MODIFICATION OF tRNA T-STEM FOR ENHANCING N-METHYL AMINO ACID INCORPORATION

Technical Field

[0001] The present invention relates to the modification of the T-stem of tRNA for enhancing the incorporation of an N-methyl amino acid.

Background Art

[0002] Peptides as middle molecules have high affinity and selectivity for target proteins having large surface recognition properties (Non-Patent Documents 1 to 3), so that they have attracted great attention as a new drug scaffold. It has been revealed that among them, naturally-occurring physiologically active peptides often have a plurality of backbone N-methyl modifications and such N-methylation enhances membrane permeability and protease resistance on peptide molecules (Non-Patent Documents 1 to 11).

In order to find novel peptide-based drugs having such ideal drug pharmacokinetic properties, it is ideal to develop a non-standard peptide library containing abundant, multiple, and respectively-different N-methyl amino acids (^{Me}AA) and thereby further improve the drug pharmacokinetic properties.

However, the ^{Me}AA is not the most suited substrate for a translation mechanism and this becomes a practical obstacle. It may cause early termination of the translation or incorporation of an inaccurate amino acid in place of a desired ^{Me}AA and deteriorate the incorporation efficiency and accuracy of N-methylated peptide synthesis (Non-Patent Documents 12 to 14). Non-Patent Document 14 discloses that only 5 ^{Me}AAs, out of 19 ^{Me}AAs, show an efficient incorporation efficiency (>80% to a proteinogenic amino acid (pAA) controls); 6 ^{Me}AAs are moderate (10 to 80%); and the remaining 8 ^{Me}AAs are poor and undetectable (<10%).

Some measures for improving the incorporation efficiency of a single ^{Me}AA in a model peptide are reported (Non-Patent Documents 12, 15, and 16), but it is still

difficult to simultaneously incorporate a plurality of different ^{Me}AAs in one peptide.

Citation List

Non-Patent Documents

[0003] Non-Patent Document 1: Passioura, T., Katoh, T., Goto, Y. & Suga, H. Selection-based discovery of druglike macrocyclic peptides. Annu. Rev. Biochem. 83, 727-752, doi:10.1146/annurev-biochem-060713-035456 (2014).

Non-Patent Document 2: Driggers, E. M., Hale, S. P., Lee, J. & Terrett, N. K. The exploration of macrocycles for drug discovery-an underexploited structural class. Nat. Rev. Drug Discov. 7, 608-624, doi:10.1038/nrd2590 (2008).

Non-Patent Document 3: Koehn, F. E. & Carter, G. T. The evolving role of natural products in drug discovery. Nat. Rev. Drug Discov. 4, 206-220, doi:10.1038/nrd1657 (2005).

Non-Patent Document 4: Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J. & Speicher, D. W. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science 226, 544-547 (1984).

Non-Patent Document 5: Takahashi, N., Hayano, T. & Suzuki, M. Peptidyl-Prolyl Cis-Trans Isomerase Is the Cyclosporin-a-Binding Protein Cyclophilin. Nature 337, 473-475, doi:DOI 10.1038/337473a0 (1989).

Non-Patent Document 6: Walsh, C. T., Zydowsky, L. D. & McKeon, F. D. Cyclosporin A, the cyclophilin class of peptidylprolyl isomerases, and blockade of T cell signal transduction. J. Biol. Chem. 267, 13115-13118 (1992).

Non-Patent Document 7: Altschuh, D., Vix, O., Rees, B. & Thierry, J. C. A conformation of cyclosporin A in aqueous environment revealed by the X-ray structure of a cyclosporin-Fab complex. Science 256, 92-94 (1992).

Non-Patent Document 8: Conradi, R. A., Hilgers, A. R., Ho, N. F. & Burton, P. S. The influence of peptide structure on transport across Caco-2 cells. II. Peptide bond modification which results in improved permeability. Pharm. Res. 9, 435-439 (1992).

Non-Patent Document 9: Haviv, F. et al. Effect of N-methyl substitution of the peptide

bonds in luteinizing hormone-releasing hormone agonists. J. Med. Chem. 36, 363-369 (1993).

Non-Patent Document 10: Chikhale, E. G., Ng, K. Y., Burton, P. S. & Borchardt, R. T. Hydrogen bonding potential as a determinant of the in vitro and in situ blood-brain barrier permeability of peptides. Pharm. Res. 11, 412-419 (1994).

Non-Patent Document 11: Miller, S. M. et al. Comparison of the Proteolytic Susceptibilities of Homologous L-Amino-Acid, D-Amino-Acid, and N-Substituted Glycine Peptide and Peptoid Oligomers. Drug Dev. Res. 35, 20-32, doi:DOI 10.1002/ddr.430350105 (1995).

Non-Patent Document 12: Roberts, R. W. & Szostak, J. W. RNA-peptide fusions for the in vitro selection of peptides and proteins. Proceedings of the National Academy of Sciences of the United States of America 94, 12297-12302 (1997).

Non-Patent Document 13: Murakami, H., Ohta, A., Ashigai, H. & Suga, H. A highly flexible tRNA acylation method for non-natural polypeptide synthesis. Nat. Methods 3, 357-359, doi:10.1038/nmeth877 (2006).

Non-Patent Document 14: Ohta, A., Murakami, H., Higashimura, E. & Suga, H. Synthesis of polyester by means of genetic code reprogramming. Chem. Biol. 14, 1315-1322, doi:10.1016/j.chembiol.2007.10.015 (2007).

Non-Patent Document 15: Goto, Y. et al. Reprogramming the translation initiation for the synthesis of physiologically stable cyclic peptides. ACS chemical biology 3, 120-129, doi:10.1021/cb700233t (2008).

Non-Patent Document 16: Goto, Y., Murakami, H. & Suga, H. Initiating translation with D-amino acids. RNA 14, 1390-1398, doi:10.1261/rna.1020708 (2008).

Summary

Technical Problem

[0004] The technical problem to be dissolved by the present invention is to develop a peptide synthesis method that enables the discovery of a highly N-methylated peptide having improved membrane permeability and improved stability

to proteolysis and the simultaneous incorporation of a plurality of different N-methyl amino acids.

Solution to Problem

[0005] As a result of keen researches, the present inventors have found that the aforesaid technical problem can be dissolved by paying attention to the T-stem of an aminoacyl-tRNA.

[0006] The present invention is disclosed in the following statements.

(1) A tRNA having a T-stem in which $N_1N_2GGN_3$ and N_4CCN_5U form a pair and encoding an N-methyl amino acid.

(N_1 is G or A; one of N_2 and N_5 is G and the other one is C; and N_3 and N_4 are any amino acids forming a pair).

Disclosed herein is a tRNA having a T-stem in which $N_1N_2GGN_3$ and N_4CCN_5U form a pair and encoding an N-methyl amino acid;

wherein N_1 is G or A; one of N_2 and N_5 is G and the other one is C; and N_3 and N_4 are any bases forming a pair.

(2) A translation system containing the tRNA as described in (1) and an EF-Tu protein.

(3) A method of producing a peptide library, including a step of translating with a cell-free translation system by using the tRNA as described in (1) or the translation system as described in (2).

(4) A method of producing a complex library of a peptide and an mRNA encoding the peptide, including a step of translating with a cell-free translation system by using the tRNA as described in (1) or the translation system as described in (3).

(5) The production method as described in (3) or (4), including a step of preparing the tRNA as described in (1) or the translation system as described in (2), wherein in the preparation step, which tRNA is charged, a tRNA having a T-stem in which $N_1N_2GGN_3$ and N_4CCN_5U form a pair or a tRNA other than the tRNA having a

T-stem in which $N_1N_2GGN_3$ and N_4CCN_5U form a pair is determined prior to charging with an N-methyl amino acid.

(6) The production method as described in (5) including a step of preparing the tRNA described in (1) or the translation system described in (2), wherein in the preparation step, charging of a tRNA having a T-stem in which $AGGGN_3$ and N_4CCCU form a pair and a tRNA having a T-stem in which $GCGGN_3$ and N_4CCGU form a pair, or a tRNA other than the said tRNAs is determined prior to charging with an N-methyl amino acid.

(7) The production method described in (5) further including a step of preparing the tRNA as described in (1) or the translation system as described in (2), wherein in the preparation step, charging of a tRNA having $AGGGG(N_4)_mCCCCU$ (SEQ ID NO: 1) and a tRNA having $GCGGG(N_4)_mCCCGU$ (SEQ ID NO: 2), or a tRNA other than the said tRNAs is determined prior to charging with an N-methyl amino acid.

(8) The production method as described in any of (3) to (8), wherein a tRNA having, as an affinity between a tRNA charged with an N-methyl amino acid and EF-Tu, ΔG (kcal/mol) of -7.5 to -10 is selected and the tRNA thus selected is charged with the N-methyl amino acid.

(9) A peptide library produced by the method as described in any of (3) and (5) to (8) or a peptide-mRNA complex library produced by the method as described in any of (4) to (8).

(10) The peptide library or peptide-mRNA complex library as described in (9), including a peptide containing two or more N-methyl amino acids in one peptide structure.

Advantageous Effects of Invention

[0007] The present invention makes it possible to provide a peptide synthesis method that enables the discovery of a highly N-methylated peptide and the simultaneous incorporation of a plurality of different N-methyl amino acids.

Brief Description of Drawings

[0008] Fig. 1 is a schematic view showing the interaction between EF-Tu and aminoacyl-tRNA. Fig. 1a shows the EF-Tu-mediated delivery, to a ribosome, of protein-constituting amino acid (proteinogenic amino acid: pAA)-tRNA. Fig. 1b schematically shows a hypothetical hindrance to the N-methyl amino acid (^{Me}AA)-tRNA interaction. It means insufficient EF-Tu affinity under competition with pAA-tRNA. Fig. 1c is a schematic view of the concept in the present invention. Fig. 1d shows used N-methyl amino acids, and they are as follows: ^{Me}G: N-methylglycine, ^{Me}S: N-methylserine, ^{Me}A: N-methylalanine, ^{Me}F: N-methylphenylalanine, ^{Me}L: N-methylleucine, ^{Me}M: N-methylmethionine, ^{Me}T: N-methylthreonine, ^{Me}Y: N-methyltyrosine, ^{Me}D: N-methylaspartic acid, ^{Me}V: N-methylvaline, ^{Me}Nl: N-methylnorleucine, ^{Me}Nv:N-methylnorvaline, ^{Me}Ym: N-methyl-p-methoxyphenylalanine, and ^{Ac}K: ε-N-acetyllysine.

Fig. 2 shows the correlation between weak EF-Tu affinity of ^{Me}AA-tRNA and inaccurate synthesis of ^{Me}AA-containing peptides. Fig. 2a shows the EF-Tu affinity of a standard pAA-tRNA. The error bars represent the standard deviation. Fig. 2b shows weak EF-Tu affinity of many ^{Me}AA-tRNA$^{AsnE2}_{GAC}$s and ^{Ac}K-tRNA$^{AsnE2}_{GAC}$. Fig. 2c shows a cause of the weak EF-Tu affinity of ^{Me}AA-tRNA. This drawing was made from the co-crystal structure (PDB ID:1TTT) of EF-Tu and Phe-tRNA^{Phe} (Reference Literature 1). Fig. 2d shows the results of the translation accuracy of peptides containing ^{Me}AA or ^{Ac}K. In each experiment, mRNA12 containing a GUC codon was translated using a FIT system containing a npAA-tRNA$^{AsnE2}_{GAC}$ of interest. The † peak corresponds to a byproduct containing Ile in place of a nonproteinogenic amino acid (non-proteinogenic amino acid: npAA).

Fig. 3 shows the reinforcement of EF-Tu affinity of an aminoacyl-tRNA by a T-stem substitution strategy. Fig. 3a shows a strategy for reinforcing the weak EF-Tu affinity of ^{Me}AA-tRNA. The T-stem sequence of ^{Me}AA-tRNA was replaced by three different sequences having respectively-different EF-Tu affinities. The original T-stem was renamed as T-stem No. 2 and the newly prepared T-stems were named as

T-stems Nos. 1, 3, and 4. Their EF-Tu affinities are supposed to increase with an increase in the number. Figs. 3b and 3c show the quantification of the EF-Tu affinity of Phe-tRNA$^{AsnE2}_{GAC}$ (b) and ^{Me}F-tRNA$^{AsnE2}_{GAC}$ (c) having different T-stem sequences, respectively. The asterisk shows that the affinity is at a not-detectable level ($\Delta G > -6$ kcal/mol). The error bars represent the standard deviation. In Fig. 3c, an uncharged tRNA was used as a control.

Fig. 4 shows that the translation accuracy and efficiency are improved by selecting an appropriate T-stem. Figs. 4a and 4b show the MALDI-TOF-MS of peptide products after a translation reaction for 15 minutes. The GUC codon was translated by Phe-tRNA$^{AsnE2}_{GAC}$ (a) and ^{Me}F-tRNA$^{AsnE2}_{GAC}$ (b) having different T-stem sequences, respectively. The † peak corresponds to byproducts containing Ile in place of Phe (a) and ^{Me}F (b), respectively. Figs. 4c and 4d respectively show time course of the peptide expression levels in the FIT systems containing Phe-tRNA$^{AsnE2}_{GAC}$ (c) and ^{Me}F-tRNA$^{AsnE2}_{GAC}$ (d).

Fig. 5 shows accurate and efficient synthesis of model peptides containing 6 respectively-different ^{Me}AAs under the optimum conditions. Fig. 5a shows the outline of EF-Tu affinity tuning. Fig. 5b shows the adjustment of the concentration of each of ^{Me}AA-tRNAs based on the flexizyme-catalyzed acylation efficiency. Fig. 5c shows the respective sequences of mRNA13 and its resultant P13 peptide. The arrow shows the relation between sequences. Fig. 5d shows a reprogrammed gene encoding 15 pAAs and 6 ^{Me}AAs. Figs. 5e and 5f show a comparison between the conventional system and the optimized system based on the analysis of a peptide product by tricine-SDS-PAGE (e) and MALDI-TOF-MS (f).

Fig. 6 shows the expression of a highly N-methylated peptide containing both 15 pAAs and 9 respectively-different ^{Me}AAs. Fig. 6a shows the respective sequences of mRNA14 and its resultant P14 peptide. The CUC, UUG, GUC, CUG, AUC, GCG, UUC, GUG, and GCC codons were translated by ^{Me}S-tRNA$^{AsnE2TS5}_{GAG}$, ^{Me}G-tRNA$^{AsnE2TS5}_{CAA}$, ^{Me}V-tRNA$^{AsnE2TS1}_{GAC}$, ^{Me}NI-tRNA$^{AsnE2TS1}_{CAG}$, ^{Me}Y-

$tRNA^{AsnE2TS3}_{GAU}$, $^{Me}A\text{-}tRNA^{AsnE2TS3}_{CGC}$, $^{Me}Ym\text{-}tRNA^{AsnE2TS1}_{GAA}$, $^{Me}Nv\text{-}tRNA^{AsnE2TS3}_{CAC}$, and $^{Me}F\text{-}tRNA^{AsnE2TS3}_{GGC}$, respectively. Fig. 6b shows a reprogrammed gene encoding 9 ^{Me}AAs and 15 pAAs. Fig. 6c shows the outline of EF-Tu affinity tuning. Fig. 6d and Fig. 6e show the expression of P14 peptides containing 9 different ^{Me}AAs. The mRNA was translated by either a FIT system containing 20 pAAs (as a control) or a FIT system containing 15 pAAs (except Phe, Leu, Ile, Val, and Ala) and 9 ^{Me}AA-tRNAs. The peptide products were analyzed by MALDI-TOF-MS (d) and tricine-SDS-PAGE (e).

Fig. 7 shows the confirmation results of the byproduct in Fig. 2d.

Fig. 8 shows the results of a control experiment for confirming that an uncharged tRNA does not bind to EF-Tu.

Fig. 9 shows the translation accuracy improved by reinforcing the EF-Tu affinity of ^{Me}AA-tRNA and ^{Ac}K-tRNA. Fig. 9a shows the respective sequences of mRNA12 and its resultant P12-npAA peptide. The GUC codon was translated by the intended npAA-$tRNA^{AsnE2}_{GAC}$. Figs. 9b to 9f are the results of studying the effect of $^{Me}G\text{-}tRNA^{AsnE2}_{GAC}$ (b), $^{Me}S\text{-}tRNA^{AsnE2}_{GAC}$ (c), $^{Me}A\text{-}tRNA^{AsnE2}_{GAC}$ (d), $^{Me}L\text{-}tRNA^{AsnE2}_{GAC}$ (e), and $^{Ac}K\text{-}tRNA^{AsnE2}_{GAC}$ (f) having respectively-different T-stem sequences on EF-Tu affinity and translation accuracy. The asterisk shows that the affinity is at a not-detectable level ($\Delta G > -6$ kcal/mol). The error bars represent the standard deviation. The † peak corresponds to a byproduct containing Ile in place of npAA.

Fig. 10 shows the reinforcement of the EF-Tu affinity for other ^{Me}AA-tRNAs. Fig. 10a to Fig. 10h respectively show the EF-Tu affinity of $^{Me}M\text{-}tRNA^{AsnE2}_{GAC}$ (a), $^{Me}T\text{-}tRNA^{AsnE2}_{GAC}$ (b), $^{Me}Y\text{-}tRNA^{AsnE2}_{GAC}$ (c), $^{Me}D\text{-}tRNA^{AsnE2}_{GAC}$ (d), $^{Me}V\text{-}tRNA^{AsnE2}_{GAC}$ (e), $^{Me}Nv\text{-}tRNA^{AsnE2}_{GAC}$ (f), $^{Me}Nl\text{-}tRNA^{AsnE2}_{GAC}$ (g), and $^{Me}Ym\text{-}tRNA^{AsnE2}_{GAC}$ (h) containing different T-stem sequences. The asterisk shows that the affinity is at a not-detectable level ($\Delta G > -6$ kcal/mol). The error bars represent the standard deviation.

Description of Embodiments

[0009] The tRNA of the present invention is a tRNA having a T-stem in which $N_1N_2GGN_3$ and N_4CCN_5U form a pair and encoding an N-methyl amino acid.

In the $N_1N_2GGN_3$ and N_4CCN_5U forming the T-stem, N_1 is G or A; one of N_2 and N_5 is G and the other one is C; and N_3 and N_4 are any amino acids forming a pair.

[0010] The present inventors assumed that the inefficient incorporation of an N-methyl amino acid occurs because of the following reasons. A ribosome incorporates an aminoacyl-tRNA, which is a tRNA charged with an amino acid, into a ribosome A site by the catalytic action of EF-Tu (Elongation Factor Thermo unstable) (Fig. 1a). The EF-Tu binds to the aminoacyl-tRNA at two different sites, that is, an amino acid site and a T-stem site. When only the interaction of the former one at the amino acid site is considered, the binding affinity differs largely depending on the kind of the amino acid. In order to complement the difference in affinity, the T-stem structure has evolved in nature so that the EF-Tu and each of 20 aminoacyl-tRNAs have equivalent affinities. Due to these equivalent affinities, EF-Tu can deliver all the 20 proteinogenic aminoacyl-tRNAs (pAA-tRNAs) to a ribosome at a similar efficiency (Fig. 1b). In contrast, if the affinity between EF-Tu and N-methylaminoacyl-tRNA (^{Me}AA-tRNA) is weak, the ^{Me}AA-tRNA cannot bind to EF-Tu under competition with the pAA-tRNA, leading to a reduction in synthesis efficiency of a ^{Me}AA-containing peptide. The present inventors have thought that if this assumption is an actual cause for the observed inefficiency, the tuning of EF-Tu affinity of ^{Me}AA-tRNA may improve the translation efficiency and various ^{Me}AAs can be incorporated in one peptide simultaneously.

In the present invention, it was experimentally confirmed that most of the ^{Me}AA-tRNAs had affinity smaller than the average affinity of pAA-tRNAs and therefore, a thought that the ^{Me}AA-tRNA cannot bind to EF-Tu with sufficient intensity was supported (Fig. 1c). Next, a method of tuning the weak EF-Tu affinity of ^{Me}AA-tRNAs to the average affinity of pAA-tRNAs was developed (Fig. 1c). The

optimized FIT system thus obtained showed improvement in the synthesis efficiency and accuracy of N-methylated peptides. Based on the aforesaid results, the new method of the present invention enables the synthesis of a library of highly N-methylated peptides and is expected to promote the future discovery of practical and non-standard peptide drugs having improved pharmacokinetic properties.

[0011] The other structures in the tRNA of the present invention, that is, acceptor stem, anticodon stem, anticodon loop, variable loop and D-arm may have any base sequence. Of these, the anticodon loop should have a base sequence corresponding to a codon to which an N-methyl amino acid is assigned.

The tRNA has a CCA sequence at the 3' end thereof and binds to an arbitrary amino acid. The tRNA of the present invention binds to an N-methyl amino acid.

The term "tRNA encodes an N-methyl amino acid" as used herein means that the tRNA binds to the N-methyl amino acid at the CCA-end of the tRNA.

[0012] In the T-stem in the tRNA of the present invention, any base may be selected as N_1 to N_5 insofar as $N_1N_2GGN_3$ and N_4CCN_5U form a base pair, but particularly in the present invention, N_1 is G or A, one of N_2 and N_5 is G and the other one is C, and N_3 and N_4 are any amino acids forming a pair.

The term "one of N_2 and N_5 is G and the other one is C" as used herein means that when N_2 is G, N_5 is C and when N_2 is C, N_5 is G.

The term "N_3 and N_4 are any amino acids forming a pair" means that N_3 and N_4 may independently be any base and N_3 and N_4 are required to be a base pair.

The $N_1N_2GGN_3$ and N_4CCN_5U forming a T-stem are preferably AN_2GGN_3 and N_4CCN_5U or GN_2GGN_3 and N_4CCN_5U, more preferably $AGGGN_3$ and N_4CCCU or $GCGGN_3$ and N_4CCGU, respectively.

The T-stem forms base pairs to form a T-loop and a T-arm.

The N_3 and N_4 are a base pair and a preferred embodiment of the T-stem in the present invention, that is, a base pair on the T-stem on which AGGG forms a base pair with CCCU and GCGG forms a base pair with CCGU, is shown as Fig. 3a.

[0013] The base sequence of the T-loop is not particularly limited insofar as it forms a loop and examples include the base sequence represented by SEQ ID NO: 3.

UUCGAAU (SEQ ID NO: 3)

The base sequence represented by SEQ ID NO: 3 forms a T-loop in tRNA and binds to $N_1N_2GGN_3$ and N_4CCN_5U forming a T-stem.

When the base sequence represented by SEQ ID NO: 3 binds to the T-stem, it may bind thereto via another base.

In the base sequence represented by SEQ ID NO: 3, one or more than one bases may be substituted, deleted, or inserted.

The term “more than one bases may be substituted” means that in the 7 bases forming the T-loop of the base sequence of SEQ ID NO: 1, 2, 3, 4, 5, 6, or 7 bases may be substituted. In the 7 bases forming the T-loop of the base sequence of SEQ ID NO: 1, 1 to 4 bases may be substituted, 1 to 3 bases may be substituted, 1 to 2 bases may be substituted, or 1 base may be substituted.

The term “more than one bases may be deleted” means that in the 7 bases forming the T-loop of the base sequence of SEQ ID NO: 1, 2, 3, 4, 5, 6, or 7 bases may be deleted. In the 7 bases forming the T-loop of the base sequence of SEQ ID NO: 1, 1 to 4 bases may be deleted, 1 to 3 bases may be deleted, 1 to 2 bases may be deleted, or 1 base may be deleted.

The term “more than one bases may be inserted” means that in the 7 bases forming the T-loop of the base sequence of SEQ ID NO: 1, 1 to 4 bases may be inserted, 1 to 3 bases may be inserted, 1 to 2 bases may be inserted, or 1 base may be inserted.

The base sequence represented by SEQ ID NO: 3 may be a base sequence having 70% or more, 80% or more, 85% or more, 90% or more, or 95% or more homology with this base sequence.

[0014] The tRNA of the present invention has a T-stem in which $N_1N_2GGN_3$ and

N_4CCN_5U form a pair and preferably, it further has a T-loop having a base sequence represented by SEQ ID NO: 3. It is to be noted that in the base sequence represented by SEQ ID NO: 3, one or more than one bases may be substituted, deleted, or inserted and it may be a base sequence having 70% or more, 80% or more, 85% or more, 90% or more, or 95% or more homology with this base sequence.

The tRNA of the present invention preferably has a T-arm having a base sequence represented by SEQ ID NO: 4, more preferably has a T-arm having a base sequence represented by SEQ ID NO: 1 or SEQ ID NO: 2, still more preferably has a T-arm having a base sequence represented by SEQ ID NO: 5 or SEQ ID NO: 6.

$N_1N_2GGN_3(N_6)_mN_4CCN_5U$ (SEQ ID NO: 4)

$AGGGG(N_6)_mCCCCU$ (SEQ ID NO: 1)

$GCGGG(N_6)_mCCCGU$ (SEQ ID NO: 2)

AGGGGUUCGAAUCCCCU (SEQ ID NO: 5)

GCGGGUUCGAAUCCCGU (SEQ ID NO: 6)

In SEQ ID NOS: 1, 2, and 4, N_1 to N_5 have the same meanings as described above, N_6 is an arbitrary base, and m is an integer of 1 or more. It is to be noted that $(N_6)_m$ is preferably a base sequence constituting a T-loop.

In SEQ ID NO: 4, $N_1N_2GGN_3$ and N_4CCN_5U may be the aforesaid preferable base sequences, respectively.

In the base sequence represented by SEQ ID NOS: 1, 2, and 4 to 6, one or more than one bases may be substituted, deleted, or inserted. Preferably, in the base sequence constituting the T-loop, one or more than one bases may be substituted, deleted, or inserted.

The term “in the base sequence represented by SEQ ID NOS: 1, 2, and 4 to 6, one or more than one bases may be substituted” means that in the base sequence forming the T-arm and T-loop having the base sequence represented by SEQ ID NOS: 1, 2, and 4 to 6, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 bases may be deleted, 1 to 10, 1

to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2 bases may be substituted, or 1 base may be substituted.

The term “in the base sequence represented by SEQ ID NOS: 1, 2, and 4 to 6, one or more than one bases may be deleted” means that in the bases forming the T-arm and T-loop of the base sequence represented by SEQ ID NOS: 1, 2, and 4 to 6, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 bases may be deleted, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2 bases may be deleted, or 1 base may be deleted.

The term “in the base sequence represented by SEQ ID NOS: 1, 2, and 4 to 6, one or more than one bases may be inserted” means that in the bases forming the T-arm and T-loop of the base sequence represented by SEQ ID NOS: 1, 2, and 4 to 6, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 bases may be inserted, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, or 1 to 2 bases may be inserted, or 1 base may be inserted.

However, when one or more than one bases are substituted, deleted, or inserted in the base sequence represented by SEQ ID NOS: 1, 2, and 4 to 6, it is preferred that no base is substituted, deleted, or inserted in the base sequence $N_1N_2GGN_3$ and N_4CCN_3U of the T-arm. In other words, the base sequence of the T-loop portion preferably has substitution, deletion, or insertion.

[0015] In SEQ ID NOS: 1, 2, and 4, m is not particularly limited insofar as $(N_6)_m$ can form a T-loop and it may be an integer of 2, 3, 4, 5, 6, 7, 8, 9, or 10, preferably an integer of 5 to 9, more preferably an integer of 6 to 8. It is still more preferred that the $(N_6)_m$ is a base sequence represented by SEQ ID NO: 3. The $(N_6)_m$ may be a sequence containing the base sequence represented by SEQ ID NO: 3. For example, the $(N_6)_m$ may be $(N_7)_{m1}UUCGAAU(N_8)_{m2}$, in which m1 and m2 may be independently an integer of 0 to 5 or may be an integer of 0 to 4, 0 to 3, 0 to 2, 0 to 1, 1, or 0. The N_7 and N_8 may be any base insofar as they do not hinder the T-loop structure formed by the base sequence represented by SEQ ID NO: 3 and may form a T-loop with the base sequence represented by SEQ ID NO: 3. The base

sequence represented by SEQ ID NO: 3 may form a T-loop, independently of $(N_7)_{m1}$ and $(N_8)_{m2}$, or with some bases.

[0016] In the tRNA used in the present invention, the base sequence other than that of the T-arm may be derived from a wild type tRNA, may be derived from an Escherichia coli-derived wild type tRNA, or may be an artificial tRNA prepared by in vitro transcription.

The tRNA in the present invention may not have a D-arm which $tRNA^{Pro1E1}$ or $tRNA^{Pro1E2}$ has. Specifically, the tRNA in the present invention may have a D-arm having a base sequence different from the base sequence of the D-arm of a tRNA known as $tRNA^{Pro1E2(CGG)}$, $tRNA^{Pro1E2(GAU)}$, $tRNA^{Pro1E2(GGU)}$, or $tRNA^{Pro1E2(GUG)}$. More specifically, it is presumed that the tRNA of the present invention does not have a D-stem in which GCGC and CGCG form a pair. Generally, it may not have a D-arm in which RNAs include a base sequence represented by SEQ ID NO: 134.

$N_9N_{10}GCN_{11}N_{12}N_{13}N_{14}N_{15}N_{16}N_{17}N_{18}N_{19}GCN_{20}N_{21}$ (SEQ ID NO: 134)

(in SEQ ID NO: 134, N_9 to N_{21} are each an arbitrary base, N_{11} to N_{19} form a D-loop, and $N_9N_{10}GC$ forms a base pair with $N_{21}N_{20}CG$).

The tRNA of the present invention may not have a D-arm which $tRNA^{GluE2(CGG)}$ has. Specifically, the tRNA of the present invention may have a D-arm having a base sequence different from the base sequence of the D-arm of a tRNA known as $tRNA^{GluE2(CGG)}$, $tRNA^{Pro1E2(GAU)}$, $tRNA^{Pro1E2(GGU)}$, or $tRNA^{Pro1E2(GUG)}$. More specifically, it is presumed that the tRNA of the present invention may not have a D-stem in which GCGC and CGCG form a pair. Generally, it may not have a D-arm in which RNAs include a base sequence represented by SEQ ID NO: 134. The base sequence represented by SEQ ID NO: 134 may alternatively be

$N_9N_{10}GCGCAGCCUGGUAGCGCN_{20}N_{21}$ (SEQ ID NO: 135)

(in SEQ ID NO: 135, N_9, N_{10}, N_{29}, and N_{21} have the same meanings as described above and it is a D-stem in which $N_9N_{10}GC$ and $N_{21}N_{20}CG$ form a base pair) or GCGCGCAGCCUGGUAGCGCGC (SEQ ID NO: 136).

The tRNA of the present invention may not have the base sequence which $tRNA^{Pro1E2(CGG)}$, $tRNA^{Pro1E2(GAU)}$, $tRNA^{Pro1E2(GGU)}$, $tRNA^{Pro1E2(GUG)}$ and $tRNA^{GluE2(CGG)}$ have.

The base sequences of these tRNAs are disclosed in WO2019/077887.

[0017] In the present invention, the translation system contains the tRNA of the present invention and the translation system including the tRNA enables the synthesis of a peptide having consecutive N-methyl amino acids which generally have difficulty in consecutive introduction. More specifically, it becomes a translation system capable of synthesizing a peptide having 2 or more consecutive N-methyl amino acids. It is also possible to prepare a peptide library with a translation system using a codon table to which 2 or more, preferably 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, or 9 or more N-methyl amino acids are simultaneously assigned.

The translation system is not particularly limited insofar as it contains the tRNA of the present invention and it contains constituents used in a cell-free translation system.

The translation system preferably contains an EF-Tu protein. In addition, it preferably contains other constituents used in a conventionally known cell-free translation system.

The translation system of the present invention contains the tRNA of the present invention and preferably contains a tRNA to be charged with a proteinogenic amino acid. The tRNA to be charged with a proteinogenic amino acid is more preferably a naturally occurring tRNA and has, as a T-arm, the T-arm of a naturally occurring tRNA present in nature. Particularly, the tRNA charged with a proteinogenic amino acid is preferably a naturally occurring tRNA charged therewith. The naturally occurring tRNA charged therewith may be a tRNA present in a natural translation system. This means that when a tRNA not subjected to artificial alteration before charging is charged with an amino acid, the tRNA charged with the

amino acid has the T-arm of a naturally occurring tRNA.

When a proteinogenic amino acid is not reconstituted in the codon table, a tRNA present in a natural translation system and charged with the proteinogenic amino acid may be used. When a proteinogenic amino acid is reconstituted in the codon table, a tRNA present in a natural translation system and corresponding to an assigned codon may be charged with the proteinogenic amino acid.

The term “charge” or “charged” as used herein has the same meaning as “bind” or “bound” or “combine” or “combined”, respectively. Therefore, “a tRNA charged with a proteinogenic amino acid” means a tRNA acylated with a proteinogenic amino acid and has the same meaning as a tRNA having an “aminoacylated” proteinogenic amino acid.

The translation system of the present invention may contain a plurality of the tRNAs of the present invention. It may contain a plurality of the tRNAs of the present invention different in N-methyl amino acids to be charged (in this case, corresponding to codons assigned to N-methyl amino acids) or may contain a plurality of the tRNAs different in T-arm.

Described specifically, the translation system may contain a plurality of tRNA selected from tRNAs having a T-arm having a base sequence represented by SEQ ID NO: 4. When the translation system contains a plurality of tRNAs having a T-arm having a base sequence represented by SEQ ID NO: 4, they may be two or more selected from the aforesaid preferable base sequences. For example, when two tRNAs having a combination of the base sequences of a T-arm, that is, $N_1N_2GGN_3$ and N_4CCN_5U as the base sequence represented by SEQ ID NO: 4 are used, examples include a combination of AN_2GGN_3 and N_4CCN_5U with GN_2GGN_3 and N_4CCN_5U and a combination of $AGGGN_3$ and N_4CCCU with $GCGGN_3$ and N_4CCGU.

More specifically, the translation system of the present invention may contain a tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1 and/or a tRNA having a T-arm having a base sequence represented by SEQ ID NO: 2; or

may contain a tRNA having a T-arm having a base sequence represented by SEQ ID NO: 5 and/or a tRNA having a T-arm having a base sequence represented by SEQ ID NO: 6.

In the present invention, the translation system may contain a tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1 and a tRNA having a T-arm having a base sequence represented by SEQ ID NO: 2; or may contain a tRNA having a T-arm having a base sequence represented by SEQ ID NO: 5 and a tRNA having a T-arm having a base sequence represented by SEQ ID NO: 6. In the translation system of the present invention, when the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1 is compared with the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 2, the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 2 has higher EF-Tu affinity, so that it is preferred to charge the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 2 with an N-methyl amino acid, which has difficulty in introduction into a peptide.

As the translation system of the present invention, the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1 and the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 2 may be charged with an N-methyl amino acid as needed, in consideration of the EF-Tu affinity.

When a tRNA aminoacylated with an N-methyl amino acid has EF-Tu affinity without using the tRNA of the present invention having a T-stem, the naturally occurring tRNA charged with an amino acid corresponding to the N-methyl amino acid (for example, an amino acid corresponding to N-methylglycine is glycine) may be charged. Alternatively, a naturally occurring tRNA corresponding to a codon to which N-methyl glycine is assigned may be charged. Examples of such an N-methyl amino acid include N-methylglycine and N-methylserine. The tRNA of the present invention may be charged with N-methylglycine or N-methylserine, but in the translation system of the present invention, N-methylglycine or N-methylserine is not

necessarily charged to the tRNA of the present invention.

[0018] In the present invention, to obtain the translation system of the present invention, a tRNA having a T-stem in which $N_1N_2GGN_3$ and N_4CCN_5U form a pair is charged with an N-methyl amino acid to prepare the tRNA of the present invention charged with an N-methyl amino acid.

Also in the present invention, in the translation in the present invention, that is, a method of producing a peptide library or a method of producing a library of a complex between a peptide and an mRNA encoding the peptide, a translation system including the tRNA of the present invention is prepared. The translation system preferably contains an EF-Tu protein.

The present invention includes a step of translating in a cell-free translation system using the tRNA or translation system of the present invention and it preferably includes a step of preparing the translation system of the present invention before translating in the cell-free translation system.

In the step of preparing the translation system of the present invention, a translation system for translating in a cell-free translation system is prepared. In the present invention, in the preparation step, it is preferred to determine, prior to charging with an N-methyl amino acid, which tRNA is charged: a tRNA having a T-stem in which $AGGGN_3$ and N_4CCCU form a pair, a tRNA having a T-stem in which $GCGGN_3$ and N_4CCGU form a pair, or a tRNA other than the aforesaid ones.

The N-methyl amino acid with which is charged to a tRNA is incorporated in an amino acid sequence of a peptide by translating the charged product in a cell-free translation system.

For efficient translation, it is preferred that the EF-Tu affinity of the tRNA charged with an N-methyl amino acid is equivalent to the EF-Tu affinity of a tRNA charged with another amino acid (including an N-methyl amino acid).

In the preparation step, therefore, it is preferred to determine, prior to charging with an N-methyl amino acid, which tRNA is charged, a tRNA having a T-stem in

which $AGGGN_3$ and N_4CCCU form a pair, a tRNA having a T-stem in which $GCGGN_3$ and N_4CCGU form a pair, or a tRNA other than the aforesaid ones, in consideration of the EF-Tu affinity.

The tRNAs charged with an amino acid (including an N-methyl amino acid) prepared in consideration of the EF-Tu affinity preferably have equivalent incorporation efficiencies when they are incorporated in an amino acid sequence of a peptide.

In the preparation step, it is preferred to select, as a tRNA to be charged with an N-methyl amino acid, a tRNA having $AGGGG(N_4)_mCCCCU$ (SEQ ID NO: 1), a tRNA having $GCGGG(N_4)_mCCCGU$ (SEQ ID NO: 2), or a tRNA other than the aforesaid ones.

[0019] The selection from the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1, the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 2 and a naturally occurring tRNA is preferably performed so that the EF-Tu affinity falls within a specific range.

As an index of the EF-Tu affinity of a tRNA, ΔG (kcal/mol) is preferably in a range of -7.5 to -10 and within this range, the upper limit is preferably -8 or less and the lower limit is preferably -9.5 or more, more preferably -9 or more.

The ΔG (kcal/mol) is preferably in a range of -7.5 to -10 and it may be -8 to -10, -7.5 to -9.5, -7.5 to -9, -8 to -9.5 or -8 to -9.

[0020] In the translation system of the present invention, reassignment of a codon using a flexizyme is performed by assigning an N-methyl amino acid to a codon selected as needed as NNN.

In the translation system of the present invention, a natural translation system may be used. In the natural translation system, tRNAs having an anticodon corresponding to each amino acid exist and these tRNAs have inherent sequences in a region other than an anticodon loop, respectively.

In the present invention, if the base sequence of the T-stem of the present

invention is introduced, the tRNA may apply sequences inherent to tRNAs in the natural translation system at the other sequences, so that a base sequence of an acceptor stem, an anticodon stem, an anticodon loop and a variable loop may be arbitrary.

[0021] In the translation system of the present invention, a flexizyme is used to reassign an arbitrary amino acid to all the NNNs (N is an arbitrary base) and in this case, all the tRNAs may be artificial or a combination of naturally occurring and artificial ones. In this case, 85% or more or 90% or more of the full length of respective elongator tRNAs corresponding to the NNNs to be added to the translation system may be composed of the same base sequence; or an elongator tRNA group having the same sequence as most of the sequences except an anticodon may be used.

The term “anticodon loop” as used herein means an anticodon-containing single-stranded loop portion of a tRNA. The sequence of the anticodon loop can be determined as needed by those skilled in the art to complement the codon-anticodon interaction.

[0022] The present invention provides a method of producing a peptide library including:

a step of preparing an mRNA library containing mRNAs encoding the respective peptides of the peptide library, the mRNAs each containing NNNs encoding one or more than one, preferably more than one N-methyl amino acids; and

a step of translating the mRNAs of the mRNA library in a cell-free translation system which contains a tRNA having an anticodon to any codon of the NNNs and charged with an N-methyl amino acid corresponding to the codon.

The translation system is preferably a translation system for which the tRNA having a T-stem or T-arm according to the present invention is selected, as described above.

Described specifically, the cell-free translation system is preferably obtained by charging, with an N-methyl amino acid, any of the naturally occurring tRNA, the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1 and the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 2. The naturally occurring tRNA may be charged with a proteinogenic amino acid and the translation system preferably contains the tRNA charged with an N-methyl amino acid and having a T-arm having a base sequence represented by SEQ ID NO: 1 and/or the tRNA charged with an N-methyl amino acid and having a T-arm having a base sequence represented by SEQ ID NO: 2, more preferably contains both of these tRNAs.

[0023] In the present invention, N-methylalanine, N-methylglycine, and N-methylserine are preferably charged to the naturally occurring tRNA; N-methylalanine may be charged to the tRNA of the present invention. It may be charged to the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1 or may be charged to the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 5.

Examples of the N-methyl amino acid which may be charged to the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1 or the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 5 include N-methyl amino acids other than N-methylglycine and N-methylserine.

An N-methylated unnatural amino acid may be charged to the tRNA of the present invention.

Examples of N-methyl amino acids which may be charged to the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 2 or the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 6 include N-methyl amino acids which are not charged to tRNAs other than the tRNA of the present invention and may be charged to the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1 or the tRNA having a T-arm having a base sequence

represented by SEQ ID NO: 5. Although not particularly limited, N-methylvaline may be charged among them.

N-methyl amino acids other than N-methylvaline, N-methylglycine and N-methylserine may be charged to the tRNA having a T-arm having a base sequence represented by SEQ ID NO: 1, 2, 5 or 6. Examples of such an N-methyl amino acid include N-methyl amino acids listed in Fig. 1d. The tRNA charged with ^{Ac}K in place of the N-methyl amino acid may be used in the translation system of the present invention.

[0024] In the present invention, the method of producing a peptide-mRNA complex library is performed by, in preparing an mRNA library by the aforesaid method of producing a peptide library, binding puromycin to the downstream region of the ORF (Open reading frame) of each mRNA. The puromycin may be bound to the mRNA via a linker composed of a peptide or a nucleic acid. When puromycin is bound to the ORF downstream region of the mRNA, a ribosome which has translated the ORF of the mRNA incorporates puromycin therein to form an mRNA-peptide complex. Such a peptide-mRNA complex can associate a genotype with a phenotype and can be applied to the in vitro display.

[0025] In the present invention, first, an NNN to which an N-methyl amino acid is assigned is selected and then, a tRNA having an anticodon of the NNN in an anticodon loop and encoding an N-methyl amino acid is prepared with a flexizyme or the like.

Then, by preparing an mRNA library of mRNAs each containing an NNN encoding one or more than one N-methyl amino acids and performing translation, peptides containing the assigned N-methyl amino acid can be expressed.

[0026] The term “NNN” as used herein means a codon designating an amino acid and three Ns forming the codon are independently selected from adenine (A), guanine (G), cytosine (C) and uracil (U). One mRNA may contain a plurality of NNNs encoding an N-methyl amino acid.

[0027] In the present invention, an arbitrary amino acid may be reassigned to an NNN not encoding an N-methyl amino acid, and a relation between a codon and an amino acid based on a natural genetic code may be applied.

In the reassignment, an amino acid different from the relation between a codon and an amino acid in the natural genetic code table may be assigned or the same one may be assigned.

The term “natural genetic code table” is a table showing amino acids assigned to the triplet (codon) of an mRNA in a living body. The table is shown below in Table 1.

[0028]

[Table 1]

Base of second letter →

Base of first letter ↓	U		C		A		G		Base of third letter ↓
	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	
U	UUU	Phenylalanine	UCU	Serine	UAU	Tyrosine	UGU	Cysteine	U
	UUC	Phenylalanine	UCC	Serine	UAC	Tyrosine	UGC	Cysteine	C
	UUA	Leucine	UCA	Serine	UAA	Stop	UGA	Stop	A
	UUG	Leucine	UCG	Serine	UAG	Stop	UGG	Tryptophan	G
C	CUU	Leucine	CCU	Proline	CAU	Histidine	CGU	Arginine	U
	CUC	Leucine	CCC	Proline	CAC	Histidine	CGC	Arginine	C
	CUA	Leucine	CCA	Proline	CAA	Glutamine	CGA	Arginine	A
	CUG	Leucine	CCG	Proline	CAG	Glutamine	CGG	Arginine	G
A	AUU	Isoleucine	ACU	Threonine	AAU	Asparagine	AGU	Serine	U
	AUC	Isoleucine	ACC	Threonine	AAC	Asparagine	AGC	Serine	C
	AUA	Isoleucine	ACA	Threonine	AAA	Lysine	AGA	Arginine	A
	AUG	Methionine	ACG	Threonine	AAG	Lysine	AGG	Arginine	G
G	GUU	Valine	GCU	Alanine	GAU	Aspartic acid	GGU	Glycine	U
	GUC	Valine	GCC	Alanine	GAC	Aspartic acid	GGC	Glycine	C
	GUA	Valine	GCA	Alanine	GAA	Glutamic acid	GGA	Glycine	A
	GUG	Valine	GCG	Alanine	GAG	Glutamic acid	GGG	Glycine	G

[0029] Assignment, to each codon, of an amino acid different from that in the natural genetic code table is achieved, for example, by codon reassignment making use of an artificially amino acylation RNA catalyst, Flexizyme. Flexizyme can make a desired amino acid bound to a tRNA having an arbitrary anticodon, so that an arbitrary amino acid can be assigned to an arbitrary codon.

[0030] The term “amino acid” as used herein includes an artificial amino acid variant or derivative as well as a proteinogenic amino acid and examples include proteinogenic L-amino acids and chemically synthesized compounds having

properties known in the art as characteristics of an amino acid.

The “proteinogenic amino acids” (proteinogenic amino acids) when expressed by a three-letter code known in the art are Arg, His, Lys, Asp, Glu, Ser, Thr, Asn, Gln, Cys, Gly, Pro, Ala, Ile, Leu, Met, Phe, Trp, Tyr and Val.

Non-proteinogenic amino acids (non-proteinogenic amino acids) other than an N-methyl amino acid may be used and the term “non-proteinogenic amino acids” means a natural or unnatural amino acid other than the proteinogenic amino acid.

Examples of the unnatural amino acid include α,α-disubstituted amino acids (such as α-methylalanine), N-alkyl-α-amino acids, D-amino acids, β-amino acids and α-hydroxy acids, each having a main chain structure different from that of natural amino acids; amino acids (such as norleucine and homohistidine) having a side-chain structure different from that of natural amino acids; amino acids (such as “homo”amino acids, homophenylalanine and homohistidine) having extra methylene in the side chain thereof; and amino acids (such as cysteic acid) obtained by substituting a carboxylic acid functional group in the side chain thereof by a sulfonic acid group. Specific examples of the unnatural amino acid include amino acids described in WO2015/030014.

The non-proteinogenic amino acid is preferably a D-amino acid, a β-amino acid or an α,α-disubstituted amino acid, more preferably a D-amino acid or a β-amino acid.

[0031] In the present invention, a peptide library containing 1×10^6 or more peptides can be produced.

The number of amino acids contained in each peptide and encoded by NNN is not particularly limited insofar as an N-methyl amino acid is contained and examples of the number may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 and 30. It may be 1 to 10. The position of the NNN encoding a non-proteinogenic amino acid in each mRNA is not particularly limited. By using the tRNA of the present invention, a peptide having consecutive non-proteinogenic amino acids is translated also from an mRNA

in which NNNs encoding a non-proteinogenic amino acid are consecutively placed, in the same level as the library synthesis of proteinogenic amino acids.

[0032] For codon reassignment, a translation system obtained by arbitrarily removing constituent factors of the translation system according to purpose and reconstituting only necessary components may be used. For example, when a translation system from which a specific amino acid has been removed is reconstituted, a codon corresponding to the amino acid becomes an empty codon that does not encode any amino acid. When an arbitrary amino acid is combined with a tRNA having an anticodon complementary to the empty codon by making use of a flexizyme or the like and translation is performed by adding it, the arbitrary amino acid is encoded by the codon and a peptide having the arbitrary amino acid introduced instead of the removed amino acid is translated.

[0033] The term “cell-free translation system” as used herein means a translation system not containing cells. As the cell-free translation system, for example, an Escherichia coli extract, a wheat germ extract, a rabbit reticulocyte extract or an insect cell extract may be used. Alternatively, a reconstituted cell-free translation system may be used, which is obtained by reconstituting a purified ribosome protein, aminoacyl-tRNA synthase (aaRS), ribosomal RNA, amino acid, rRNA, GTP, ATP, translation initiation factor (IF), elongation factor (EF), release factor (RF) and ribosome regeneration factor (RRF), and another factor necessary for translation.

The system may contain RNA polymerase for simultaneously performing transcription from DNA. Examples of a commercially available cell-free translation system include Escherichia-coli derived systems such as “RTS-100“ (trade mark) of Roche Diagnostics, reconstituted translation systems such as “PURESYSTEM” (trade mark) of PGI and “PURExpressR In Vitro Protein Synthesis Kit” of New England BioLabs and systems using a wheat germ extract such as those of ZOEGENE Corporation or CellFree Sciences.

As a system using a ribosome of Escherichia coli, for example, a technology

described in the following documents is known: H. F. Kung et al., 1977. The Journal of Biological Chemistry Vol. 252, No. 19, 6889-6894; M. C. Gonza et al., 1985, Proceeding of National Academy of Sciences of the United States of America Vol. 82, 1648-1652; M. Y. Pavlov and M. Ehrenberg, 1996, Archives of Biochemistry and Biophysics Vol. 328, No. 1, 9-16; Y. Shimizu et al., 2001, Nature Biotechnology Vol. 19, No. 8, 751-755; and H. Ohashi et al., 2007, Biochemical and Biophysical Research Communications Vol. 352, No. 1, 270-276.

By using the cell-free translation system, a high-purity expression product can be obtained without purifying.

The cell-free translation system of the present invention may be used not only for translation, but also for transcription after adding a factor necessary for transcription.

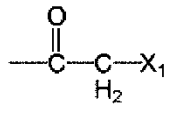
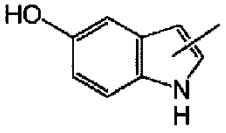
[0034] The peptide obtained in the present invention may be a cyclic peptide and it may be, for example, a cyclic peptide in which an amino acid having Functional group 1 and an amino acid having Functional group 2 corresponding thereto shown below in Table 2 are cyclized.

Either Functional group 1 or Functional group 2 may be placed on the N-terminal side; they may be placed at the N terminal and C terminal sides, respectively; one may be a terminal amino acid and the other one may be a non-terminal amino acid; or both groups may be a non-terminal amino acid.

The bond formed by Functional group 1 and Functional group 2 may be a chemically crosslinked structure for forming a molecular ring structure in a cyclic peptide.

[0035]

[Table 2]

	Functional group1	Functional group2
(A)	 (A−1)	HS— (A−2)
(B)	—C≡C—H (B−1)	N_3— (B−2)
(C)	—Ar—CH_2NH_2 (C−1)	 (C−2)
(D)	—C≡C—CH_2—X_1 (D−1)	HS— (D−2)
(E)	—Ar—CH_2—X_1 (E−1)	HS— (E−2)

In the formula, X_1 is a leaving group, for example, the leaving group is a halogen atom such as Cl, Br, or I; and Ar is an aromatic ring which may have a substituent.

[0036] As an amino acid having Functional group (A-1), for example, a chloroacetylated amino acid can be used. Examples of the chloroacetylated amino acid include N-chloroacetyl-L-alanine, N-chloroacetyl-L-phenylalanine, N-chloroacetyl-L-tyrosine, N-chloroacetyl-L-tryptophan, N-3-(2-chloroacetamido)benzoyl-L-phenylalanine, N-3-(2-chloroacetamido)benzoyl-L-tyrosine, N-3-(2-chloroacetamido)benzoyl-L-tryptophan, β-N-chloroacetyl-L-diaminopropanoic acid, γ-N-chloroacetyl-L-diaminobutyric acid, δ-N-chloroacetyl-L-ornithine, and ε-N-chloroacetyl-L-lysine, and D-amino acid derivatives corresponding thereto.

As the amino acid having Functional group (A-1), N-chloroacetyl-L-tryptophan and N-chloroacetyl-L-tyrosine are preferred, with D-forms being more preferred.

The present specification sometimes clearly describes that the amino acid used is an L-form, but it may be either an L-form or a D-form, or it may be a mixture of an L-form and a D-form mixed at any ratio. Even when the present specification does not clearly describe that the amino acid used is an L-form or a D-form, it means that it may be either an L-form or a D-form, or it may be a mixture of an L-form and a D-form at any ratio.

[0037] Examples of an amino acid having Functional group (A-2) include cysteine, homocysteine, mercaptonorvaline, mercaptonorleucine, 2-amino-7-mercaptoheptanoic acid, and 2-amino-8-mercaptooctanoic acid.

As the amino acid having Functional group (A-2), cysteine is preferred.

[0038] Examples of a cyclization method with the amino acid having Functional group (A-1) and the amino acid having Functional group (A-2) include methods described in Kawakami, T. et al., Nature Chemical Biology 5, 888-890 (2009); Yamagishi, Y. et al., ChemBioChem 10, 1469-1472 (2009); Sako, Y. et al., Journal of American Chemical Society 130, 7932-7934 (2008); Goto, Y. et al., ACS Chemical Biology 3, 120-129 (2008); Kawakami T. et al., Chemistry & Biology 15, 32-42 (2008); and WO2008/117833.

[0039] Examples of an amino acid having Functional group (B-1) include propargylglycine, homopropargylglycine, 2-amino-6-heptynoic acid, 2-amino-7-octynoic acid, and 2-amino-8-nonynoic acid.

A 4-pentynoylated or 5-hexynoylated amino acid may also be used.

Examples of the 4-pentynoylated amino acid include N-(4-pentenoyl)-L-alanine, N-(4-pentenoyl)-L-phenylalanine, N-(4-pentenoyl)-L-tyrosine, N-(4-pentenoyl)-L-tryptophan, N-3-(4-pentynoylamido)benzoyl-L-phenylalanine, N-3-(4-pentynoylamido)benzoyl-L-tyrosine, N-3-(4-pentynoylamido)benzoyl-L-tryptophan, β-N-(4-pentenoyl)-L-diaminopropanoic acid, γ-N-(4-pentenoyl)-L-diaminobutyric acid, σ-N-(4-pentenoyl)-L-ornithine and ε-N-(4-pentenoyl)-L-lysine, and D-amino acid derivatives corresponding thereto.

Examples of the 5-hexynoylated amino acid include amino acids obtained by substituting the 4-pentynoyl group of the compounds mentioned above as the 4-pentynoylated amino acids by a 5-hexynoyl group.

[0040] Examples of an amino acid having Functional group (B-2) include azidoalanine, 2-amino-4-azidobutanoic acid, azidoptonorvaline, azidonorleucine, 2-amino-7-azidoheptanoic acid, and 2-amino-8-azidooctanoic acid.

In addition, azidoacetylated or 3-azidopentanoylated amino acids can also be used.

Examples of the azidoacetylated amino acids include N-azidoacetyl-L-alanine, N-azidoacetyl-L-phenylalanine, N-azidoacetyl-L-tyrosine, N-azidoacetyl-L-tryptophan, N-3-(4-pentynoylamido)benzoyl-L-phenylalanine, N-3-(4-pentynoylamido)benzoyl-L-tyrosine, N-3-(4-pentynoylamido)benzoyl-L-tryptophan, β-N-azidoacetyl-L-diaminopropanoic acid, γ-N-azidoacetyl-L-diaminobutyric acid, σ-N-azidoacetyl-L-ornithine and ε-N-azidoacetyl-L-lysine, and D-amino acid derivatives corresponding thereto.

Examples of the 3-azidopentanoylated amino acids include amino acids obtained by substituting the azidoacetyl group of the compounds mentioned above as the azidoacetylated amino acids by a 3-azidopentanoyl group.

[0041] Examples of a cyclization method with the amino acid having Functional group (B-1) and the amino acid having Functional group (B-2) include those described in Sako, Y. et al., Journal of American Chemical Society 130, 7932-7934 (2008) and WO2008/117833.

[0042] Examples of an amino acid having Functional group (C-1) include N-(4-aminomethyl-benzoyl)-phenylalanine (AMBF) and 3-aminomethyltyrosine.

Examples of an amino acid having Functional group (C-2) include 5-hydroxytryptophan (WOH).

Examples of a cyclization method with the amino acid having Functional group (C-1) and the amino acid having Functional group (C-2) include those described in Yamagishi, Y. et al., ChemBioChem 10, 1469-1472 (2009) and WO2008/117833.

[0043] Examples of an amino acid having Functional group (D-1) include 2-amino-6-chloro-hexynoic acid, 2-amino-7-chloro-heptynoic acid and 2-amino-8-chloro-octynoic acid.

Examples of an amino acid having Functional group (D-2) include cysteine, homocysteine, mercaptonorvaline, mercaptonorleucine, 2-amino-7-

mercaptoheptanoic acid and 2-amino-8- mercaptooctanoic acid.

Examples of a cyclization method with the amino acid having Functional group (D-1) and the amino acid having Functional group (D-2) include that described in WO2012/074129.

[0044] Examples of an amino acid (E-1) include N-3-chloromethylbenzoyl-L-phenylalanine, N-3-chloromethylbenzoyl-L-tyrosine and N-3-chloromethylbenzoyl-L-tryptophane, and D-amino acid derivatives corresponding thereto.

Examples of an amino acid (E-2) include cysteine, homocysteine, mercaptonorvaline, mercaptonorleucine, 2-amino-7-mercaptoheptanoic acid and 2-amino-8- mercaptooctanoic acid.

A cyclization method with the amino acid having Functional group (E-1) and the amino acid having Functional group (E-2) can be carried out with reference to, for example, the cyclization method with (A-1) and (A-2) or the cyclization method with (D-1) and (D-2).

[0045] As a ring-forming amino acid, a combination of the amino acid having Functional group (A-1) with the amino acid having Functional group (A-2) is preferred; a combination of N-acetyltryptophan whose H has been substituted by a leaving group with cysteine is more preferred; and a combination of an N-haloacetyl-D-tyrosine or an N-haloacetyl-D-tryptophan, preferably N-chloroacetyl-D-tyrosine or N-chloroacetyl-D-tryptophan with cysteine (Cys) is still more preferred.

[0046] The present invention also provides a peptide library or a peptide-mRNA complex library.

Producing a peptide library with the tRNA of the present invention enables the synthesis of a peptide containing an N-methyl amino acid at an expression level equal to that of a peptide composed of an L-amino acid, so that a peptide library more abundant in variety in the N-methyl amino acid introduction in one peptide than a conventional peptide library can be formed.

Particularly, a library containing a peptide containing two or more N-methyl

amino acids can be formed.

Producing a peptide-mRNA complex library with the tRNA of the present invention enables the synthesis of a peptide containing an N-methyl amino acid at an expression level equal to that of a peptide composed of an L-amino acid, so that a peptide-mRNA complex library more abundant in variety in the N-methyl amino acid introduction than a conventional peptide-mRNA complex library can be formed.

[0047] The present invention also provides a screening method for identifying a peptide which binds to a target substance by using the peptide library produced with the tRNA of the present invention.

The screening method includes a step of bringing the peptide library produced with the tRNA of the present invention into contact with a target substance and incubating the resulting mixture.

The target substance is not particularly limited and examples include low molecular compounds, high molecular compounds, nucleic acids, peptides, proteins, sugars and lipids.

The screening method further includes a step of selecting a peptide bound to the target substance. The selection of a peptide bound to the target substance is performed, for example, by labeling the peptide to be detectable by a known method, washing the surface of a solid phase carrier with a buffer after the aforesaid contact step, and then detecting a compound bound to the target substance.

Examples of the detectable label include enzymes such as peroxidase and alkaline phosphatase, radioactive substances such as 125I, 131I, 35S and 3H, fluorescent substances such as fluorescein isothiocyanate, rhodamine, dansyl chloride, phycoerythrin, tetramethyl rhodamine isothiocyanate and near infrared fluorescent materials, light-emitting substances such as luciferase, luciferin and aequorin, and nanoparticles such as gold colloid and quantum dot. When the enzyme is used, detection can be achieved by adding a substrate of the enzyme to develop a color. Detection can also be achieved by binding biotin to a peptide and

then binding avidin or streptavidin labeled with the enzyme or the like to the biotin-bound peptide.

[0048] Screening using the peptide-mRNA complex library can be carried out by applying the TRAP display method.

In this case, after the peptide-mRNA complex library is subjected to a reverse transcription reaction, the resulting library is brought into contact with a target substance. A complex that binds to the target substance is selected and its DNA is amplified by PCR. By adding the resulting DNA to a TRAP reaction system, a peptide-mRNA complex library is formed again. Then, a similar operation is repeated.

Since a peptide-mRNA complex having high affinity for the target substance is concentrated by the aforesaid operation, a peptide that binds to the target substance can be identified efficiently by analyzing the DNA sequence of the concentrated complex.

[0049] Disclosure of all the Non-Patent Documents and Reference Literatures cited herein is incorporated herein by reference in their entirety.

Examples

[0050] The present invention will hereinafter be described specifically based on Examples but the present invention is not limited to them. The present invention can be changed into various modes by those skilled in the art without departing from the significance of the present invention and such a change is also embraced within the scope of the present invention.

[0051]

Preparation of tRNA, flexizyme, mDNA and mRNA

The oligonucleotides listed in Table 3 were all purchased from Eurofins Genomics (Japan). In Table 3, G(Me) means 2'-O-methylguanosine. The sequence of a tRNA transcript prepared in the present Example are shown in Table 4. Double-stranded DNA templates encoding tRNAs were prepared by primer

extension and then, PCR in the following manner: appropriate Forward primer and Reverse primer (each, 1 μM, refer to Table 5 as to the primers used) were mixed in 100 μL of a PCR mixture (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, each dNTP, 0.1% (v/v) Triton X-100, and 45 nM Taq DNA polymerase). The primer extension was performed by denaturation (at 95°C for 1 minute), 5 cycles of annealing (at 50°C for 1 minute), and extension (at 72°C for 1 minute). After 1 μL of the reaction mixture thus obtained was diluted 200-fold with 199 μL of a PCR mixture containing appropriate Forward and Reverse primers (each, 0.5 μM, refer to Table 5 as to the primers used) and PCR was performed by 12 cycles of denaturation (at 95°C for 40 seconds), annealing (at 50°C for 40 seconds), and extension (at 72°C for 40 seconds). The amplification of the resulting PCR product was confirmed by 3% agarose gel electrophoresis and ethidium bromide staining. The DNA thus obtained was purified by phenol/chloroform extraction and ethanol precipitation and then, the purified product was dissolved in 20 μL of water. The concentration of the tRNA was determined using A_{260} of a 10-fold diluted solution.

The transcription reaction was performed by incubating 200 μL of a transcription mixture (40 mM Tris-HCl (pH 8.0), 1 mM spermidine, 0.01% (v/v) Triton X-100, 10 mM DTT, 22.5 mM $MgCl_2$, each NTP, 5 mM GMP, 22.5 mM KOH, 10% (v/v) DNA template, and 120 nM T7 RNA polymerase) overnight at 37°C. The transcription mixture was mixed with $MnCl_2$ (100 mM, 4 μL) and RQ1 RNase-free DNase (1 U/μL, 1 μL, Promega) and the resulting mixture was incubated at 37°C for 30 minutes. The tRNA transcript thus obtained was precipitated in isopropanol and then, dissolved in water. The tRNA transcript was purified by 8% denaturing PAGE and ethanol precipitation and was then dissolved in 10 μL of water.

Flexizymes (dFx and eFx) were prepared in accordance with the method described in Non-Patent Document 13 and Reference Literature 2. ADDIN EN. CITE ADDIN EN.CITE.DATA

Double stranded DNA templates encoding mRNAs (refer to Table 6 as to the

mDNA and mRNA sequences) were prepared by primer extension and PCR (refer to Table 7 as to the primers used). The mDNAs thus obtained were purified by phenol/chloroform extraction and ethanol precipitation and the purified product was then dissolved in 10 μL of water. The concentration was determined by 8% native PAGE and ethidium bromide staining with 100 bp Quick-Load DNA Ladders (New England BioLabs) as a control.

An mRNA was prepared by in vitro transcription as follows. A transcription mixture (200 μL) (40 mM Tris-HCl (pH 8.0), 1 mM spermidine, 0.01% (v/v) Triton X-100, 10 mM DTT, 30 mM $MgCl_2$, 5 mM each NTPs, 30 mM KOH, 10% (v/v) DNA template, and 120 nM T7 RNA polymerase) was incubated overnight at 37°C. The resulting transcription mixture was mixed with $MnCl_2$ (100 mM, 4 μL) and RQ1 RNase-free DNase (1 U/μL, 1 μL, Promega) and the resulting mixture was incubated at 37°C for 30 minutes. The resulting mRNA was precipitated by isopropanol and then dissolved in water. The tRNA transcript was purified by 8% denaturing PAGE and ethanol precipitation and then dissolved in 10 μL of water. The concentration of the mRNA was determined using A_{260} of a 10-fold diluted solution.

[0052]

[Table 3]

Name	Sequence
O-197	5'-TAATACGACTCACTATAGCCCGGATAGCTCAGTCGGTAGAGCAGGGGACTGAAAATCCCCGTGTCC-3'
O-198	5'-GTGCCCGGACTCGGAATCGAACCAAGGACACGGGGATTTTCA-3'
O-199	5'-GGCGTAATACGACTCACTATAG-3'
O-200	5'-GG(Me)TGCCCGGACTCGG-3'
O-201	5'-TG(Me)GTGCCCGGACTCGG-3'
O-202	5'-GGCGTAATACGACTCACTATAGGTGGGGTTCCCGAGCGGCCAAAGGGAGCAGACTGTAAATCTGCCG-3'
O-203	5'-GGTGGTGGGGGAAGGATTCGAACCTTCGAAGTCTGTGACGGCAGATTTACAGTCTG-3'
O-204	5'-GG(Me)TGGTGGGGGAAGGAT-3'
O-205	5'-TG(Me)GTGGTGGGGGAAGGAT-3'
O-206	5'-GGCGTAATACGACTCACTATAGGAGAGATGCCGGAGCGGCTGAACGGACCGGTCTCGAAAACCGGAG-3'
O-207	5'-GGCGGAGAGAGGGGGATTTGAACCCCCGGTAGAGTTGCCCCTACTCCGGTTTTCGAGACC-3'
O-208	5'-GG(Me)CGGAGAGAGGGGGA-3'
O-209	5'-TG(Me)GCGGAGAGAGGGGGA-3'
O-210	5'-GTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGG-3'
O-211	5'-GCGGCTCTGCAATGACTCGAACACTGGACATACGGATTGTCAGTCCGCCGTTCTACCG-3'
O-212	5'-GG(Me)CGGCTCTGCAATG-3'
O-213	5'-GCGGCTCTGACTGGACTCGAACCAGTGACATACGGATTGTCAGTCCGCCGTTCTACCG-3'
O-214	5'-GG(Me)CGGCTCTGACTGG-3'
O-215	5'-GCGGCTCTGAGGGGACTCGAACCCCTGACATACGGATTGTCAGTCCGCCGTTCTACCG-3'
O-216	5'-GG(Me)CGGCTCTGAGGGG-3'
O-217	5'-GCGGCTCTGACGGGACTCGAACCCGCGACATACGGATTGTCAGTCCGCCGTTCTACCG-3'
O-218	5'-GG(Me)CGGCTCTGACGGG-3'
O-219	5'-TG(Me)GCGGCTCTGCAATG-3'
O-220	5'-TG(Me)GCGGCTCTGACTGG-3'
O-221	5'-TG(Me)GCGGCTCTGAGGGG-3'
O-222	5'-TG(Me)GCGGCTCTGACGGG-3'
O-223	5'-GTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGAAGAACGGCGG-3'
O-224	5'-GCGGCTCTGCAATGACTCGAACACTGGACATACGGATTTTCAGTCCGCCGTTCTTCCG-3'
O-225	5'-GCGGCTCTGACTGGACTCGAACCAGTGACATACGGATTTTCAGTCCGCCGTTCTTCCG-3'
O-226	5'-GCGGCTCTGAGGGGACTCGAACCCCTGACATACGGATTTTCAGTCCGCCGTTCTTCCG-3'
O-227	5'-GCGGCTCTGACGGGACTCGAACCCGCGACATACGGATTTTCAGTCCGCCGTTCTACCG-3'
O-228	5'-GCGGCTCTGCAATGACTCGAACACTGGACATACGGATTTTGAGTCCGCCGTTCTACCG-3'
O-229	5'-GCGGCTCTGACTGGACTCGAACCAGTGACATACGGATTTTGAGTCCGCCGTTCTACCG-3'
O-230	5'-GCGGCTCTGAGGGGACTCGAACCCCTGACATACGGATTTTGAGTCCGCCGTTCTACCG-3'
O-231	5'-GCGGCTCTGACGGGACTCGAACCCGCGACATACGGATTTTGAGTCCGCCGTTCTACCG-3'
O-232	5'-GCGGCTCTGCAATGACTCGAACACTGGACATACGGAACCTCAATCCGCCGTTCTACCG-3'
O-233	5'-GCGGCTCTGACTGGACTCGAACCAGTGACATACGGAACCTCAATCCGCCGTTCTACCG-3'
O-234	5'-GCGGCTCTGAGGGGACTCGAACCCCTGACATACGGAACCTCAATCCGCCGTTCTACCG-3'
O-235	5'-GCGGCTCTGACGGGACTCGAACCCGCGACATACGGAACCTCAATCCGCCGTTCTACCG-3'
O-236	5'-GCGGCTCTGCAATGACTCGAACACTGGACATACGGAACCTGAATCCGCCGTTCTACCG-3'
O-237	5'-GCGGCTCTGACTGGACTCGAACCAGTGACATACGGAACCTGAATCCGCCGTTCTACCG-3'
O-238	5'-GCGGCTCTGAGGGGACTCGAACCCCTGACATACGGAACCTGAATCCGCCGTTCTACCG-3'
O-239	5'-GCGGCTCTGACGGGACTCGAACCCGCGACATACGGAACCTGAATCCGCCGTTCTACCG-3'
O-240	5'-GCGGCTCTGCAATGACTCGAACACTGGACATACGGATTATCAGTCCGCCGTTCTACCG-3'
O-241	5'-GCGGCTCTGACTGGACTCGAACCAGTGACATACGGATTATCAGTCCGCCGTTCTACCG-3'
O-242	5'-GCGGCTCTGAGGGGACTCGAACCCCTGACATACGGATTATCAGTCCGCCGTTCTACCG-3'
O-243	5'-GCGGCTCTGACGGGACTCGAACCCGCGACATACGGATTATCAGTCCGCCGTTCTACCG-3'
O-244	5'-GCGGCTCTGCAATGACTCGAACACTGGACATACGGAGTGTGAGTCCGCCGTTCTACCG-3'
O-245	5'-GCGGCTCTGACTGGACTCGAACCAGTGACATACGGAGTGTGAGTCCGCCGTTCTACCG-3'
O-246	5'-GCGGCTCTGAGGGGACTCGAACCCCTGACATACGGAGTGTGAGTCCGCCGTTCTACCG-3'
O-247	5'-GCGGCTCTGACGGGACTCGAACCCGCGACATACGGAGTGTGAGTCCGCCGTTCTACCG-3'
O-248	5'-GCGGCTCTGCAATGACTCGAACACTGGACATACGGACTGCCAGTCCGCCGTTCTACCG-3'
O-249	5'-GCGGCTCTGACTGGACTCGAACCAGTGACATACGGACTGCCAGTCCGCCGTTCTACCG-3'
O-250	5'-GCGGCTCTGAGGGGACTCGAACCCCTGACATACGGACTGCCAGTCCGCCGTTCTACCG-3'
O-251	5'-GCGGCTCTGACGGGACTCGAACCCGCGACATACGGACTGCCAGTCCGCCGTTCTACCG-3'
O-252	5'-GCGGCTCTGCAATGACTCGAACACTGGACATACGGATTGCGAATCCGCCGTTCTACCG-3'
O-253	5'-GCGGCTCTGACTGGACTCGAACCAGTGACATACGGATTGCGAATCCGCCGTTCTACCG-3'
O-254	5'-GCGGCTCTGAGGGGACTCGAACCCCTGACATACGGATTGCGAATCCGCCGTTCTACCG-3'
O-255	5'-GCGGCTCTGACGGGACTCGAACCCGCGACATACGGATTGCGAATCCGCCGTTCTACCG-3'
O-256	5'-GCGGCTCTGCAATGACTCGAACACCGGACATACGGATTGTCAGTCCGCCGTTCTACCG-3'
O-257	5'-GCGGCTCTGCAATGACTCGAACACCGGACATACGGATTTAGAGTCCGCCGTTCTACCG-3'
O-258	5'-TAATACGACTCACTATAGGGTTAACTTTAACAAGGAGAAAAACATGAAGAAGAAG-3'
O-259	5'-CGAAGCTTACTTGTCGTCGTCGTCCTTGTAGTCGTAGACCTTCTTCTTCATGTTTTTCTC-3'
O-260	5'-CGAAGCTTACTTGTCGTC-3'
O-261	5'-GAGAAAAACATGAAGAAGAAGACAAATTTCGGTATCCGTGCCTCTGTCATGGA-3'
O-262	5'-GTCGTCCTTGTAGTCGTATGGATGGAGCAGTTGCCATTCCATGACAGAGGCACG-3'
O-263	5'-CGAAGCTTACTTGTCGTCGTCGTCCTTGTAGTC-3'
O-264	5'-GAGAAAAACATGAAGAAGAAGCTCCATATGTTGGTCACAGAACTGTCTATCGCGCAAAA-3'
O-265	5'-GTCGTCCTTGTAGTCTGGGGCACGCACCCAACCGAAATTTTGCGCGATAGACAGT-3'
O-266	5'-TAATACGACTCACTATAGGGTTAACTTTAACAAGGAGAAAAACATGGACTACAAGGACGACGACGACAAG-3'
O-267	5'-CGAAGCTTACTTCTTCTTGATGAAGGCGACCAGGAGCTTGTCGTCGTCGTCCT-3'
O-268	5'-CGAAGCTTACTTCTTCTT-3'

[0053]

[Table 4]

tRNA	Anticodon	T-stem	tRNA sequence
Phe without 3'-A	GAA	-	5'-GCCCGGAUAGCUCAGUCGGUAGAGCAGGGGACUGAAAAUCCCCGUGUCCUUGGUUCGAUUCCGAGUCCGGGCACC-3'
Tyr without 3'-A	GUA	-	5'-GGUGGGGUUCCCGAGCGGCCAAAGGGAGCAGACUGUAAAAUCUGCCGUCACAGACUUCGAAGGUUCGAAUCCUUCCCCCACCAC-3'
Ser without 3'-A	CGA	-	5'-GGAGAGAUGCCGGAGCGGCUGAACGGACCGGUCUCGAAAACCGGAGUAGGGGCAACUCUACCGGGGGUUCAAAUCCCCCUCUCCGCC-3'
AsnE2 without 3'-A	GAC	No.1	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAC AUUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCC-3'
AsnE2 without 3'-A	GAC	No.2	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAC AUUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCC-3'
AsnE2 without 3'-A	GAC	No.3	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAC AUUCCGUAUGUCGCGGUUCGAGUCCCGUCAGAGCCGCC-3'
AsnE2 without 3'-A	GAC	No.4	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAC AUUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCC-3'
AsnE2	GAC	No.1	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAC AUUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCCA-3'
AsnE2	GAC	No.2	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAC AUUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCCA-3'
AsnE2	GAC	No.3	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAC AUUCCGUAUGUCAGGGGUUCGAGUCCCCUCAGAGCCGCCA-3'
AsnE2	GAC	No.4	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAC AUUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCCA-3'
AsnE2	GAA	No.1	5'-GGCUCUGUAGUUUCAGUCGGAAGAACGGCGGACU GAA AAUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCCA-3'
AsnE2	GAA	No.2	5'-GGCUCUGUAGUUUCAGUCGGAAGAACGGCGGACU GAA AAUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCCA-3'
AsnE2	GAA	No.3	5'-GGCUCUGUAGUUUCAGUCGGAAGAACGGCGGACU GAA AAUCCGUAUGUCAGGGGUUCGAGUCCCCUCAGAGCCGCCA-3'
AsnE2	GAA	No.4	5'-GGCUCUGUAGUUUCAGUCGGAAGAACGGCGGACU GAA AAUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCCA-3'
AsnE2	CAA	No.1	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAA AAUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCCA-3'
AsnE2	CAA	No.2	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAA AAUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCCA-3'
AsnE2	CAA	No.3	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAA AAUCCGUAUGUCAGGGGUUCGAGUCCCCUCAGAGCCGCCA-3'
AsnE2	CAA	No.4	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAA AAUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCCA-3'
AsnE2	GAG	No.1	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAG GUUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCCA-3'
AsnE2	GAG	No.2	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAG GUUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCCA-3'
AsnE2	GAG	No.3	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAG GUUCCGUAUGUCAGGGGUUCGAGUCCCCUCAGAGCCGCCA-3'
AsnE2	GAG	No.4	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAG GUUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCCA-3'
AsnE2	CAG	No.1	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU CAG GUUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCCA-3'
AsnE2	CAG	No.2	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU CAG GUUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCCA-3'
AsnE2	CAG	No.3	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU CAG GUUCCGUAUGUCAGGGGUUCGAGUCCCCUCAGAGCCGCCA-3'
AsnE2	CAG	No.4	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU CAG GUUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCCA-3'
AsnE2	GAU	No.1	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAU AAUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCCA-3'
AsnE2	GAU	No.2	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAU AAUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCCA-3'
AsnE2	GAU	No.3	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAU AAUCCGUAUGUCAGGGGUUCGAGUCCCCUCAGAGCCGCCA-3'
AsnE2	GAU	No.4	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAU AAUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCCA-3'
AsnE2	CAC	No.1	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAC ACUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCCA-3'
AsnE2	CAC	No.2	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAC ACUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCCA-3'
AsnE2	CAC	No.3	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAC ACUCCGUAUGUCAGGGGUUCGAGUCCCCUCAGAGCCGCCA-3'
AsnE2	CAC	No.4	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU CAC ACUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCCA-3'
AsnE2	GGC	No.1	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU GGC AGUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCCA-3'
AsnE2	GGC	No.2	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU GGC AGUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCCA-3'
AsnE2	GGC	No.3	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU GGC AGUCCGUAUGUCAGGGGUUCGAGUCCCCUCAGAGCCGCCA-3'
AsnE2	GGC	No.4	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGACU GGC AGUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCCA-3'
AsnE2	CGC	No.1	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU CGC AAUCCGUAUGUCCAGUGUUCGAGUCCGUCAUUGCAGAGCCGCCA-3'
AsnE2	CGC	No.2	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU CGC AAUCCGUAUGUCACUGGUUCGAGUCCGUCAGUCAGAGCCGCCA-3'
AsnE2	CGC	No.3	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU CGC AAUCCGUAUGUCAGGGGUUCGAGUCCCCUCAGAGCCGCCA-3'
AsnE2	CGC	No.4	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU CGC AAUCCGUAUGUCGCGGGUUCGAGUCCCGUCAGAGCCGCCA-3'
AsnE2	GAC	No.0	5'-GGCUCUGUAGUUUCAGUCGGUAGAACGGCGGAUU GAC AUUCCGUAUGUCCGGUUCGAGUCAUUGCAGAGCCGCCA-3'

[0054]

[Table 5]

tRNA	Anticodon	T-stem	Extension		PCR	
			Forward primer	Reverse primer	Forward primer	Reverse primer
Phe without 3'-A	GAA	Native	O-197	O-198	O-199	O-200
Phe	GAA	Native	O-197	O-198	O-199	O-201
Tyr without 3'-A	GUA	Native	O-202	O-203	O-199	O-204
Tyr	GUA	Native	O-202	O-203	O-199	O-205
Ser without 3'-A	CGA	Native	O-206	O-207	O-199	O-208
Ser	CGA	Native	O-206	O-207	O-199	O-209
AsnE2 without 3'-A	GAC	No.1	O-210	O-211	O-199	O-212
AsnE2 without 3'-A	GAC	No.2	O-210	O-213	O-199	O-214
AsnE2 without 3'-A	GAC	No.3	O-210	O-215	O-199	O-216
AsnE2 without 3'-A	GAC	No.4	O-210	O-217	O-199	O-218
AsnE2	GAC	No.1	O-210	O-211	O-199	O-219
AsnE2	GAC	No.2	O-210	O-210	O-199	O-220
AsnE2	GAC	No.3	O-210	O-212	O-199	O-221
AsnE2	GAC	No.4	O-210	O-214	O-199	O-222
AsnE2	GAA	No.1	O-223	O-224	O-199	O-219
AsnE2	GAA	No.2	O-223	O-225	O-199	O-220
AsnE2	GAA	No.3	O-223	O-226	O-199	O-221
AsnE2	GAA	No.4	O-223	O-227	O-199	O-222
AsnE2	CAA	No.1	O-210	O-228	O-199	O-219
AsnE2	CAA	No.2	O-210	O-229	O-199	O-220
AsnE2	CAA	No.3	O-210	O-230	O-199	O-221
AsnE2	CAA	No.4	O-210	O-231	O-199	O-222
AsnE2	GAG	No.1	O-210	O-232	O-199	O-219
AsnE2	GAG	No.2	O-210	O-233	O-199	O-220
AsnE2	GAG	No.3	O-210	O-234	O-199	O-221
AsnE2	GAG	No.4	O-210	O-235	O-199	O-222
AsnE2	CAG	No.1	O-210	O-236	O-199	O-219
AsnE2	CAG	No.2	O-210	O-237	O-199	O-220
AsnE2	CAG	No.3	O-210	O-238	O-199	O-221
AsnE2	CAG	No.4	O-210	O-239	O-199	O-222
AsnE2	GAU	No.1	O-210	O-240	O-199	O-219
AsnE2	GAU	No.2	O-210	O-241	O-199	O-220
AsnE2	GAU	No.3	O-210	O-242	O-199	O-221
AsnE2	GAU	No.4	O-210	O-243	O-199	O-222
AsnE2	CAC	No.1	O-210	O-244	O-199	O-219
AsnE2	CAC	No.2	O-210	O-245	O-199	O-220
AsnE2	CAC	No.3	O-210	O-246	O-199	O-221
AsnE2	CAC	No.4	O-210	O-247	O-199	O-222
AsnE2	GGC	No.1	O-210	O-248	O-199	O-219
AsnE2	GGC	No.2	O-210	O-249	O-199	O-220
AsnE2	GGC	No.3	O-210	O-250	O-199	O-221
AsnE2	GGC	No.4	O-210	O-251	O-199	O-222
AsnE2	CGC	No.1	O-210	O-252	O-199	O-219
AsnE2	CGC	No.2	O-210	O-253	O-199	O-220
AsnE2	CGC	No.3	O-210	O-254	O-199	O-221
AsnE2	CGC	No.4	O-210	O-255	O-199	O-222
AsnE2	GAC	No.0	O-210	O-256	O-199	O-219
Non-functional	CUA	No.0	O-210	O-257	O-199	O-219

[0055]

[Table 6]

Name	Corresponding mDNA	mRNA sequence
mRNA12	mDNA12	5'-GGGCUUUAAUAAGGAGAAAAACAUGAAGAAGGUCUACGACUACAAGGACGACGACAA GUAAGCUUCG-3'
mRNA13	mDNA13	5'-GGGCUUUAAUAAGGAGAAAAACAUGAAGAAGGACAAAUUUCGGUAUCCGUGCCUCUGUCAU GGAAUGGCAACUGCUCCAUCCAUACGACUACAAGGACGACGACAAGUAAGCUUCG-3'
mRNA14	mDNA14	5'-GGGCUUUAAUAAGGAGAAAAACAUGAAGAAGAAGCUCCAUAUGUUGGUCACAGAACUGUCUAU CGCGCAAAAUUUCGGUUGGGUGCGUGCCCCAGACUACAAGGACGACGACGACAAGUAAGCUUCG-3'

[0056]

[Table 7]

Name	Corresponding mRNA	Extension		1st PCR		2nd PCR	
		Forward primer	Reverse primer	Forward primer	Reverse primer	Forward primer	Reverse primer
mRNA12	mDNA12	O-258	O-259	O-199	O-260	-	-
mRNA13	mDNA13	O-261	O-262	O-258	O-263	O-199	O-260
mRNA14	mDNA14	O-264	O-265	O-258	O-263	O-199	O-260

[0057] The base sequences listed in Table 3 correspond to SEQ ID NOS: 8 to 78 in the order of mention, respectively. The base sequences listed in Table 4 correspond to SEQ ID NOS: 79 to 122 in the order of mention. The base sequence

of the tRNA of the present invention may have a base sequence listed as No. 3 or No. 4 in Table 4.

[0058]

Synthesis of aminoacyl-tRNA by flexizymes

Amino acids activated with an appropriate ester group (Phe-CME, Tyr-CME, Ser-DBE, ^{Me}G-DBE, Me S-DBE, ^{Me}A-DBE, ^{Me}F-CME, ^{Me}L-DBE, ^{Me}M-DBE, ^{Me}T-DBE, ^{Me}Y-CME, ^{Me}D-DBE, ^{Me}V-DBE, ^{Me}Nl-DBE, ^{Me}Ym-CME, ^{Me}Nv-DBE, and ^{Ac}K-DBE in which CME means a cyanomethyl ester and DBE means a 3,5-dinitrobenzyl ester) were synthesized in accordance with the method described in Non-Patent Documents 13 and 15, and Reference Literature 3.

An aminoacyl-tRNA was prepared in the following manner. ADDIN EN. CITE ADDIN EN. CITE. DATA. After 12 μL of a HEPES-KOH buffer (pH 7.5, 83 mM) containing 42 μM tRNA and 42 μM flexizyme (eFx for a CME-activated amino acid and dFx for a DBE activated amino acid) was heated at 95°C for 2 minutes, the reaction mixture was cooled to 25°C for 5 minutes. Then, 3 M $MgCl_2$ (4 μL) was added, and the resulting mixture was incubated at 25°C for 5 minutes.

The reaction was started by addition of an activated amino acid substrate in 25 mM DMSO (4 μL), followed by incubation on ice for acylation (incubation time: 2 hours for Phe-CME, Tyr-CME, ^{Me}G-DBE, ^{Me}A-DBE, and ^{Ac}K-DBE; 6 hours for Ser-DBE, ^{Me}S-DBE, ^{Me}F-CME, ^{Me}L-DBE, ^{Me}M-DBE, ^{Me}M-DBE, and ^{Me}Ym-CME; and 10 hours for ^{Me}T-DBE, ^{Me}D-DBE, ^{Me}V-DBE, ^{Me}V-DBE^{Me}Nl-DBE, and ^{Me}Nv-DBE). After the acylation, the reaction was terminated by the addition of 80 μL of 0.3 M sodium acetate (pH 5.2) and the RNA was precipitated by ethanol. The pellet was washed twice with 70% ethanol containing 0.1 M sodium acetate (pH 5.2) and once with 70% ethanol. The aminoacyl-tRNA thus obtained was dissolved in 1 mM sodium acetate (pH 5.2) immediately before addition to a translation solution.

[0059]

Measurement of the efficiency of flexizyme-catalyzed aminoacylation

The pellet of ethanol-precipitated 50 pmol tRNA precharged with pAA or npAA was dissolved in 0.52 μL of 10 mM sodium acetate (pH 5.2) and mixed with 5.0 μL of acid PAGE loading buffer (83% (v/v) formamide, 150 mM sodium acetate (pH 5.2), and 10 mM EDTA). The resulting solution was loaded on an acid denaturing polyacrylamide gel (12% (w/v) acrylamide/bisacrylamide (19:1), 8 M urea, and 50 mM sodium acetate (pH 5.2)) and electrophoresis was performed at 300 V (approximately 10 V/cm) for 20 hours. The resulting gel was stained with ethidium bromide and analyzed using a Typhoon FLA 7000 (GE Healthcare). The aminoacylation efficiency was calculated based on the band intensity of the aminoacyl-tRNA (A) and free tRNA (T) and presented as (A)/[(A)+(T)].

[0060]

Radiolabeling of 5′-end or 3′-end of tRNA

The 5′-end of the tRNA was radiolabeled in the following manner. A dephosphorylated reaction mixture (4 μL) (1 μM tRNA, 50 mM Bis-Tris-Propane-HCl (pH 6.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 0.5 U Antarctic Phosphatase (New England BioLabs)) was incubated at 37°C for 30 minutes and then incubated at 70°C for 5 minutes. The solution (4 μL) was mixed with 9.1 μL of water, 2.0 μL of a 10× buffer (700 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, and 50 mM DTT), 2.9 μL of 1.7 μM [γ-^{32}P]-ATP, and 2.0 μL of 10 U/μL T4 Polynucleotide Kinase (New England BioLabs). The resulting 20 μL Kinase reaction mixture was incubated at 37°C for 60 minutes. The ^{32}P labeled tRNA (4 pmol) was mixed with an unlabeled tRNA (121 pmol). The tRNA was purified by phenol/chloroform extraction and ethanol precipitation and then, dissolved in 10 μL of water. The concentration of the tRNA was determined using A_{260} of a 50-fold diluted solution.

The 3′-end of the tRNA was radiolabeled in the following manner. After 7.5 μL of 16.7 μM tRNA lacking a 3′-end adenosine was incubated at 80°C for 3 minutes, it was placed on ice for 10 minutes. Then, 25 μL of a CCA-added reaction mixture (120 mM Gly-NaOH (pH 9.0), 75 mM $MgCl_2$, 30 mM DTT, 5 μM of the tRNA lacking

the 3′-end adenosine prepared above, 10 mM non-radiolabeled ATP, 0.67 μM [α-^{32}P]-ATP and 200 nM CCA-adding enzyme) was incubated at 37°C for 20 minutes. The tRNA was purified by phenol/chloroform extraction, Micro Bio-Spin 30 column (Bio-Rad), and ethanol precipitation and then, dissolved in 5 μL of water. The concentration of the tRNA was determined using A_{260} of a 100-fold diluted solution.

[0061]

Quantification of affinity between EF-Tu and aminoacyl-tRNA

The aminoacyl-tRNA was prepared as described above except that the acylation reaction was performed using a mixture of the 3′-radiolabeled tRNA (10%) and a non-radiolabeled tRNA (90%). The aminoacyl-tRNA pellets thus obtained were dissolved in 3.2 μL of 10 mM sodium acetate (pH 5.2). The concentration of the aminoacyl-tRNA was adjusted as follows. The aminoacyl-tRNA solution (0.7 μL) was diluted 100-fold with 69.3 μL of 10 mM sodium acetate (pH 5.2) and the concentration of the tRNA and flexizyme was determined using A_{260} of the diluted solution. The concentration of the aminoacyl-tRNA was calculated based on the tRNA concentration and the flexizyme-catalyzed acylation efficiency. The concentration of the aminoacyl-tRNA was adjusted to 2.0 μM by adding an adequate amount of 10 mM sodium acetate (pH 5.2). Immediately before use, the concentration of the aminoacyl-tRNA was adjusted to 100 nM by mixing the 2.0 μM aminoacyl-tRNA solution with a buffer A (50 mM HEPES-KOH (pH 7.6), 100 mM KOAc, 12 mM $Mg(OAc)_2$, 1 mM GTP, 1 mM DTT, 20 mM creatine phosphate, 2 mM spermidine, 3 mM phosphoenolpyruvic acid, and 0.1 μg/μL of pyruvate kinase from rabbit muscle (Sigma-Aldrich)).

The EF-Tu stored in a GDP-bound form was converted into a GTP-bound form by incubating an EF-Tu solution in the buffer A at 37°C for 30 minutes. Samples usually containing EF-Tu at concentrations different in a range of 1.5 nM to 25 μM were prepared on ice by twofold serial dilution. The EF-Tu solution (9.6 μL) was mixed with 2.4 μL of 100 nM aminoacyl-tRNA and the resulting mixture was

incubated on ice for 20 minutes. Under equilibrium binding conditions, 10 μL of each of the solutions was mixed on ice with 1 μL of 1 mg/mL RNase A (Sigma-Aldrich). Twenty seconds later, 50 μL of 10% (v/v) trichloroacetic acid (TCA) containing 0.1 mg/mL of an unfractionated yeast tRNA was added onto ice to quench the reaction. The precipitate was filtered through a nitrocellulose membrane which had a pore size of 0.45 μM and was assembled in a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories), followed by washing 6 times with 200 μL of 5% (v/v) TCA. The membrane was soaked in 95% (v/v) ethanol for 5 minutes and the dried membrane was analyzed by autoradiography using Typhoon FLA 7000 (GE Healthcare). To correct for the background signal derived from an aminoacyl-tRNA that would remain after 20-second treatment with RNase A, the membrane was analyzed concurrently with one without EF-Tu, and the radioactivity of it was subtracted from the experiment data. The resulting radioactivity was converted into a concentration of a ternary complex by using a conversion coefficient determined using a calibration aminoacyl-tRNA sample in respective buffers A containing 20, 4, 0.8 and 0.16 nM tRNA. The equilibrium dissociation constant was determined by fitting the binding data to the following formula (3) by using KaleidaGraph Program (Hulinks Inc.).

The K_D formula is defined as follows and in the formula, aatRNA, EFTu, and aatRNA-EFTu represent aminoacyl-tRNA, EF-Tu and complexes thereof, respectively.

[0062]

Formula (1)

[Equation 1]

$$K_D = \frac{[\text{aatRNA}]_{eq} \times [\text{EFTu}]_{eq}}{[\text{aatRNA}\cdot\text{EFTu}]_{eq}}$$

[0063]

Formula (2)

[Equation 2]

$$K_D = \frac{([aatRNA]_{input} - [aatRNA \cdot EFTu]_{eq}) \times ([EFTu]_{input} - [aatRNA \cdot EFTu]_{eq})}{[aatRNA \cdot EFTu]_{eq}}$$

[0064]

Formula (3)

[Equation 3]

$$[aatRNA \cdot EFTu]_{eq} = \frac{\left([aatRNA]_{input} + [EFTu]_{input} + K_D - \sqrt{([aatRNA]_{input} + [EFTu]_{input} + K_D)^2 - 4 \times [aatRNA]_{input} \times [EFTu]_{input}} \right)}{2}$$

[0065] The concentration of the aminoacyl-tRNA varied slightly in every experiment due to its multistep preparation, so that both K_D and the aminoacyl-tRNA concentration were set as variables in the fitting analysis.

[0066]

<u>In Vitro translation</u>

The reconstituted cell-free translation system (Reference Literature 4) contained all the components necessary for translation except for RF1. The concentrations of the translation components are optimized as follows (Reference Literature 2) insofar as each drawing does not include a particular description: 50 mM HEPES-KOH (pH 7.6), 100 mM KOAc, 2 mM GTP, 2 mM ATP, 1 mM CTP, 1 mM UTP, 20 mM creatine phosphate, 12 to 20 mM $Mg(OAc)_2$, 2 mM spermidine, 1 mM DTT, 100 μM 10-formyl-5,6,7,8-tetrahydrofolate, 1.2 μM ribosome, 2.7 μM IF1, 0.4 μM IF2, 1.5 μM IF3, 10 μM EF-Tu, 10 μM EF-Ts, 0.26 μM EF-G, 0.25 μM RF2, 0.17 μM RF3, 0.5 μM RRF, 0.6 μM MTF, 4 μg/mL creatine kinase, 3 μg/mL myokinase, 0.1 μM pyrophosphatase, 0.1 μM nucleoside diphosphate kinase, 0.1 μM T7 RNA polymerase, 0.73 μM AlaRS, 0.03 μM ArgRS, 0.38 μM AsnRS, 0.13 μM AspRS, 0.02 μM CysRS, 0.06 μM GlnRS, 0.23 μM GluRS, 0.09 μM GlyRS, 0.02 μM HisRS, 0.4 μM IleRS, 0.04 μM LeuRS, 0.11 μM LysRS, 0.03 μM MetRS, 0.68 μM PheRS, 0.16 μM ProRS, 0.04 μM SerRS, 0.09 μM ThrRS, 0.03 μM TrpRS, 0.02 μM TyrRS, 0.02 μM ValRS, 1.5 mg/mL native E. coli tRNA mixture (Roche), 200 μM each 3 to 20

pAA, 10-50 μM $tRNA^{AsnE2}$s charged with each npAA and either 0.25 μM mDNA or 6.0 μM mRNA.

The translation products were analyzed by MALDI-TOF-MS analysis and/or autoradiography after tricine-SDS-PAGE. In MALDI-TOF MS analysis, 1.0 to 10 μL of the translation reaction mixture was incubated at 37°C for 3 to 120 minutes. After the reaction, the mixture was diluted with the same volume of a FLAG purification buffer (100 mM Tris-HCl (pH 7.6) and 300 mM NaCl). The expressed peptide was immobilized on anti-FLAG M2 agarose beads (Sigma-Aldrich) by incubating at 25°C for 1 hour. After washing the beads with 25 μL of wash buffer (50 mM Tris-HCl (pH 7.6) and 150 mM NaCl), the immobilized peptide was eluted with 15 μL of 0.2% TFA. After the purification, the peptide was desalted with SPE C-TIP (Nikkyo Technos) and eluted with 1 μL of 80% acetonitrile, 0.5% acetic acid solution 50% saturated with the matrix (R)-cyano-4-hydroxycinnamic acid (Bruker Daltonics). The MALDI-TOF MS measurement was performed using a ultraflextreme (Bruker Daltonics) under reflector/positive-mode and externally calibrated with peptide calibration standard II (Bruker Daltonics) and/or protein calibration standard I (Bruker Daltonics). For the autoradiographic analysis, the translation reaction was performed in the presence of 50 μM [^{14}C]-Asp instead of 200 μM nonradioactive Asp. The translation product was analyzed by 15% tricine-SDS-PAGE and autoradiography using Typhoon FLA 7000 (GE Healthcare) without FLAG purification. The amount of the peptide product was quantified based on the relative band intensity of the peptide product to the sum intensities present in the lane (that is, the sum intensities of unreacted free Asp and peptide product).

The method of preparing a ^{Me}AA-tRNA was changed as follows for the translation of mRNAs 12 to 14 (Figs. 2, 4 to 7, and 9). First, the ethanol precipitate after flexizyme-catalyzed acylation was dissolved in 300 μL of 0.3 M sodium acetate (pH 5.2) and precipitated by second ethanol precipitation. This precipitation was performed to wash away a coprecipitated Mg salt. The pellet was washed twice

with 70% ethanol containing 0.1 M sodium acetate (pH 5.2) and once with 70% ethanol. The resulting aminoacyl-tRNA was dissolved in 1 mM sodium acetate (pH 5.2) immediately before addition to a translation solution. The concentration of the aminoacyl-tRNA was adjusted as follows: 0.25 μL of the aminoacyl-tRNA solution was diluted 5000-fold with 1250 μL of 1 mM sodium acetate (pH 5.2) and the concentration of the tRNA and the flexizyme was determined using A_{260} of the diluted solution. The concentration of the aminoacyl-tRNA was calculated based on the tRNA concentration and the flexizyme-catalyzed acylation efficiency.

[0067]

Experimental evidence showing that many ^{Me}AA-tRNAs do not bind to EF-Tu with sufficient intensity

First, EF-Tu affinities of standard pAA-tRNA and those of ^{Me}AA-tRNA were compared. As a control, three standard pAA-tRNAs (Phe-tRNA^{Phe}, Tyr-tRNA^{Tyr}, and Ser-tRNA^{Ser}) were prepared using a flexizyme (Non-Patent Document 13) catalyst, that is, an artificial aminoacylation ribozyme and their EF-Tu affinities were quantified by RNase A protection assay. As reported previously, they showed similar affinity in a range of -8 to -9.4 kcal/mol (Fig. 2a). The affinity values observed were slightly lower than those previously reported (-9.5 to -10.5 kcal/mol) (Reference Literature 5). The difference was thought to occur due to a difference in the composition of a buffer or the presence of a His6 tag at the C terminal of the EF-Tu used in the present study. For the determination of the EF-Tu affinity of ^{Me}AA-tRNA, 14 kinds of ^{Me}AAs were charged to orthogonal tRNA$^{AsnE2}_{GAC}$ and the body sequence of it was engineered to avoid aminoacylation by endogenous AARS (Non-Patent Document 14 and Reference Literatures 2 and 6) by using flexizyme-catalyzed aminoacylation. In contrast to the standard pAA-tRNAs, many ^{Me}AA-tRNA$^{AsnE2}_{GAC}$s other than ^{Me}G-tRNA$^{AsnE2}_{GAC}$, ^{Me}S-tRNA$^{AsnE2}_{GAC}$ and ^{Me}A-tRNA$^{AsnE2}_{GAC}$ showed an undetectably weak EF-Tu affinity level (Fig. 2b). Considering that the α-amino group of the charged amino acid is housed in a pocket composed of an EF-Tu residue (Fig. 2c),

the N-methyl modification is presumed to cause steric hindrance between the ^{Me}AA and the pocket, leading to deterioration in EF-Tu affinity. The three exceptions, that is, ^{Me}G-tRNA$^{AsnE2}_{GAC}$, ^{Me}S-tRNA$^{AsnE2}_{GAC}$, and ^{Me}A-tRNA$^{AsnE2}_{GAC}$C have a possibility of alleviating the steric hindrance by locating their side chains (-H, -CH_3, and -OH groups) in the pocket.

Next, the ribosomal synthesis of a model peptide (Fig. 2d) containing a single ^{Me}AA was performed using six kinds of ^{Me}AA-tRNA$^{AsnE2}_{GAC}$s by an improved in vitro translation system called FIT (Flexible In vitro Translation) system (Non-Patent Documents 13 to 16 and Reference Literatures 2 and 3). The system depends on a reconstituted E. coli cell-free translation system (Reference Literature 4) supplemented with a synthetic tRNA pre-charged with npAA by the catalytic action of flexizyme. The mRNA template was designed to determine the translation accuracy by MALDI-TOF-MS after purification by an anti-FLAG M2 agarose. The genetic code of a GUC codon was reprogrammed by excluding the corresponding Val from the FIT system and supplementing one of ^{Me}AA-tRNA$^{AsnE2}_{GAC}$s instead. When ^{Me}G-tRNA$^{AsnE2}_{GAC}$ and ^{Me}S-tRNA$^{AsnE2}_{GAC}$ showing strong EF-Tu affinity were used, an correct peptide was observed as a single major peak in MALDI-TOF-MS. On the other hand, when ^{Me}AA-tRNA$^{AsnE2}_{GAC}$ having weak EF-Tu affinity was used, an undesirable byproduct containing either one of Leu or Ile instead of ^{Me}AA was detected in addition to the correct peptide product in MALDI-TOF-MS (Fig. 2d). As an exception, ^{Me}F-tRNA$^{AsnE2}_{GAC}$ did not show a detectable level of EF-Tu affinity, but a single major peak was observed for ^{Me}F. In a supplementary experiment, a byproduct was observed only when Ile was supplied in the FIT system, which has revealed that the misincorporated amino acid was not Leu but Ile (Fig. 7). Similar weak EF-Tu affinity and translation inaccuracy were observed in another type npAA, ε-N-acetyllysine (^{Ac}K), which was examined to confirm the universality of the present study (Figs. 2b and 2d). In conclusion, the present experiment indicated that the EF-Tu affinities of many ^{Me}AA-tRNA$^{AsnE2}_{GAC}$s were significantly weaker than the

mean value of standard pAA-tRNAs and this weak EF-Tu affinity often caused translation inaccuracy during ribosomal polypeptide synthesis.

[0068]

Development of a method for reinforcing the weak EF-Tu affinity of ^{Me}AA-tRNA

Since the fact that many ^{Me}AA-tRNAs did not bind to EF-Tu with sufficient intensity was discovered, development of a novel method for reinforcing the weak EF-Tu affinity of ^{Me}AA-tRNA was encouraged. It has been reported that binding affinity between EF-Tu and Phe-tRNA^{Phe} changes depending on its T-stem sequence (Reference Literature 7), so that the present inventors have thought that the EF-Tu affinity of ^{Me}AA-tRNA can be changed by using a different T-stem sequence. Based on this concept, three T-stem sequences (T-stems No. 1, No. 3, and No.4) were selected (Fig. 3a, Reference Literature 7). The original T-stem shown in Fig. 3a was renamed as T-stem No. 2. Based on the previous studies, the present inventors considered that the EF-Tu affinity of those four ^{Me}AA-tRNAs showed an increase with an increase in the number of the T-stems from No. 1, 2 to 4.

The feasibility of the affinity reinforcement by T-stem substitution was confirmed using Phe-tRNA$^{AsnE2}_{GAC}$ and ^{Me}F-tRNA$^{AsnE2}_{GAC}$. When the T-stem sequence of Phe-tRNA$^{AsnE2}_{GAC}$ was substituted by another T-stem sequence, the EF-Tu affinity changed in an expected order (Fig. 3b). In ^{Me}F-tRNA$^{AsnE2}_{GAC}$, a similar change was also observed in the EF-Tu affinity of T-stem sequences No. 2, No. 3, and No. 4 (Fig. 3c). A control test using an uncharged tRNA$^{AsnE2}_{GAC}$ has revealed that the aforesaid tRNAs do not bind to EF-Tu insofar as they are not charged with an amino acid (Fig. 8).

The effect of EF-Tu affinity reinforcement on the translation accuracy and efficiency was studied using Phe-tRNA$^{AsnE2}_{GAC}$ and ^{Me}F-tRNA$^{AsnE2}_{GAC}$. An mRNA template was designed to determine the peptide expression level by tricine-SDS-PAGE (autoradiographic detection of [^{14}C]-Asp in a C-terminal FLAG peptide region) and decoding accuracy was evaluated by MALDI-TOF-MS after purification using an

anti-FLAG M2 agarose (Figs. 4a and 4b). When the T-stem No. 1 was used, a byproduct containing Ile instead of Phe and ^{Me}F was observed in MALDI-TOF-MS, but only an expected peptide was obtained when the tRNAs having the relatively strong T-stem were used (Fig. 4a and Fig. 4b). When the translation efficiency of a Phe-containing peptide was quantified using an Ile-free FIT system, a synthesis rate decreased with an increase in the EF-Tu affinity (Fig. 4c). The decrease in synthesis rate is presumed to occur because (1) Phe-tRNA$^{AsnE2}_{GAC}$ having a stronger T-stem might bind to EF-Tu in preference and disturb the binding of canonical pAA-tRNA and thereby decrease the synthesis rate of a full-length peptide; or (2) the too strong interaction between EF-Tu and the substituted T-stem might disturb the rapid delivery of aminoacyl-tRNA from EF-Tu to a ribosome as observed in the previous study (Reference Literatures 8 and 9). To specify a correct reason, further research is necessary. In the case of ^{Me}F-tRNA$^{AsnE2}_{GAC}$, the synthesis rate was highest when the T-stems No. 2 and No. 3 were used, compared with the use of T-stem No. 1 or No. 4 (Fig. 4d). This suggests that the T-stem No. 1 is too weak for ^{Me}F-tRNA$^{AsnE2}_{GAC}$. In conclusion, the present results have proved that the EF-Tu affinity of the aminoacyl-tRNA can be drastically changed by the T-stem substitution strategy and selection of the best T-stem from four candidates enables improvement in translation accuracy and efficiency. The influence of the EF-Tu affinity on the translation accuracy was also studied in the other four ^{Me}AA-tRNA$^{AsnE2}_{GAC}$ and ^{Ac}K-tRNA$^{AsnE2}_{GAC}$ (Fig. 9). In all the examples, the reinforcement of the EF-Tu affinity was achieved by the T-stem substitution, and therefore, the translation accuracy was improved. Particularly, when ^{Me}L and ^{Ac}K were incorporated, a byproduct was observed as a major peak when T-stem No. 2 was used, but production of an undesirable byproduct was suppressed by substituting the T-stem sequence by No. 4 sequence. The reinforcement of the EF-Tu affinity was confirmed also in the other eight kinds of ^{Me}AA-tRNA$^{AsnE2}_{GAC}$s (Fig. 10).

[0069]

Expression of highly N-methylated peptide via tuning of EF-Tu affinity of ^{Me}AA-tRNA

Next, to prove the improved the N-methyl peptide synthesis by the EF-Tu affinity tuning strategy, expression of highly N-methylated peptides respectively containing 6 different ^{Me}AAs was attempted. In the present experiment, two improvements were performed. First, the EF-Tu affinity of ^{Me}AA-tRNAs was adjusted to fall in a range of that of pAA-tRNAs by selecting the T-stem sequence suited for individual ^{Me}AA-tRNAs (Fig. 5a). Under the conditions, EF-Tu can bind to any of pAA-tRNAs and ^{Me}AA-tRNAs with equivalent affinity. Secondly, the concentration of each of the ^{Me}AA-tRNAs was adjusted based on the flexizyme catalyzed acylation efficiency (typically, 20 to 50%) and the concentration of the pre-charged ^{Me}AA-tRNAs was adjusted to 10 μM, while in a conventional method, 50 μM of each of the pre-charged tRNAs with ^{Me}AA was added without considering the acylation efficiency (Fig. 5b). In the present experiment, a model peptide composed of 14 pAAs and 6 ^{Me}AAs was expressed in the FIT system, and 6 ^{Me}AA-tRNAs (^{Me}Y-tRNA$^{AsnE2TS3}_{GAA}$, ^{Me}A-tRNA$^{AsnE2TS3}_{GAU}$, ^{Me}F-tRNA$^{AsnE2TS3}_{GGC}$, ^{Me}V-tRNA$^{AsnE2TS1}_{GAC}$, ^{Me}G-tRNA$^{AsnE2TS5}_{CAG}$, and ^{Me}S-tRNA$^{AsnE2TS5}_{GAG}$) were used instead of the excluded 5 pAAs (Phe, Leu, Ile, Val, and Ala) (Fig. 5c and Fig. 5d). When the conventional system (Reference Literature 6) was used for the translation, a mixture of a desired peptide containing pAA instead of ^{Me}AA with various byproducts was synthesized, and an expression level of the mixture was 0.7 μM in total (Fig. 5e and Fig. 5f). On the other hand, the optimized system developed in the present study produced only a desired peptide having an improved expression level 1.8 μM. The results have shown that a translation disorder may be caused by poor EF-Tu affinity of ^{Me}AA-tRNA and that the affinity tuning strategy drastically improves the synthesis efficiency and accuracy of N-methyl peptides.

Next, the number of the ^{Me}AAs was increased further to 9, and a 32-mer model peptide P14 composed of 9 respectively different ^{Me}AAs and 14 pAAs were expressed (Fig. 6a to Fig. 6c). In the present experiment, 3 kinds of ^{Me}AAs having

an unnatural side chain were added to the repertoire. Compared with control translation using 20 pAAs, the P14 peptide having 9 N-methyl modifications was expressed at 34% efficiency relative to the control having maintained translation accuracy, as confirmed by MALDI-TOF-MS (Fig. 6d and Fig. 6e). The results have proved that the EF-Tu affinity tuning strategy drastically increases the number of simultaneously usable ^{Me}AAs to at most 9 and thereby enables the expression of a highly N-methylated peptide.

[0070]

As shown in the results of the present Examples, the present invention can provide a novel method which achieves ribosomal synthesis of a non-standard peptide containing many kinds of ^{Me}AAs.

First, the present Example has quantitatively proved that the binding affinity between EF-Tu and many ^{Me}AA-tRNAs was weak (Fig. 2). This has supported our assumption that inefficient ^{Me}AA incorporation is due to the disorder in EF-Tu mediated delivery of ^{Me}AA-tRNA to a ribosome. The present Example has also indicated that the weak EF-Tu affinity of ^{Me}AA-tRNA can be reinforced by the T-stem substitution strategy (Fig. 3, Fig. 9, and Fig. 10). Particularly when the EF-Tu binding affinity was changed to a too intense value, a synthesis efficiency of a corresponding peptide decreased (Fig. 4), which was consistent with the previous report on the study of pAAs and fluorescent npAA (Reference Literatures 8 and 9).

Tuning of the affinity between EF-Tu and each ^{Me}AA-tRNA has successfully improved the synthesis efficiency and accuracy of model peptides containing 6 respectively-different ^{Me}AAs (Fig. 5). Further, the translation system developed herein has achieved the synthesis of a highly N-methylated peptide containing even 9 respectively-different ^{Me}AAs (Fig. 6). This has increased the number of simultaneously usable ^{Me}AAs from 3 to 9 (Reference Literatures 6 and 10). Particularly, 9 ^{Me}AAs include ^{Me}V which is reported as a substrate unsuited for ribosome polypeptide synthesis (Reference Literature 3). The diversity of 24

constitution blocks (15 pAAs and 9 ^{Me}AAs) achieved by the present Example has overcome the previous restriction of 23 constitution blocks (20 pAAs and 3 npAAs) (Reference Literatures 11 and 12). These facts shown by the present Examples have clearly proved the improvement in synthesis efficiency and accuracy of a highly N-methylated peptide.

The npAA incorporation inefficiency is presumed to occur due to (1) the disorder in the EF-Tu-mediated delivery of a corresponding npAA-tRNA, (2) a slow peptidyl transfer reaction with which npAA is involved, and (3) the hindered ejection of a newly-formed npAA-containing polypeptide through an exit tunnel. In the related art, which process is a real cause for inefficient incorporation has not been identified, but the present invention has shown that the synthesis efficiency and accuracy of N-methylated peptide can be improved drastically by tuning the insufficient EF-Tu affinity of ^{Me}AA-tRNA. This fact means that the same strategy is useful for promoting the efficient incorporation of abundant and multiple npAAs as well as ^{Me}AAs. In the present Example, such an example is proved for ^{Ac}K (Fig. 9).

The peptide translation system according to the present invention enables the synthesis of a highly N-methylated peptide library with sufficient accuracy and efficiency. Such integration of a peptide library and a peptide screening technique is expected to promote the discovery of practical non-standard peptide drugs having desirable pharmacological properties in future.

[0071]

Reference Literature 1: Nissen, P. et al. Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu, and a GTP analog. Science 270, 1464-1472 (1995).
Reference Literature 2: Goto, Y., Katoh, T. & Suga, H. Flexizymes for genetic code reprogramming. Nat Protoc 6, 779-790, doi:10.1038/nprot.2011.331 (2011).
Reference Literature 3: Kawakami, T., Murakami, H. & Suga, H. Messenger RNA-programmed incorporation of multiple N-methyl amino acids into linear and cyclic peptides. Chem. Biol. 15, 32-42, doi:10.1016/j.chembiol.2007.12.008 (2008).

Reference Literature 4: Shimizu, Y. et al. Cell-free translation reconstituted with purified components. Nat. Biotechnol. 19, 751-755, doi:10.1038/90802 (2001).
Reference Literature 5: LaRiviere, F. J., Wolfson, A. D. & Uhlenbeck, O. C. Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. Science 294, 165-168, doi:10.1126/science.1064242 (2001).
Reference Literature 6: Yamagishi, Y. et al. Natural product-like macrocyclic N-methyl-peptide inhibitors against a ubiquitin ligase uncovered from a ribosome-expressed de novo library. Chem. Biol. 18, 1562-1570, doi:10.1016/j.chembiol.2011.09.013 (2011).
Reference Literature 7: Schrader, J. M., Chapman, S. J. & Uhlenbeck, O. C. Understanding the sequence specificity of tRNA binding to elongation factor Tu using tRNA mutagenesis. J. Mol. Biol. 386, 1255-1264 (2009).
Reference Literature 8: Schrader, J. M., Chapman, S. J. & Uhlenbeck, O. C. Tuning the affinity of aminoacyl-tRNA to elongation factor Tu for optimal decoding. Proceedings of the National Academy of Sciences of the United States of America 108, 5215-5220, doi:10.1073/pnas.1102128108 (2011).
Reference Literature 9: Mittelstaet, J., Konevega, A. L. & Rodnina, M. V. A kinetic safety gate controlling the delivery of unnatural amino acids to the ribosome. J. Am. Chem. Soc. 135, 17031-17038, doi:10.1021/ja407511q (2013).
Reference Literature 10: Subtelny, A. O., Hartman, M. C. & Szostak, J. W. Ribosomal synthesis of N-methyl peptides. J. Am. Chem. Soc. 130, 6131-6136, doi:10.1021/ja710016v (2008).
Reference Literature 11: Ohtsuki, T., Manabe, T. & Sisido, M. Multiple incorporation of non-natural amino acids into a single protein using tRNAs with non-standard structures. FEBS Lett. 579, 6769-6774, doi:10.1016/j.febslet.2005.11.010 (2005).
Reference Literature 12: Iwane, Y. et al. Expanding the amino acid repertoire of ribosomal polypeptide synthesis via the artificial division of codon boxes. Nat Chem 8, 317-325, doi:10.1038/nchem.2446 (2016).

Sequence Listing Free Text

[0072]

SEQ ID NOS: 1, 2 and 4 to 6 represent the base sequences of a T-arm.

SEQ ID NO: 3 represents the base sequences of a T-loop.

SEQ ID NOS: 123 to 125 represent the base sequences of mRNAs listed in Table 6.

SEQ ID NOS: 126 to 128 represent the base sequences of mRNAs in a translation region described in the drawing.

SEQ ID NOS: 129 to 133 represent the amino acid sequences of translated peptides.

SEQ ID NOS: 134 to 136 represent the base sequences of a D-arm.

What is claimed is:

1. A tRNA having a T-stem in which $N_1N_2GGN_3$ and N_4CCN_5U form a pair and encoding an N-methyl amino acid;

wherein N_1 is G or A; one of N_2 and N_5 is G and the other one is C; and N_3 and N_4 are any bases forming a pair.

2. A translation system, comprising the tRNA as claimed in Claim 1 and an EF-Tu protein.

3. A method of producing a peptide library, comprising a step of translating with a cell-free translation system by using the tRNA as claimed in Claim 1 or the translation system as claimed in Claim 2.

4. A method of producing a complex library of a peptide and an mRNA encoding the peptide, comprising a step of translating with a cell-free translation system by using the tRNA as claimed in Claim 1 or the translation system as claimed in Claim 2.

5. The production method according to Claim 3 or 4, further comprising a step of preparing the tRNA as claimed in Claim 1 or the translation system as claimed in Claim 2, wherein in the preparation step, which tRNA is charged, a tRNA having a T-stem in which $N_1N_2GGN_3$ and N_4CCN_5U form a pair or a tRNA other than the tRNA having a T-stem in which $N_1N_2GGN_3$ and N_4CCN_5U form a pair is determined prior to charging with an N-methyl amino acid.

6. The production method according to Claim 5, comprising a step of preparing the tRNA as claimed in Claim 1 or the translation system as claimed in Claim 2, wherein in the preparation step, which tRNA is charged, a tRNA having a T-stem in which $AGGGN_3$ and N_4CCCU form a pair and a tRNA having a T-stem in which $GCGGN_3$ and N_4CCGU form a pair, or a tRNA other than the said tRNAs is determined prior to charging with an N-methyl amino acid.

7. The production method according to Claim 5, further comprising a step of preparing the tRNA as claimed in Claim 1 or the translation system as claimed in

Claim 2, wherein in the preparation step, which tRNA is charged, a tRNA having AGGGG(N_6)$_m$CCCCU (SEQ ID NO: 1) and a tRNA having GCGGG(N_6)$_m$CCCGU (SEQ ID NO: 2), wherein N_6 is an any base and m is an integer of 1 or more, or a tRNA other than the said tRNAs is determined prior to charging with an N-methyl amino acid.

8. The production method according to any one of Claims 3 to 7, wherein a tRNA having, as an affinity between a tRNA charged with an N-methyl amino acid and EF-Tu, ΔG (kcal/mol) of -7.5 to -10 is selected and the tRNA thus selected is charged with the N-methyl amino acid.

9. A peptide library produced by the method as claimed in any one of Claims 3 and 5 to 8 or a peptide-mRNA complex library produced by the method as claimed in any one of Claims 4 to 8.

10. The peptide library or peptide-mRNA complex library according to Claim 9, comprising a peptide containing two or more N-methyl amino acids in one peptide structure.