-methylaminoacyl-$tRNA^{AsnE2}$ to EF-Tu and showed that most of them have significantly weaker affinities ($\Delta G > -7.0$ kcal/mol for $^{Me}$Ala, $^{Me}$Thr, $^{Me}$Phe, $^{Me}$Tyr, $^{Me}$Val, $^{Me}$Leu, $^{Me}$Met, $^{Me}$Asp, *N*-methylnorvaline, *N*-methylnorleucine, and *N*-methyl-*O*-methyltyrosine) than those of

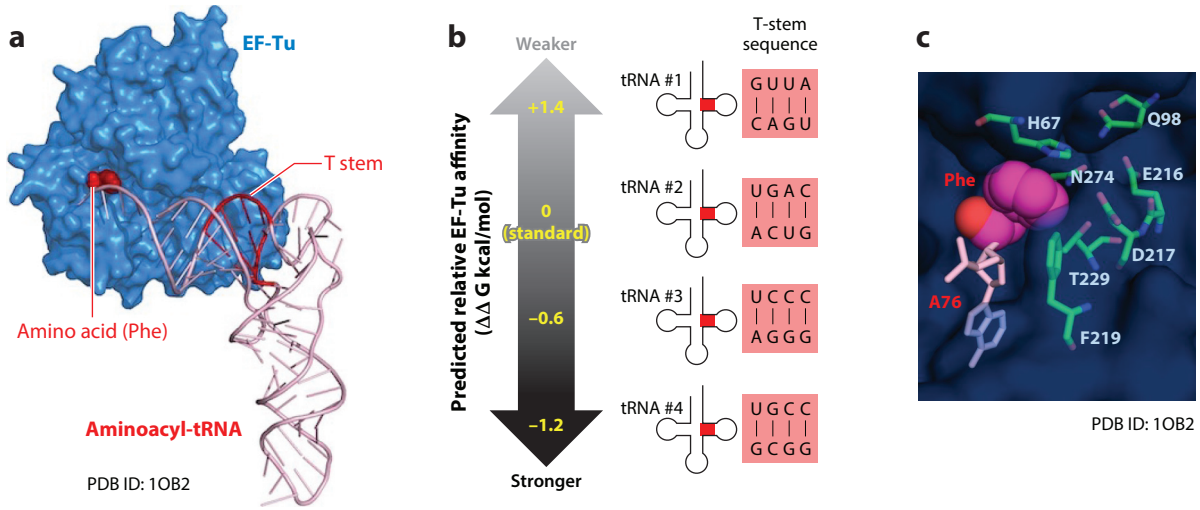



**Figure 4**

Accommodation of aminoacyl-tRNA by EF-Tu. (*a*) Structure of *Saccharomyces cerevisiae* Phe-tRNA$^{Phe}$ bound to *Escherichia coli* EF-Tu (PDB ID: 1OB2). The amino acid moiety (Phe) and T-stem of Phe-tRNA$^{Phe}$ are indicated in red. (*b*) Predicted relative EF-Tu affinity of four tRNA variants (#1–#4) with different T-stem sequences. (*c*) Structure of the amino acid binding pocket of *E. coli* EF-Tu (PDB ID: 1OB2). Abbreviation: PDB ID, Protein Data Bank identifier.

pAA-tRNA$^{AsnE2}$ (from −8.0 to −9.4 kcal/mol for Phe, Ser, and Tyr), except for $^{Me}$Gly (−8.4 kcal/mol) and $^{Me}$Ser (−8.4 kcal/mol). In order to compensate for the weak affinity of those *N*-methylamino acids, a series of engineered tRNA$^{AsnE2}$ with four T-stem variations (**Figure 4*b***) were devised, and their affinities were analyzed. Note that tRNA #2 is identical to the original tRNA$^{AsnE2}$ and that the affinities of these tRNAs increase as the number increases from #1 to #4. Consequently, the affinity of $^{Me}$Phe-tRNA was enhanced from an undetectable level (with tRNA #2) to −7.9 and −9.0 kcal/mol by using tRNA #3 and #4, respectively. This result indicated that the affinity of *N*-methylaminoacyl-tRNA can be fine-tuned by choosing an appropriate T-stem structure for each amino acid. Indeed, the use of *N*-methylaminoacyl-tRNAs with fine-tuned EF-Tu affinities improved the expression of peptides containing multiple *N*-methylamino acids with high fidelity and expression levels. By means of this strategy, a macrocyclic peptide containing 9 distinct *N*-methylamino acids, 14 different kinds of pAAs, and an *N*-chloroacetyltyrosine ($^{ClAc}$Tyr) has been cleanly expressed. $^{ClAc}$Tyr was introduced at the N terminus so that spontaneous thioether bond formation occurred between the chloroacetyl group and a thiol group of the downstream Cys to give a macrocyclic structure. Notably, simply maximizing the affinities by replacing all tRNAs with the strongest tRNA (#4) was not effective at optimizing the translation of such highly *N*-methylated peptides, because tRNAs with overly strong affinities, such as $^{Me}$Gly-tRNA #4 and $^{Me}$Ser-tRNA #4, might hinder their release from EF-Tu at the ribosomal A site (63) or bind to EF-Tu so dominantly as to exclude other aminoacyl-tRNAs.

The same strategy can also be applied to other inefficient substrates such as D- and β-amino acids. tRNA$^{GluE2}$ was designed based on the structure of *E. coli* tRNA$^{Glu}$, whose T-stem is identical to that of tRNA #3, to compensate for the weak binding affinity of npAAs (64). The expression levels of model peptides containing D-Ala or β-homomethionine (β-hMet) were significantly improved by using tRNA$^{GluE2}$ compared to the use of weaker tRNA$^{AsnE2}$ (tRNA #2) (54–56).

Development of EF-Tu mutants with higher binding affinities for npAAs is another approach to improve the slow accommodation rate of npAA-tRNA. Doi et al. (65) developed EF-Tu mutants

that efficiently bind to bulky npAAs like 1-pyrenylalanine ($Ala^{1pyr}$) and 9-anthrylalanine ($Ala^{9ant}$) (**Figure 1*a***). Since the side chains of these npAAs are much bulkier than those of pAAs, the amino acid–binding pocket of EF-Tu, located around E216 and D217, was enlarged for tighter binding (**Figure 4*c***). Two mutant EF-Tus, E216A and D217A, showed greater affinities toward $Ala^{1pyr}$-tRNA and $Ala^{9ant}$-tRNA, leading to improved incorporation of these npAAs into streptavidin at a CGGG four-base codon. Note that E216 and D217 were referred to as E215 and D216, respectively, in the original paper but have been changed in this review to standardize the numbering of all related studies.

Since the amino acid–binding pocket of EF-Tu contains the negatively charged residues, E216 and D217, its affinity for negatively charged amino acids is generally low due to electrostatic repulsion. To incorporate negatively charged *O*-phosphoserine (Sep) (**Figure 1*a***), Park et al. (66) developed a mutant EF-Tu, named EF-Sep, in which six residues around the binding pocket were mutated (H67R, E216N, D217G, F219Y, T229S, and N274W). Consequently, EF-Sep showed a higher binding affinity for Sep-tRNA and resulted in improved Sep incorporation efficiency. To incorporate L-phosphotyrosine (pTyr) (**Figure 1*a***), Fan et al. (67) developed a similar EF-Tu mutant, named EF-pY, that bears three mutations (E216V, D217G, and F219G) and resulted in improved pTyr incorporation efficiency. Selenocysteine (Sec) (**Figure 1*a***) is also negatively charged under physiological conditions, so the affinity of Sec-tRNA for EF-Tu is expected to be weak. Haruna et al. (68) developed an EF-Tu variant named EF-Sel1, containing five mutations (H67R, Q98W, E216N, D217K, and N274R), which showed good binding affinity for Sec-tRNA. Importantly, in all of these EF-Tu mutants that efficiently bind to negatively charged npAAs, the negatively charged residues E216 and D217 were substituted with neutral or positively charged residues such as Asn, Gly, Val, or Lys.

## 4.2. Acceleration of Peptide-Bond Formation Between npAAs by Mutant Ribosomes

The slow peptidyl transfer of npAA could be attributed to the incompatibility of the npAA's structure and the peptidyl transferase center (PTC) of the ribosome. The carbonyl group of the P-site peptidyl-tRNA and the amino group of the A-site aminoacyl-tRNA should be aligned properly in the PTC so that the peptidyl transfer reaction proceeds efficiently. However, the reaction is difficult if the peptidyl- and/or aminoacyl-tRNAs are charged with npAAs whose structure is incompatible with the PTC. For instance, in the case of D-amino acid incorporation, if the amino group of the A-site D-aminoacyl-tRNA is properly positioned for its nucleophilic attack onto the carbonyl group of the P-site peptidyl-tRNA, the β-carbon of the A-site D-amino acid clashes with the base of the conserved U2506 of 23S rRNA in the PTC (69). The P-site peptidyl-D-aminoacyl-tRNA has also been reported to stabilize the ribosome in an inactive form by altering the conformations of A2058, A2059, A2062, A2063, G2505, and U2506 of 23S rRNA (70). These observations indicate that both the A-site D-aminoacyl-tRNA and P-site peptidyl-D-aminoacyl-tRNA slow down peptidyl transfer. Therefore, consecutive incorporation of two or more D-amino acids should be even slower than single incorporation (45, 53, 70–72).

Since the PTC is located at domain V of 23S rRNA, efforts to develop mutant ribosomes by introducing mutations into domain V have been made for efficient D- and β-amino acid incorporation (**Figure 5*a***). To develop ribosomes compatible with D-amino-acid incorporation, Dedkova et al. (52, 73) introduced mutations into nucleotides 2447–2450 and 2457–2462 of domain V. Variants named A4 (2447-UGGC-2450) and B25 (2457-GCUGAU-2462) showed significantly improved incorporation of D-Met into dihydrofolate reductase (DHFR): 22.9% and 19.7% (relative to wild-type DHFR expression), compared with 10.1% for the wild-type ribosome

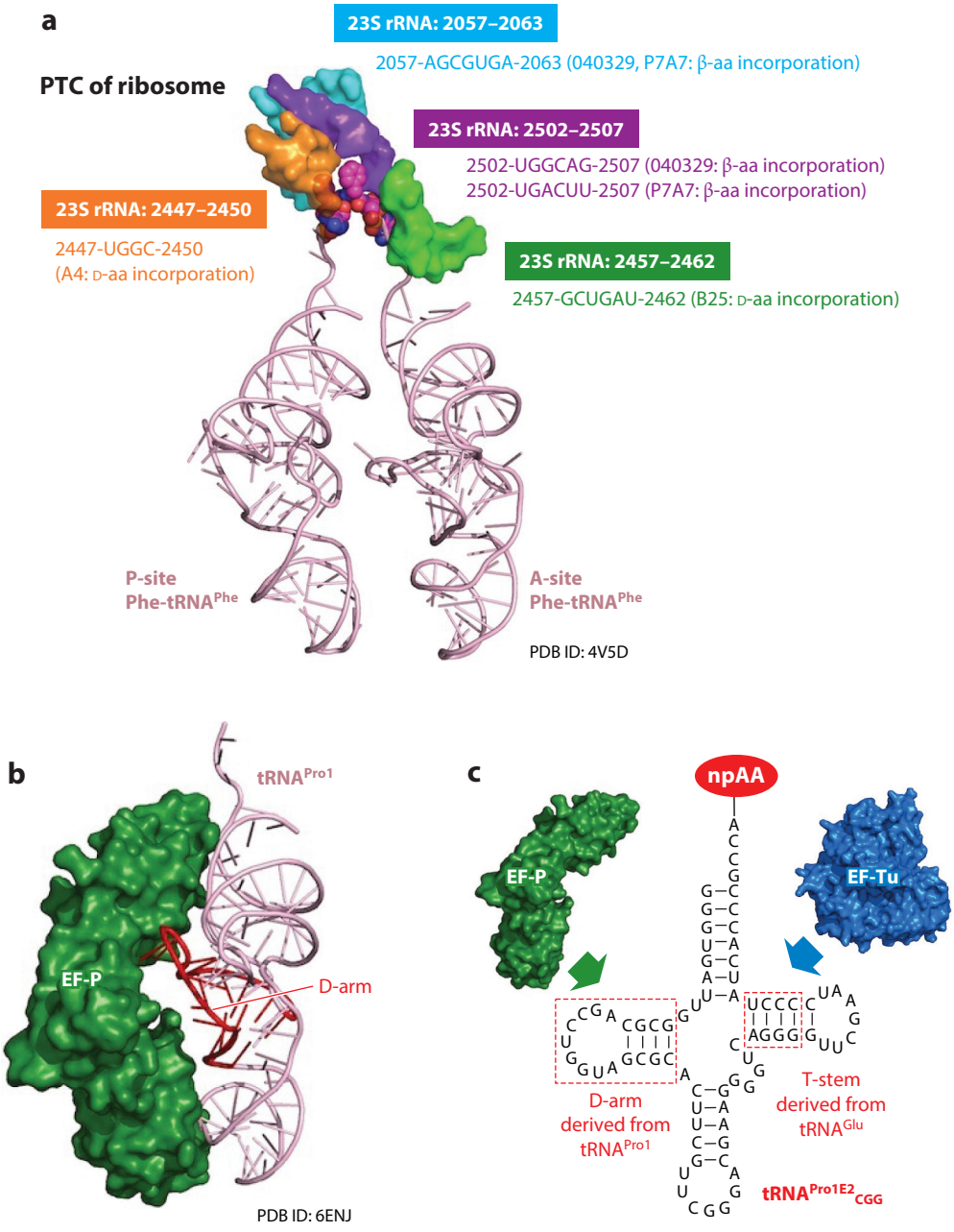



**Figure 5**

Acceleration of peptidyl transfer by engineered translation systems. (*a*) Structures of *Escherichia coli* P- and A-site Phe-tRNA$^{Phe}$ bound to *Thermus thermophilus* 70S ribosome (PDB ID: 4V5D). Nucleobases of 23S rRNA that were mutated to enhance incorporation of D- and β-amino acids are also indicated. Mutants A4 and B25 were developed for D-amino acid incorporation and 040329 and P7A7 for β-amino acid incorporation. (*b*) Structure of *E. coli* tRNA$^{Pro1}$ bound to EF-P (PDB ID: 6ENJ). The D-arm of the tRNA is recognized by EF-P and indicated in red. (*c*) Secondary structure of tRNA$^{Pro1E2}{}_{CGG}$, which has specific D-arm and T-stem motifs for tight binding to EF-P and EF-Tu, respectively. Abbreviations: npAA, nonproteinogenic amino acid; PDB ID, Protein Data Bank identifier; PTC, peptidyl transferase center.

(**Figure 5*a***). Dedkova and colleagues (74–76) also developed similar mutant ribosomes compatible with β-amino acid incorporation. A variant named 040329 bearing the mutant sequences 2057-AGCGUGA-2063 and 2502-UGGCAG-2507 showed significantly higher incorporation efficiency of β-alanine (β-Ala): 12.3% (relative to L-α-Val incorporation), compared with 4.0% for the wild-type ribosome (**Figures 1*b*** and **5*a***). Czekster et al. (77) further optimized the sequence of 040329 and established variant P7A7, bearing 2057-AGCGUGA-2063 and 2502-UGACUU-2507, which showed improved β-(p-bromophenyl)glycine incorporation efficiency (**Figure 5*a***). These results suggest that the local structures of the PTC could enhance the incorporation of some npAAs such as D-Met and β-Ala. However, the experiments described here showed only their single incorporation, not multiple or consecutive incorporations, and therefore, it is still unclear whether mutant ribosomes can effectively incorporate other npAAs and perform consecutive elongations.

## 4.3. Acceleration of Peptide Bond Formation by EF-P

Among the 20 pAAs, Pro is the only secondary amino acid, and its peptidyl transfer is extremely slow compared to the other pAAs. Therefore, a translation factor named EF-P is used to accelerate peptidyl transfer of Pro (78, 79). EF-P recognizes Pro-tRNA$^{Pro}$ by the specific D-arm motif of tRNA$^{Pro}$ isoacceptors, which consists of a nine-nucleotide D-loop closed by a stable 4-bp D-stem with two G/C base pairs at positions 12/23 and 13/22 (**Figure 5*b*,*c***) (80, 81). Katoh et al. (55) used one of the *E. coli* tRNA$^{Pro}$ isoacceptors, tRNA$^{Pro1}$, to incorporate not only Pro but also D-amino acids in an in vitro translation system containing EF-P and found that EF-P accelerates peptidyl transfer of D-amino acids, such as D-Ala, D-Ser, D-His, and D-Ser, by recognizing the D-arm motif of tRNA$^{Pro1}$. This EF-P-dependent enhancement effect was significantly decreased by the introduction of a point mutation at position 13, C13G, indicating the importance of the D-arm motif for recognition. Notably, incorporation of α,α-disubstituted amino acids and β-amino acids charged on tRNA$^{Pro1}$, as well as D-amino acids, could be improved by EF-P (55, 56).

Since EF-Tu recognizes the T-stem of tRNA, as explained in Section 4.1, if the T-stem of tRNA$^{Pro1}$ is fine-tuned to have strong affinity to EF-Tu, accommodation of npAA-tRNA and peptidyl transfer could be accelerated at the same time. On this basis, Katoh et al. (55) developed an engineered tRNA, named tRNA$^{Pro1E2}$, that has both the D-arm and the T-stem motifs required for efficient recruitment of EF-P and EF-Tu, respectively (**Figure 5*c***). The T-stem of tRNA$^{Pro1E2}$ is identical to those of tRNA #3 and tRNA$^{GluE2}$ (**Figure 4*b***). By using tRNA$^{Pro1E2}$ in the presence of EF-P, incorporation of two consecutive D-Ala or β-hMet residues was enhanced 18- and 28-fold, respectively, compared to the use of a conventional tRNA$^{AsnE2}$ that has neither the D-arm nor the T-stem motif. This enhancement effect was significantly higher than that observed using the original tRNA$^{Pro1}$ or tRNA$^{GluE2}$ (55, 56).

2-Abz and its derivatives, such as 3-aminopyridine-4-carboxylic acid (Apy), 3-aminothiophene-2-carboxylic acid (Atp), and 5-aminothiazole-4-carboxylic acid (Atz), are variations of β-amino acids in which amino nitrogen and carbonyl carbon are both directly attached to an aromatic ring (**Figure 1*b***). Since the amino group of 2-Abz derivatives is a poor nucleophile due to the resonance effect (the p$K_a$ value of the ammonium form of 2-Abz is 4.9), the reactivity of 2-Abz derivatives in peptide-bond formation is low. Indeed, their elongation is much less efficient than that of nonaromatic β-amino acids. However, the use of tRNA$^{Pro1E2}$ improved the elongation of 2-Abz 3.7-fold in the presence of EF-P compared to its absence (50). Likewise, elongation of Apy, Atp, and Atz was improved 1.8-, 1.4-, and 1.5-fold, respectively. Although γ-amino acids are much less efficient substrates than β-amino acids, and thus, ribosomal elongation of γ-amino acids had never been reported previously, the use of tRNA$^{Pro1E2}$ enabled the elongation of some cyclic γ-amino acids, such as 3-aminocyclobutane carboxylic acid (3-ACBC), 3-aminocyclopentane carboxylic acid (3-ACPC), and even 3-aminobenzoic acid (3-Abz) (**Figure 1*b***), for the first time (31, 50).

### 4.4. Peptide Truncation Caused by Mistranslocation

Slow accommodation of aminoacyl-tRNA and peptide-bond formation eventually induce ribosomal stalling and peptidyl-tRNA drop-off from the ribosomal P site (**Figure 3**). For example, elongation of inefficient npAAs such as D- and β-amino acids often suffers from severe peptidyl-tRNA drop-off, leading to the synthesis of a truncated peptide lacking the C-terminal region (45, 49). If the ribosome that lost the P-site peptidyl-tRNA is still active, translation reinitiates by migration of the remaining A-site aminoacyl-tRNA to the P site, followed by accommodation of a new aminoacyl-tRNA into the empty A site. Eventually, a truncated peptide lacking the N-terminal region is also generated by translation reinitiation, which is referred to as the drop-off-reinitiation event (82). Indeed, three related papers published by the Suga group (45, 82, 83) showed the generation of such truncated peptides lacking the N-terminal region.

Since canonical translocation is mediated by EF-G, the drop-off-reinitiation event that requires the migration of A-site aminoacyl-tRNA could also be triggered by EF-G. Moreover, since it has also been reported that EF-G is involved in the release of peptidyl-tRNAs from the stalled ribosome (84), EF-G should be responsible for both drop-off and translation reinitiation at the same time. Both the P-site peptidyl-tRNA and A-site aminoacyl-tRNA could be translocated to the E site and P site, respectively, by EF-G, if translocation precedes completion of peptidyl transfer (**Figure 3**, step 3, mistranslocation). Indeed, excessive EF-G (>0.1 μM) significantly promoted the drop-off-reinitiation event when introducing two consecutive D-Ala (82). Therefore, suppression of the drop-off-reinitiation event by optimizing EF-G concentration is critical for the efficient expression of such a noncanonical peptide (54).

## 5. INCREASING THE NUMBER OF AVAILABLE AMINO ACIDS IN THE REPROGRAMMED CODON TABLES

Another important issue to be addressed is the limited number of available amino acids in the reprogrammed codon table. In the canonical codon table, the number of available pAAs is 20. In the case of genetic code reprogramming, the number of available amino acids is no more than 20 in general, because one pAA should be sacrificed to assign one additional npAA. To overcome this issue, Iwane et al. (85) developed a method to artificially divide a codon box into two, to which one pAA and one npAA are assigned so that no pAA is sacrificed. Consequently, the authors succeeded in increasing the number of available amino acids up to 23 (3 npAAs and 20 pAAs) and translating a 32-mer peptide with 23 different amino acids. Since 11 npAAs can be theoretically added using this method without sacrificing any pAAs, up to 31 different amino acids (11 npAAs and 20 pAAs) are available in one codon table.

Using nonsense codon suppression, there are only three stop codons available for suppression, and at least one of them should be retained for translation termination. Therefore, only one or two additional npAA(s) can be installed in a codon table, i.e., the maximum number of amino acids is 22 (2 npAAs and 20 pAAs). Similarly, with quadruplet codon suppression, the number of available amino acids cannot be easily increased because the number of rare codons is also limited. Recently, Dunkelmann et al. (86) combined nonsense codon suppression and quadruplet codon suppression and assigned one npAA at the amber codon and two npAAs at two quadruplet codons. Theoretically, artificial division of codon boxes could also be combined with these methods.

Genetic alphabet expansion theoretically has the greatest potential to increase the number of npAAs for incorporation because 152 additional codons can be created by adding one artificial base pair. However, the orthogonality of artificial bases to the natural bases is insufficient due to mispair formation, e.g., mispairing between isoG and T (87). In addition, whether multiple npAA-tRNAs containing artificial bases can be incorporated with high enough fidelities has

not yet been determined. Therefore, high hurdles for the practical use of artificial base pairs in translation still remain.

## 6. DEVELOPMENT OF DE NOVO FUNCTIONAL PEPTIDES BY GENETIC CODE REPROGRAMMING

Genetic code reprogramming methods can be applied to ribosomal synthesis of a random peptide library containing diverse npAAs. Such peptide libraries are compatible with display-based screening methods, such as mRNA display (88, 89), ribosome display (90), and phage display (91), to obtain specific peptide ligands that bind to specific target molecules. In particular, the combination of mRNA display with a random peptide library containing npAAs prepared by genetic code reprogramming is referred to as the random nonstandard peptides integrated discovery (RaPID) system (**Figure 6**) (44). Introduction of npAAs into such peptide ligands often leads to improvements in peptidase resistance, structural rigidity, binding affinity, and membrane

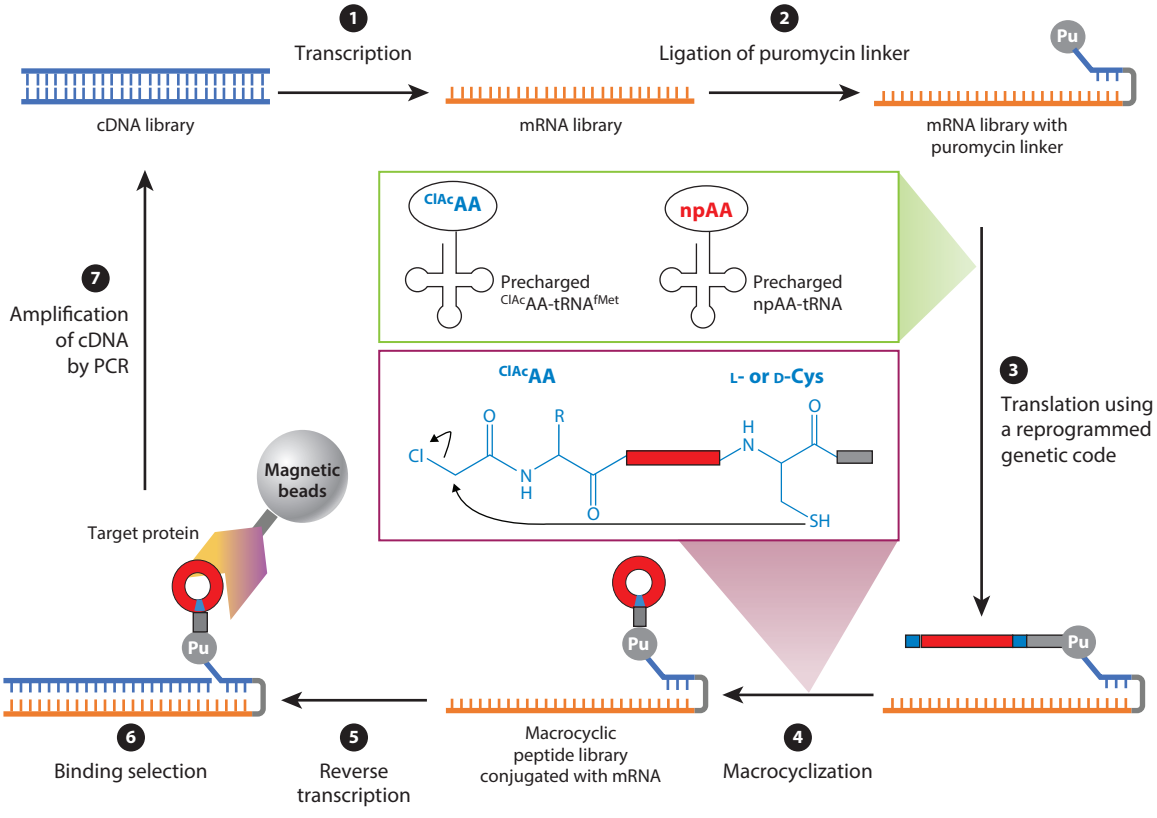



**Figure 6**

Schematic depiction of the RaPID system using a macrocyclic peptide library. ① Transcription of the cDNA library to make the mRNA library. ② Ligation of a puromycin linker at the 3′ end of the mRNA library. ③ Translation of a peptide library using a reprogrammed genetic code. ④ Spontaneous macrocyclization of the peptide library. The N-terminally incorporated $^{ClAc}$AA is used for macrocyclization by forming a thioether bond with a thiol group of the downstream Cys. ⑤ Reverse transcription of mRNA into cDNA. ⑥ Selection for binding of the peptide library to the target protein immobilized on magnetic beads. ⑦ Amplification of cDNA by PCR. Abbreviations: $^{ClAc}$AA, *N*-chloroacetylamino acid; npAA, nonproteinogenic amino acid; PCR, polymerase chain reaction; Pu, puromycin; RaPID, random nonstandard peptides integrated discovery.

permeability. For instance, peptides consisting of only pAAs are rapidly degraded within a few minutes to a few hours (92); however, the introduction of several D-amino acids into peptides usually improves their peptidase resistance if appropriately arranged (93–96).

Imanishi et al. (97) took advantage of a ribosomally synthesized macrocyclic peptide library containing five kinds of D-amino acids, ClAc-D-Tyr, D-Ser, D-His, D-Tyr, and D-Ala, for the RaPID selection of epidermal growth factor receptor (EGFR) inhibitors. The N-terminally incorporated ClAc-D-Tyr was used for macrocyclization of peptides via formation of a thioether bond with a thiol group from the downstream L-Cys (**Figure 6**) (98). The library contained a repeat of 4–15 random residues encoded by NNU codons, in which D-Ser, D-His, D-Tyr, and D-Ala randomly appeared. Deep sequencing of the cDNA after five rounds of affinity selection revealed that the library was enriched with several families of peptides. Among them, peptides 2D and 18D contained five and six D-amino acid residues, respectively, and were able to inhibit protein–protein interactions between EGFR and its ligand, EGF (**Figure 7*a***). Moreover, their serum stabilities were extremely high, and no significant degradation was observed even after 24 h incubation in human serum at 37°C, whereas their L-configuration counterparts, 2L and 18L, were significantly degraded (their half-lives in human serum were 5.0 h and 15.5 h, respectively).

β-Amino acids induce unique rigid folding structures in peptides, known as foldamers, by forming stable helix and turn structures (99–104). Cyclic $\beta^{2,3}$-amino acids (cβAAs), such as 2-aminocyclohexanecarboxylic acid (2-ACHC) and 2-aminocyclopentanecarboxylic acid (2-ACPC), act as particularly strong helix/turn inducers due to their constrained cyclic structures, leading to the formation of designer drug–like β-peptides (99, 105–107). Katoh and others (49, 107–108) developed a macrocyclic peptide library containing three types of cβAA, (1*S*,2*S*)-2-ACHC, (1*R*,2*R*)-2-ACPC, and (1*S*,2*S*)-2-ACPC, and used it to screen for human FXIIa inhibitors. The peptide library was cyclized via a thioether bond between an N-terminal ClAc-D-Tyr and a downstream D-Cys. One of the resulting peptides, F3, which contains two (1*S*,2*S*)-2-ACHC residues, exhibited strong binding affinity (0.98 nM $K_D$) and inhibitory activity (1.02 nM $K_i$) against FXIIa (**Figure 7*b***). The X-ray crystal structure of F3 bound to FXIIa revealed that one of the two (1*S*,2*S*)-2-ACHC residues (ACHC8) induces two γ-turns and folds the peptide into an antiparallel β-sheet. Moreover, the half-life of F3 in human serum at 37°C was 59 h, whereas substitution of the two (1*S*,2*S*)-2-ACHC residues with Ala shortened its half-life to 13 h, indicating the contribution of (1*S*,2*S*)-2-ACHC to the peptide's high serum stability. Not only aliphatic cβAA but also aromatic cβAAs, such as 2-Abz and Atp, could be introduced into a random macrocyclic peptide library for in vitro selection of FXIIa inhibitors and IFNGR1 inhibitors (109). One of the IFNGR1 inhibitor peptides, ArβI-1, contains 2-Abz and showed remarkably strong binding affinity (0.44 nM $K_D$) and inhibitory activity (9.7 nM $IC_{50}$).

Peptide libraries containing multiple *N*-methylamino acids have also been used for RaPID display (44, 110). aIL6R-1 and $CM_{11}$-1 were obtained by screening for binding to IL6R and E6AP, respectively, and contain two and four *N*-methylamino acids, respectively (**Figure 7*c*,*d***). In contrast to the high binding affinity of $CM_{11}$-1 ($K_D$ = 0.6 nM), a negative control variant of $CM_{11}$-1 lacking *N*-methylation, named $CP_{11}$-1, completely lost its ability to bind to E6AP, indicating the importance of *N*-methylation to the peptide's binding ability.

## 7. SUMMARY AND OUTLOOK

The genetic code reprogramming methods described here have enabled the ribosomal incorporation of diverse npAAs into peptides and proteins. Since incorporation of npAAs is sometimes extremely inefficient or even impossible, particularly their consecutive incorporation, efforts to engineer EF-Tu, rRNA, and tRNA have been made to improve the efficiency. As a result, not only

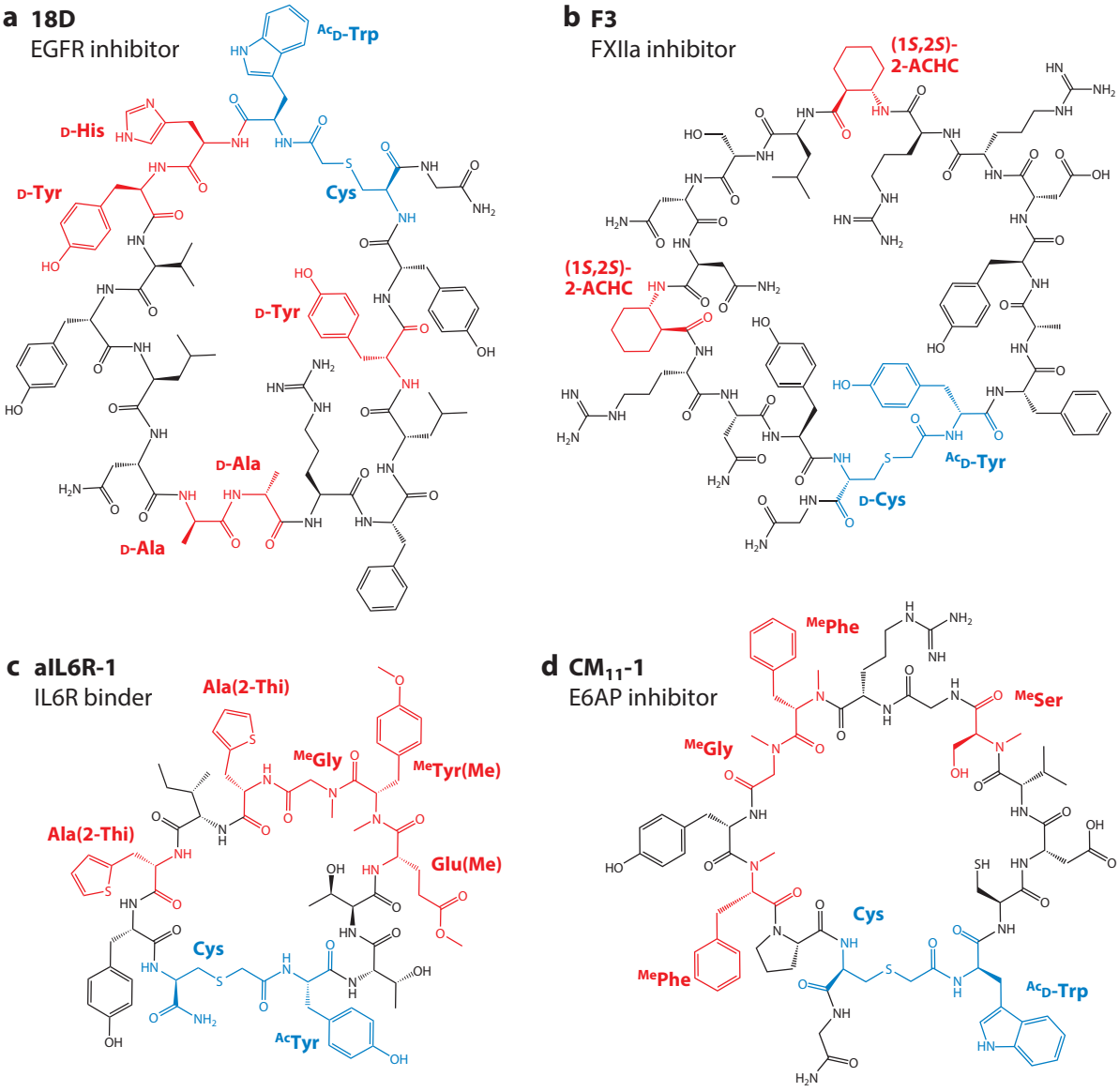



**Figure 7**

Examples of macrocyclic peptides containing nonproteinogenic amino acids discovered by the random nonstandard peptides integrated discovery system. (*a*) 18D, EGFR inhibitor; (*b*) F3, FXIIa inhibitor; (*c*) aIL6R-1, IL6R inhibitor; (*d*) $CM_{11}$-1, E6AP inhibitor.

L-α-amino acids with noncanonical side chains but also npAAs with noncanonical backbones, such as *N*-methyl-α-amino, D-α-amino, β-amino, and γ-amino acids, can be introduced. In canonical translation, the ribosome is responsible only for amide bond formation; however, non–amino acid substrates like α-hydroxy acid (6, 8), α-thio acid (47), and α-aminocarbothionic *O*-acid (48) can be used for ribosomal formation of ester, thioester, and thioamide bonds, respectively (**Figure 1*c***). Therefore, the use of ribosomal translation to allow the formation of a wide range of chemical bonds would be an interesting extension of this work. The development of novel

mutant ribosomes; suppressor tRNAs; and translation factors, such as EF-Tu and EF-P, that are able to enhance the formation of such chemical bonds would be important.

Genetic code reprogramming has enabled the ribosomal synthesis of random peptide libraries containing diverse kinds of npAAs. Since npAAs provide beneficial characteristics to the resulting peptides, such as peptidase resistance, structural rigidity, binding affinity, and membrane permeability, the application of random peptide/protein libraries containing npAAs to display-based screening methods is a promising approach for developing de novo bioactive peptides and proteins. To date, libraries bearing L-amino acids with noncanonical side chains, *N*-methyl-α-amino acids, D-α-amino acids, and β-amino acids have been developed by means of the FIT system and used for screening against proteins of interest in the RaPID system. Because of the nearly infinite combinations of npAAs that can be assigned to the genetic code, peptide libraries can be expanded further to produce extraordinary numbers of de novo bioactive peptides in the future.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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