

[DESCRIPTION]

[Title of Invention]

COMPOSITION FOR TRANSLATION, AND METHOD FOR PRODUCING PEPTIDE

5 [Technical Field]

[0001]

The present disclosure relates to compositions for translation and methods for producing peptides.

10 [Background Art]

[0002]

Cell-free translation systems artificially reconstituted by mixing together only the factors involved in protein translation (Non-Patent Literature (NPL) 1) are being used in a wide range of fields from elucidation of life phenomena to development of new drugs. Since cell-free translation systems do not use microorganisms and cells, they can be used to synthesize highly toxic proteins. Furthermore, since components such as amino acids, tRNAs, and aminoacyl-tRNA synthetases can be removed or added depending on the purpose, correspondence between codons and amino acids can be changed (reprogramming of the genetic code). Because of such features, cell-free translation systems are being applied to synthesis of proteins containing unnatural amino acids, and to construction of display libraries introduced with various building blocks. Cell-free translation systems are thus very useful techniques, but sometimes generate unintended peptides through codon misreading. For example, it has been reported that when RF1 was inactivated and an unnatural amino acid was assigned to the amber codon (UAG), misincorporation of tyrosine (UAU, UAC), lysine (AAG), and glutamine (GAG) occurred at this codon at a certain rate (NPL 2).

[0003]

Examples of reprogramming the genetic code by artificially dividing codon boxes have been reported. In NPL 3, assignment of one unnatural amino acid and one natural amino acid to C-G-C/G, G-U-C/G, and G-G-C/G was attempted. This literature shows, as a measure for reducing misreading of the GGC codon by tRNA^{Gly}_{CCC} attached with Gly, stopping the translation reaction before depletion of a tRNA that carries the anticodon complementary to GGC, *i.e.*, GCC, and that is precharged with an amino acid; however, it does not show what kind of composition for translation can reduce the codon misreading. Artificial division of codon boxes has also been undertaken in NPL 4 to 7 and in Patent Literature (PTL) 1 and 2, but these documents are silent on methods for reducing codon misreading.

[0004]

tRNAs are known to have specific conserved bases at positions 32 and 38 in the anticodon loop depending on the anticodon triplets. According to a report using tRNA(Ala)_{GGC} carrying the GGC anticodon, the use of the bases conserved in natural tRNA(Ala)_{GGC} at positions 32 and 38 caused less misreading of the GUC codon; on the other hand, alteration of the bases at positions 32 and 38 was shown to cause this tRNA to misread the GUC codon, leading to misincorporation of alanine (GCC) in translation (NPL 8). This literature only studied misreading of the GUC codon by tRNA(Ala)_{GGC} using the natural genetic code table, and does not mention codon misreading that may take place due to reprogramming of the genetic code table.

[Citation List]

[Patent Literature]

[0005]

[PL 1] WO2019/139126

[PL 2] WO2012/026566

[Non-patent Literature]

[0005]

[NPL 1] Shimizu et al., Nat. Biotechnol. 2001 Aug; 19(8): 751-755

[NPL 2] Gan et al., Biochem. Biophys. Acta, 2017 Dec; 1861; 3047-3052.

[NPL 3] Iwane et al., Method Mol. Biol., 2018, 1728, 17-47.

[NPL 4] Passioura et al., J Am. Chem. Soc., 2018, 140, 11551-11555.

[NPL 5] Passioura et al., Cell Chem. Biol., 2018, 25, 906-915.

[NPL 6] Iwane et al., Nat. Chem., 2016, 8, 317-325.

[NPL 7] Azusa et al., Peptide Science 2009, 2010 Mar; 17-18.

[NPL 8] urakami et al., Nat. Struct. Mol. Biol., 2009 Mar; 16; 353-358.

[Summary of Invention]

[Technical Problem]

[0007]

The present invention was achieved in view of the above circumstances. An objective of the present invention is to provide compositions for translation that enable reducing the rate of mistranslation into unintended amino acids attributable to misreading of codons by tRNAs, and methods for producing peptides using the compositions for translation.

[Solution to Problem]

[0008]

The present inventors discovered that the above problem can be solved by selecting specific bases in the anticodon loop of the tRNA used for translation. The present disclosure is based on such findings, and specifically encompasses the embodiments exemplified below:

[1] a composition for translation, comprising a first tRNA to which a first amino acid is attached and a second tRNA to which a second amino acid is attached, wherein a combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is:

(1) 32U, 33U, 37G, and 38A;

(2) 32A, 33U, 37G, and 38U;

(3) 32A, 33U, 37A, and 38U;

(4) 32U, 33U, 37G, and 38U;

(5) 32U, 33U, 37A, and 38U; or

(6) 32C, 33U, 37G, and 38A;

bases at the first letters of the anticodons in the first tRNA and the second tRNA are different from each other,

bases at the second letters of the anticodons in the first tRNA and the second tRNA are the same,

bases at the third letters of the anticodons in the first tRNA and the second tRNA are the same, and

at least one of the first amino acid and the second amino acid is an unnatural amino acid, and wherein A is adenine, C is cytosine, G is guanine, and U is uracil;

[2] the composition of [1], wherein the anticodon in the first tRNA is $N_{11}N_{12}N_{13}$ (herein, N_{11} , N_{12} , and N_{13} are each independently A, C, G, or U);

[3] the composition of [1] or [2], wherein the anticodon in the second tRNA is $N_{21}N_{22}N_{23}$

(herein, N_{21} , N_{22} , and N_{23} are each independently A, C, G, or U);

[4] the composition of any one of [1] to [3], wherein a tRNA body of the first tRNA is a chimeric tRNA body;

[5] the composition of any one of [1] to [4], wherein the base at the first letter of the anticodon in the first tRNA is A or G, and the base at the first letter of the anticodon in the second tRNA is C or U; or the base at the first letter of the anticodon in the first tRNA is C or U, and the base at the first letter of the anticodon in the second tRNA is A or G;

[6] the composition of any one of [1] to [5], wherein the base at the first letter of the anticodon in the first tRNA is A or G, and the base at the first letter of the anticodon in the second tRNA is C or U;

[7] the composition of any one of [1] to [6], wherein the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is C;

the base at the first letter of the anticodon in the first tRNA is C, and the base at the first letter of the anticodon in the second tRNA is A;

the base at the first letter of the anticodon in the first tRNA is G, and the base at the first letter of the anticodon in the second tRNA is C;

5 the base at the first letter of the anticodon in the first tRNA is C, and the base at the first letter of the anticodon in the second tRNA is G;

the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is U;

the base at the first letter of the anticodon in the first tRNA is U, and the base at the first letter of the anticodon in the second tRNA is A;

10 the base at the first letter of the anticodon in the first tRNA is G, and the base at the first letter of the anticodon in the second tRNA is U; or

the base at the first letter of the anticodon in the first tRNA is U, and the base at the first letter of the anticodon in the second tRNA is G;

15 [8] the composition of any one of [1] to [7], wherein the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is C;

the base at the first letter of the anticodon in the first tRNA is C, and the base at the first letter of the anticodon in the second tRNA is A;

the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is U;

20 the base at the first letter of the anticodon in the first tRNA is U, and the base at the first letter of the anticodon in the second tRNA is A;

the base at the first letter of the anticodon in the first tRNA is G, and the base at the first letter of the anticodon in the second tRNA is U; or

25 the base at the first letter of the anticodon in the first tRNA is U, and the base at the first letter of the anticodon in the second tRNA is G;

[9] the composition of any one of [1] to [7], wherein the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is C;

the base at the first letter of the anticodon in the first tRNA is C, and the base at the first letter of the anticodon in the second tRNA is A;

30 the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is U;

the base at the first letter of the anticodon in the first tRNA is G, and the base at the first letter of the anticodon in the second tRNA is U; or

35 the base at the first letter of the anticodon in the first tRNA is G, and the base at the first letter of the anticodon in the second tRNA is C;

[10] the composition of any one of [1] to [7], wherein the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is C; the base at the first letter of the anticodon in the first tRNA is C, and the base at the first letter of the anticodon in the second tRNA is A;

5 the base at the first letter of the anticodon in the first tRNA is G, and the base at the first letter of the anticodon in the second tRNA is C; or the base at the first letter of the anticodon in the first tRNA is C, and the base at the first letter of the anticodon in the second tRNA is G;

10 [11] the composition of any one of [1] to [10], wherein the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is C; or the base at the first letter of the anticodon in the first tRNA is C, and the base at the first letter of the anticodon in the second tRNA is A;

[12] the composition of any one of [1] to [7] and [10], wherein the base at the first letter of the anticodon in the first tRNA is G, and the base at the first letter of the anticodon in the second

15 tRNA is C; or the base at the first letter of the anticodon in the first tRNA is C, and the base at the first letter of the anticodon in the second tRNA is G;

[13] the composition of any one of [1] to [8], wherein the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is U; or

20 the base at the first letter of the anticodon in the first tRNA is U, and the base at the first letter of the anticodon in the second tRNA is A;

[14] the composition of any one of [1] to [8], wherein the base at the first letter of the anticodon in the first tRNA is G, and the base at the first letter of the anticodon in the second tRNA is U; or the base at the first letter of the anticodon in the first tRNA is U, and the base at the first letter of the anticodon in the second tRNA is G;

25 [15] the composition of any one of [1] to [11], wherein the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is C;

[16] the composition of any one of [1] to [15], wherein a combination of bases at the second and third letters of the anticodons in the first and the second tRNAs is:

30 (i) G at the second letter and G at the third letter;

(ii) A at the second letter and G at the third letter;

(iii) C at the second letter and C at the third letter;

(iv) G at the second letter and C at the third letter;

(v) A at the second letter and C at the third letter;

35 (vi) G at the second letter and U at the third letter;

(vii) G at the second letter and A at the third letter; or

(viii) C at the second letter and G at the third letter;
 [17] the composition of any one of [1] to [16], wherein a combination of bases at the second and third letters of the anticodons in the first and the second tRNAs is

(i) G at the second letter and G at the third letter;
 5 (ii) A at the second letter and G at the third letter;
 (iii) C at the second letter and C at the third letter;
 (iv) G at the second letter and C at the third letter;
 (v) A at the second letter and C at the third letter;
 (vi) G at the second letter and U at the third letter; or
 10 (vii) G at the second letter and A at the third letter;
 [18] the composition of any one of [1] to [17], wherein a combination of bases at the second and third letters of the anticodons in the first and the second tRNAs is:

(i) G at the second letter and G at the third letter;
 (ii) A at the second letter and G at the third letter; or
 15 (iii) C at the second letter and C at the third letter;
 [19] the composition of any one of [1] to [18], wherein the base is G at the second letter and the base is G at the third letter of the anticodons in the first and the second tRNAs;
 [20] the composition of any one of [1] to [18], wherein the base is A at the second letter and the base is G at the third letter of the anticodons in the first and the second tRNAs;
 20 [21] the composition of any one of [1] to [18], wherein the base is C at the second letter and the base is C at the third letter of the anticodons in the first and the second tRNAs;
 [22] the composition of any one of [1] to [21], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the first tRNA are not derived from any of the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA
 25 having at least one base sequence selected from the group consisting of SEQ ID NOs: 275, 294, 295, 296, 302, 303, and 304;
 [23] the composition of any one of [1] to [22], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the first tRNA are derived from the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having
 30 at least one base sequence selected from the group consisting of (a) SEQ ID NO: 253, (b) SEQ ID NO: 255, and (c) SEQ ID NO: 254;
 [24] the composition of any one of [1] to [23], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the first tRNA are the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having a base
 35 sequence set forth in at least one selected from the group consisting of (a) SEQ ID NO: 253, (b) SEQ ID NO: 255, and (c) SEQ ID NO: 254;

[25] the composition of any one of [1] to [24], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the first tRNA have sequence identity of 80% or more, preferably 90% or more, and more preferably 95% or more to the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having the base sequence of any one of (a) to (c) of [24];

[26] the composition of any one of [1] to [25], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the second tRNA are not derived from any of the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having at least one base sequence selected from the group consisting of SEQ ID NOs: 275, 294, 295, 296, 302, 303, and 304;

[27] the composition of any one of [1] to [26], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the second tRNA are derived from the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having at least one base sequence selected from the group consisting of (a) SEQ ID NO: 253, (b) SEQ ID NO: 255, and (c) SEQ ID NO: 254;

[28] the composition of any one of [1] to [27], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the second tRNA are the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having at least one base sequence selected from the group consisting of (a) SEQ ID NO: 253, (b) SEQ ID NO: 255, and (c) SEQ ID NO: 254;

[29] the composition of any one of [1] to [28], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the second tRNA have sequence identity of 80% or more, preferably 90% or more, and more preferably 95% or more to the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having the base sequence of any one of (a) to (c) of [28];

[30] the composition of any one of [1] to [29], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the first tRNA and the second tRNA are identical or have sequence identity of 80% or more, preferably 90% or more, and more preferably 95% or more;

[31] the composition of any one of [1] to [30], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the first tRNA and the second tRNA are identical;

[32] the composition of any one of [4] to [31], wherein the chimeric tRNA body is a chimeric tRNA body in which the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) and the base sequences of the other portions have different origins;

[33] the composition of any one of [1] to [32], wherein the base sequence of positions 32 to 38

- (tRNA numbering rule) in the first tRNA is different from the base sequence of positions 32 to 38 (tRNA numbering rule) in a wild-type tRNA from *Escherichia coli*;
- [34] the composition of any one of [1] to [33], wherein the first tRNA is a tRNA having a chimeric anticodon loop;
- 5 [35] the composition of any one of [1] to [34], wherein the second amino acid is an unnatural amino acid;
- [36] the composition of any one of [1] to [35], wherein the first amino acid is an unnatural amino acid;
- [37] the composition of any one of [1] to [36], wherein either one or both of the first amino acid
- 10 and the second amino acid are N-substituted amino acids;
- [38] the composition of any one of [1] to [37], wherein the first tRNA is an artificial tRNA;
- [39] the composition of any one of [1] to [38], wherein the second tRNA is an artificial tRNA;
- [40] the composition of any one of [1] to [39], wherein the base sequence of positions 75 and 76 (tRNA numbering rule) in the first tRNA is CA;
- 15 [41] the composition of any one of [1] to [40], wherein the base sequence of positions 75 and 76 (tRNA numbering rule) in the second tRNA is CA;
- [42] the composition of any one of [1] to [41], wherein the first tRNA is a tRNA not containing a modified base;
- [43] the composition of any one of [1] to [42], wherein the second tRNA is a tRNA not
- 20 containing a modified base;
- [44] the composition of any one of [1] to [43], wherein the first amino acid is attached to the 3' end of the first tRNA;
- [45] the composition of any one of [1] to [44], wherein the second amino acid is attached to the 3' end of the second tRNA;
- 25 [46] the composition of any one of [1] to [45], wherein the first tRNA is a tRNA having a base sequence consisting of A, U, G, and C;
- [47] the composition of any one of [1] to [46], wherein the second tRNA is a tRNA having a base sequence consisting of A, U, G, and C;
- [48] the composition of any one of [1] to [47], wherein the first tRNA is a tRNA to which an
- 30 amino acid is attached outside a translation system;
- [49] the composition of any one of [1] to [48], wherein the second tRNA is a tRNA to which an amino acid is attached outside a translation system;
- [50] the composition of any one of [1] to [49], wherein the first tRNA and the second tRNA are *E. coli*-derived tRNAs;
- 35 [51] the composition of any one of [1] to [50], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is:

- (1) 32U, 33U, 37G, and 38A;
- (2) 32A, 33U, 37G, and 38U;
- (3) 32A, 33U, 37A, and 38U; or
- (4) 32U, 33U, 37G, and 38U;

5 [52] the composition of any one of [1] to [51],

wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is:

- (1) 32U, 33U, 37G, and 38A;
- (2) 32A, 33U, 37G, and 38U; or
- 10 (3) 32A, 33U, 37A, and 38U;

[53] the composition of any one of [1] to [52],

wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is:

- (1) 32U, 33U, 37G, and 38A;
- 15 (3) 32A, 33U, 37A, and 38U; or
- (4) 32U, 33U, 37G, and 38U;

[54] the composition of any one of [1] to [53], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is either 32U, 33U, 37G, and 38A, or 32U, 33U, 37G, and 38U;

20 [55] the composition of any one of [1] to [54], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is 32U, 33U, 37G, and 38A;

[56] the composition of any one of [1] to [52], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is 32A, 33U, 37G, and 38U;

25 [57] the composition of any one of [1] to [53], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is 32A, 33U, 37A, and 38U;

[58] the composition of any one of [1] to [51], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is 32U, 33U, 37G, and 38U;

[59] the composition of any one of [1] to [50], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is 32U, 33U, 37A, and 38U;

30 [60] the composition of any one of [1] to [50], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is 32C, 33U, 37G, and 38A;

[61] the composition of any one of [1] to [60], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the second tRNA is:

- (1) 32U, 33U, 37G, and 38A;
- 35 (2) 32A, 33U, 37G, and 38U;
- (3) 32A, 33U, 37A, and 38U;

(4) 32U, 33U, 37G, and 38U;
 (5) 32U, 33U, 37A, and 38U; or
 (6) 32C, 33U, 37G, and 38A;
 [62] the composition of any one of [1] to [61], wherein the combination of bases at positions 32,
 5 33, 37, and 38 (tRNA numbering rule) in the second tRNA is:
 (1) 32U, 33U, 37G, and 38A;
 (2) 32A, 33U, 37G, and 38U;
 (3) 32A, 33U, 37A, and 38U; or
 (4) 32U, 33U, 37G, and 38U;
 10 [63] the composition of any one of [1] to [62], wherein a tRNA body of the second tRNA is a
 chimeric tRNA body;
 [64] the composition of any one of [1] to [63], which is a composition for cell-free translation;
 [65] the composition of any one of [1] to [64], which is a composition for reconstituted cell-free
 translation;
 15 [66] the composition of any one of [1] to [65], which is a composition for cell-free translation
 that has been reconstituted by an *E. coli*-derived factor;
 [67] the composition of any one of [1] to [66], comprising an *E. coli*-derived ribosome;
 [68] the composition of any one of [1] to [67], comprising an mRNA that has a codon
 complementary to an anticodon in the second tRNA;
 20 [69] the composition of any one of [1] to [68], comprising an mRNA that has a codon
 complementary to an anticodon in the first tRNA and a codon complementary to an anticodon in
 the second tRNA, wherein these complementary codons may optionally be included in the same
 or different mRNAs;
 [70] the composition of any one of [1] to [69], which does not comprise an aminoacyl-tRNA
 25 synthetase (aaRS) that can attach an amino acid to the first tRNA or the second tRNA;
 [71] the composition of any one of [1] to [70] (provided that, when the first tRNA and the second
 tRNA have a chimeric tRNA body which has the combination of bases specified in (4) of the
 above [1] at positions 32, 33, 37, and 38 (tRNA numbering rule), and which at positions 1 to 31
 and positions 39 to 74 (tRNA numbering rule) has the base sequences of positions 1 to 31 and
 30 positions 39 to 74 (tRNA numbering rule) of a tRNA having the base sequence of SEQ ID NO:
 255, a composition in which the combination of the anticodons in the first tRNA and the second
 tRNA is GCG and CCG, and a composition in which the combination of the anticodons in the
 first tRNA and the second tRNA is CCG and GCG, are excluded);
 [72] the composition of any one of [1] to [71] (provided that, when the first tRNA and the second
 35 tRNA have a chimeric tRNA body which has the combination of bases specified in (4) of the
 above [1] at positions 32, 33, 37, and 38 (tRNA numbering rule), a composition in which the

combination of the anticodons in the first tRNA and the second tRNA is GCG and CCG, and a composition in which the combination of the anticodons in the first tRNA and the second tRNA is CCG and GCG, are excluded);

[73] the composition of any one of [1] to [72] (provided that, when the first tRNA and the second tRNA have a chimeric tRNA body which has the combination of bases specified in (4) of the above [1] at positions 32, 33, 37, and 38 (tRNA numbering rule), a composition in which the combinations of the anticodons in the first tRNA and the second tRNA is AAG and UAG, a composition in which the combination of the anticodons in the first tRNA and the second tRNA is AAG and CAG, and a composition in which the combination of the anticodons in the first tRNA and the second tRNA is UAG and CAG, are excluded);

[74] the composition of any one of [1] to [73] (provided that, when the first tRNA and the second tRNA have a chimeric tRNA body which has the combination of bases specified in (4) of the above [1] at positions 32, 33, 37, and 38 (tRNA numbering rule), a composition in which both bases at the second letter of the anticodons in the first tRNA and the second tRNA are C and both bases at the third letter of the anticodons in the first tRNA and the second tRNA are G, is excluded);

[75] the composition of any one of [1] to [74] (provided that, when the first tRNA and the second tRNA have a chimeric tRNA body which has the combination of bases specified in (4) of the above [1] at positions 32, 33, 37, and 38 (tRNA numbering rule), a composition in which both bases at the second letter of the anticodons in the first tRNA and the second tRNA are A and both bases at the third letter of the anticodons in the first tRNA and the second tRNA are G, is excluded);

[76] the composition of any one of [1] to [75] (provided that a composition in which both bases at the second letter of the anticodons in the first tRNA and the second tRNA are C and both bases at the third letter of the anticodons in the first tRNA and the second tRNA are G, is excluded);

[77] the composition of any one of [1] to [76] (provided that, when the first tRNA has the combination of bases specified in (4) of the above [1] at positions 32, 33, 37, and 38 (tRNA numbering rule), a composition in which the base at the second letter of the anticodon in the first tRNA is A and the base at the third letter of the anticodon in the first tRNA is G, is excluded);

[78] the composition of any one of [1] to [77] (provided that, when the first tRNA has the combination of bases specified in (1) of the above [1] at positions 32, 33, 37, and 38 (tRNA numbering rule), compositions in which the anticodons in the first tRNA are UGG and CGG, are excluded);

[79] the composition of any one of [1] to [78] (provided that, when the first tRNA has the combination of bases specified in (2) of the above [1] at positions 32, 33, 37, and 38 (tRNA

numbering rule), compositions in which the anticodons in the first tRNA are AGG and GGG, are excluded);

[80] the composition of any one of [1] to [79] (provided that, when the first tRNA has a chimeric tRNA body which has the combination of bases specified in (6) of the above [1] at positions 32, 33, 37, and 38 (tRNA numbering rule), compositions in which the anticodons in the first tRNA are GCG and CCG, are excluded);

[81] the composition of any one of [1] to [80] (provided that, when the first tRNA has a chimeric tRNA body which has the combination of bases specified in (3) of the above [1] at positions 32, 33, 37, and 38 (tRNA numbering rule), compositions in which the anticodons in the first tRNA are AGC and GGC, are excluded);

[82] the composition of any one of [1] to [81], wherein the first tRNA and the second tRNA are elongator tRNAs;

[83] the composition of any one of [37] to [82], wherein the N-substituted amino acid is an N-methyl amino acid;

[84] the composition of any one of [1] to [83], wherein the amino acid is a translatable amino acid;

[85] the composition of any one of [1] to [84], wherein the second tRNA is a tRNA having a chimeric anticodon loop;

[86] a method for producing the composition for translation of any one of [1] to [85], which comprises preparing the first tRNA by attaching the first amino acid to a tRNA outside a translation system, and/or preparing the second tRNA by attaching the second amino acid to a tRNA outside a translation system;

[87] the method of [86], wherein the first tRNA and/or the second tRNA are prepared by at least one method selected from the group consisting of the pCpA method, the pdCpA method, a method using a flexizyme, and a method involving aaRS;

[88] the method of [86] or [87], comprising preparing the first tRNA by *in vitro* transcription; and

[89] the method of any one of [86] to [88], comprising preparing the second tRNA by *in vitro* transcription.

[0009]

[A1] a method for producing a peptide, comprising translating a nucleic acid using the composition for translation of any one of [1] to [85] or a composition for translation produced by the method of any one of [86] to [89];

[A2] a method for reducing misreading of a codon complementary to the anticodon in the second tRNA by the first tRNA, comprising translating a nucleic acid using the composition for translation of any one of [1] to [85] or a composition for translation produced by the method of

any one of [86] to [89];

[A3] the method of [A1] or [A2], wherein the nucleic acid is an mRNA carrying a codon complementary to an anticodon in the second tRNA;

[A4] the method of [A3], wherein the nucleic acid is an mRNA carrying a codon complementary to an anticodon in the first tRNA, and optionally, the codon complementary to an anticodon in the first tRNA and the codon complementary to an anticodon in the second tRNA may be included in the same or different mRNAs;

[A5] the method of any one of [A1] to [A4], comprising obtaining a peptide library by translating a nucleic acid library containing the above nucleic acids;

[A6] the method of any one of [A1] to [A5], wherein the codon complementary to an anticodon in the first tRNA is a codon that forms Watson-Crick base pairs at all three bases with the anticodon;

[A7] the method of any one of [A1] to [A6], wherein the codon complementary to an anticodon in the second tRNA is a codon that forms Watson-Crick base pairs at all three bases with the anticodon;

[A8] the method of any one of [A1] to [A5], wherein the third letter of the codon complementary to an anticodon in the first tRNA forms a wobble base pair with the first letter of the anticodon;

[A9] the method of any one of [A1] to [A6] or [A8], wherein the third letter of the codon complementary to the anticodon in the second tRNA forms a wobble base pair with the first letter of the anticodon;

[A10] the method of any one of [A1] to [A9], wherein the nucleic acid is mRNA;

[A11] a peptide or a peptide library produced by the method of any one of [A1] to [A10];

[A12] a peptide produced by using the composition for translation of any one of [1] to [85] or the composition for translation produced by the method of any one of [86] to [89];

[A13] a peptide library produced by using the composition for translation of any one of [1] to [85] or the composition for translation produced by the method of any one of [86] to [89]; and

[A14] a method for identifying a peptide that binds to a target molecule comprising: producing a peptide library using the method of any one of [A1] to [A10]; and contacting the target molecule with the peptide library.

[0010]

[B1] a method for reducing misreading of a second codon by a tRNA carrying an anticodon complementary to a first codon, comprising substituting at least one base selected from the group consisting of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the tRNA, wherein the bases at the first letters of the first codon and the second codon are the same,

the bases at the second letters of the first codon and the second codon are the same, and

the bases at the third letters of the first codon and the second codon are different from each

other;

[B2] the method of [B1], wherein, a combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) after the substitution is:

(1) 32U, 33U, 37G, and 38A;

5 (2) 32A, 33U, 37G, and 38U;

(3) 32A, 33U, 37A, and 38U;

(4) 32U, 33U, 37G, and 38U;

(5) 32U, 33U, 37A, and 38U; or

(6) 32C, 33U, 37G, and 38A;

10 and wherein, A is adenine, C is cytosine, G is guanine, and U is uracil;

[B3] the method of [B1] or [B2], comprising substituting at least one base selected from the group consisting of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in a tRNA carrying an anticodon complementary to the first codon;

[B4] the method of any one of [B1] to [B3], wherein the first codon is M_1M_2X and the second
15 codon is M_1M_2Y ,

the above M_1 and M_2 are each independently A, C, G, or U, and

the above X and Y are each selected from A, C, G, and U, and are different from each other;

[B5] the method of [B4], wherein the combination of the bases of the above X and Y is any one selected from the following group:

20 (a1) U and G; (a2) G and U; (a3) U and A; (a4) A and U; (a5) C and A; (a6) A and C; (a7) C and G; and (a8) G and C;

[B6] the method of [B4] or [B5], wherein the combination of the bases of the above X and Y is any one selected from the following group:

(a1) U and G; (a2) G and U; (a3) U and A; (a5) C and A; and (a7) C and G;

25 [B7] the method of [B5], wherein the combination of bases of the above X and Y is (a1) U and G or (a2) G and U;

[B8] the method of any one of [B5] to [B7], wherein the combination of bases of the above X and Y is (a1) U and G;

[B9] the method of any one of [B5] to [B7], wherein the combination of bases of the above X
30 and Y is (a2) G and U;

[B10] the method of [B5] or [B6], wherein the combination of bases of the above X and Y is (a3) U and A;

[B11] the method of [B5] or [B6], wherein the combination of bases of the above X and Y is (a5) C and A;

35 [B12] the method of [B5] or [B6], wherein the combination of bases of the above X and Y is (a7) C and G;

[B13] the method of any one of [B4] to [B12], wherein the above M_1M_2 is a base sequence of any one selected from the following group:

(b1) CC; (b2) CU; (b3) GG; (b4) GU; (b5) GC; (b6) UC; (b7) CG; and (b8) AC;

[B14] the method of [B13], wherein the above M_1M_2 is any one base sequence selected from the following group:

(b1) CC; (b2) CU; and (b3) GG;

[B15] the method of [B13] or [B14], wherein the base sequence of the above M_1M_2 is (b1) CC;

[B16] the method of [B13] or [B14], wherein the base sequence of the above M_1M_2 is (b2) CU;

[B17] the method of [B13] or [B14], wherein the base sequence of the above M_1M_2 is (b3) GG;

[B18] the method of any one of [B1] to [B17], wherein the first codon and the anticodon in the tRNA form Watson-Crick base pairs at all three bases;

[B19] the method of any one of [B1] to [B17], wherein the base at the third letter of the first codon and the base at the first letter of the anticodon in the tRNA form a wobble base pair;

[B20] the method of any one of [B1] to [B19], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the tRNA are derived from the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having the base sequence of at least one selected from the group consisting of (a) SEQ ID NO: 253, (b) SEQ ID NO: 255, and (c) SEQ ID NO: 254;

[B21] the method of any one of [B1] to [B20], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the tRNA are the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having the base sequence of at least one selected from the group consisting of (a) SEQ ID NO: 253, (b) SEQ ID NO: 255, and (c) SEQ ID NO: 254;

[B22] the method of any one of [B1] to [B21], wherein the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in the tRNA have sequence identity of 80% or more, preferably 90% or more, and more preferably 95% or more to the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) in a tRNA having the base sequence of any one of (a) to (c) of [B20];

[B23] the method of any one of [B1] to [B22], comprising attaching an amino acid to the tRNA outside a translation system;

[B24] the method of [B23], wherein attaching the amino acid to the tRNA outside a translation system is performed by at least one method selected from the group consisting of the pCpA method, the pdCpA method, a method using a flexizyme, and a method involving aaRS;

[B25] the method of [B23] or [B24], wherein the amino acid is an unnatural amino acid;

[B26] the method of [B23] to [B25], wherein the unnatural amino acid is an N-substituted amino acid;

[B27] the method of any one of [B1] to [B26], further comprising synthesizing the tRNA from a template nucleic acid by *in vitro* transcription;

[B28] the method of any one of [B1] to [B27], wherein the base at position 37 (tRNA numbering rule) before the engineering of the tRNA is A;

5 [B29] the method of any one of [B1] to [B28], wherein the base at position 33 (tRNA numbering rule) before the engineering of the tRNA is U;

[B30] the method of any one of [B1] to [B29], wherein the base at position 32 (tRNA numbering rule) before the engineering of the tRNA is C or U;

[B31] the method of any one of [B1] to [B30], wherein the base at position 32 (tRNA numbering rule) before the engineering of the tRNA is C;

10 [B32] the method of any one of [B1] to [B30], wherein the base at position 32 (tRNA numbering rule) before the engineering of the tRNA is U;

[B33] the method of any one of [B1] to [B32], wherein the base at position 38 (tRNA numbering rule) before the engineering of the tRNA is A or C;

15 [B34] the method of any one of [B1] to [B33], wherein the base at position 38 (tRNA numbering rule) before the engineering of the tRNA is A;

[B35] the method of any one of [B1] to [B33], wherein the base at position 38 (tRNA numbering rule) before the engineering of the tRNA is C;

[B36] the method of any one of [B1] to [B35], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) after the substitution is:

20 (1) 32U, 33U, 37G, and 38A;

(2) 32A, 33U, 37G, and 38U;

(3) 32A, 33U, 37A, and 38U; or

(4) 32U, 33U, 37G, and 38U;

25 [B37] the method of any one of [B1] to [B36], wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) after the substitution is:

(1) 32U, 33U, 37G, and 38A;

(2) 32A, 33U, 37G, and 38U; or

(3) 32A, 33U, 37A, and 38U;

30 [B38] the method of any one of [B1] to [B37] (provided that, when the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) after the substitution is the combination specified in (4) of [B2], and the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) of the tRNA are the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) of a tRNA having the base sequence of SEQ ID NO: 255, a method

35 in which the combination of the first codon and the second codon is CGC and CGG, and a method in which the combination of the first codon and the second codon is CGG and CGC, are

excluded);

[B39] the method of any one of [B1] to [B38], further comprising translating a nucleic acid in a translation system comprising a tRNA subjected to base substitution;

[B40] the method of any one of [B26] to [B39], wherein the N-substituted amino acid is an N-methylamino acid; and

[B41] the method of any one of [B23] to [B40], wherein the amino acid is a translatable amino acid.

[Effects of the Invention]

[0011]

Compositions for translation containing a tRNA that has specific bases at positions 32, 33, 37, and 38 (tRNA numbering rule), methods for producing a peptide comprising translating a nucleic acid using this tRNA, and such were provided in the present disclosure. Using the compositions, methods, and such of the present disclosure enables reducing the rate of mistranslation into unintended amino acids attributable to misreading of codons by tRNAs during peptide synthesis.

[Brief Description of Drawings]

[0012]

Fig. 1 shows the rate of misreading of the CCG codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the CCG codon (see Table 6 for specific amount of translation) in the coexistence of tRNAs carrying cgg and agg as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 242) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 241) (μM) x 100

Fig. 2 shows the rate of misreading of the CCU codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the CCU codon (see Table 7 for specific amount of translation) in the coexistence of tRNAs carrying agg and cgg as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated

peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 242) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 241) (μM) x 100

Fig. 3 shows the rate of misreading of the GGG codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the GGG codon (see Table 8 for specific amount of translation) in the coexistence of tRNAs carrying ccc and acc as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243) (μM) x 100

Fig. 4 shows the rate of misreading of the GGU codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the GGU codon (see Table 9 for specific amount of translation) in the coexistence of tRNAs carrying acc and ccc as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243) (μM) x 100

Fig. 5 shows the rate of misreading of the CUG codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the CUG codon (see Table 10 for specific amount of translation) in the coexistence of tRNAs carrying cag and aag as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239) (μM) x 100

Fig. 6 shows the rate of misreading of the CUU codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the CUU codon (see Table 11 for specific

amount of translation) in the coexistence of tRNAs carrying aag and cag as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239) (μM) x 100

Fig. 7 shows the rate of misreading of the CUG codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(AsnE2). The values in the figure are based on the results of translating an mRNA carrying the CUG codon (see Table 12 for specific amount of translation) in the coexistence of tRNAs carrying cag and aag as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239) (μM) x 100

Fig. 8 shows the rate of misreading of the CUU codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(AsnE2). The values in the figure are based on the results of translating an mRNA carrying the CUU codon (see Table 13 for specific amount of translation) in the coexistence of tRNAs carrying aag and cag as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239) (μM) x 100

Fig. 9 shows the rate of misreading of the CUG codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Asp1). The values in the figure are based on the results of translating an mRNA carrying the CUG codon (see Table 14 for specific amount of translation) in the coexistence of tRNAs carrying cag and aag as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240) (μM)/ amount of translated peptide corresponding to the sequence

BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239) (μM) $\times 100$

Fig. 10 shows the rate of misreading of the CUU codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Asp1). The values in the figure are based on the results of translating an mRNA carrying the CUU codon (see Table 15 for specific amount of translation) in the coexistence of tRNAs carrying aag and cag as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240) (μM) / amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239) (μM) $\times 100$

Fig. 11 shows the rate of misreading of the GGA codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the GGA codon (see Table 16 for specific amount of translation) in the coexistence of tRNAs carrying ucc and acc as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244) (μM) / amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243) (μM) $\times 100$

Fig. 12 shows the rate of misreading of the GGA codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the GGA codon (see Table 17 for specific amount of translation) in the coexistence of tRNAs carrying ucc and gcc as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244) (μM) / amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243) (μM) $\times 100$

Fig. 13 shows the rate of misreading of the GGG codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the GGG codon (see Table 18 for specific amount of translation) in the coexistence of tRNAs carrying ccc and gcc as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis,

was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244) (μM)/ amount of translated peptide corresponding to the sequence

5 BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243) (μM) x 100

Fig. 14 shows the rate of misreading of the GGG codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the GGG codon (see Table 21 for specific amount of translation) in the coexistence of tRNAs carrying acc and ccc as anticodons, and the
10 percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:Pic2:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 245) (μM)/ amount of translated peptide corresponding to the sequence

15 BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243) (μM) x 100

Fig. 15 shows the rate of misreading of the GGG codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the GGG codon (see Table 22 for specific amount of translation) in the coexistence of tRNAs carrying acc and ccc as anticodons, and the
20 percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeG:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 246) (μM)/ amount of translated peptide corresponding to the sequence

25 BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243) (μM) x 100

Fig. 16 shows the rate of misreading of the GGG codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the GGG codon (see Table 23 for specific amount of translation) in the coexistence of tRNAs carrying acc and ccc as anticodons, and the
30 percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Leu:Phe:dA:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 248) (μM)/ amount of translated peptide corresponding to the sequence

35 BdpF:Thr:Phe:Ile:Ile:Leu:Phe:nBuG:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 247) (μM) x 100

Fig. 17 shows the rate of misreading of the CCG codon according to differences in the

combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the CCG codon (see Table 24 for specific amount of translation) in the coexistence of tRNAs carrying cgg and agg as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 242) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 249) (μM) x 100

Fig. 18 shows the rate of misreading of the CCU codon according to differences in the combination of bases at positions 32, 33, 37, and 38 of tRNA(Glu2). The values in the figure are based on the results of translating an mRNA carrying the CCU codon (see Table 25 for specific amount of translation) in the coexistence of tRNAs carrying agg and cgg as anticodons, and the percentage (%) of misread peptides relative to an intended product, which is on the vertical axis, was calculated from the following equation:

percentage of misread peptides relative to the intended product (%) = amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 242) (μM)/ amount of translated peptide corresponding to the sequence BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 249) (μM) x 100

[Description of Embodiments]

[0013]

For the purpose of interpreting this specification, the following definitions will apply and whenever applicable, terms used in the singular will also include the plural, and vice versa. It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. If any of the following definitions conflict with any document incorporated herein by reference, the following definitions shall control.

[0014]

“Codon” refers to a set of three bases (triplet) that corresponds to each amino acid, when genetic information in a living body is translated to a protein. For DNA, four bases, adenine (A), guanine (G), cytosine (C), and thymine (T), are used. For mRNA, four bases, adenine (A), guanine (G), cytosine (C) and uracil (U), are used. The table showing the correspondence between each codon and amino acid is called the genetic code table or codon table, and 20 amino acids are assigned to 61 codons excluding the stop codon (Table 1). The genetic code table shown in Table 1 is used commonly for almost all eukaryote and prokaryote (eubacteria and archaea); therefore, it is called the standard genetic code table or the universal

genetic code table. In the present disclosure, a genetic code table used for naturally-occurring organisms is referred to as the natural genetic code table, and it is distinguished from an artificially reprogrammed genetic code table (the correspondence between codons and amino acids is engineered). In the genetic code table, generally, four codons which are the same in the first and second letters and which differ only in the third letter are grouped into one box, and this group is called a codon box. In the present disclosure, a specific codon box may be represented by positioning “M” referring to any base selected from A, C, G, and U, after the bases at the first and second letters of the codon. For example, the codon box assigned to Ser in the natural genetic code table, in which U is at the first letter and C is at the second letter of the codons, is denoted as “UCM”, and the codon box assigned to Pro is denoted as “CCM”.

[Table 1]

	U		C		A		G		
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC		UCC		UAC		UGC		C
	UUA	Leu	UCA		UAA	Stop	UGA	Stop	A
	UUG		UCG		UAG		UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	Gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	Lys	AGA	Arg	A
	AUG	Met	ACG		AAG		AGG		G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	Glu	GGA		A
	GUG		GCG		GAG		GGG		G

[0015]

In the present disclosure, a codon in mRNA may be expressed as “M₁M₂M₃”. Here, M₁, M₂, and M₃ represent the bases for the first letter, the second letter, and the third letter of the codon, respectively.

[0016]

“Anticodon” refers to three consecutive bases on tRNA that correspond to a codon on the mRNA. Similar to mRNA, four bases, adenine (A), guanine (G), cytosine (C), and uracil

(U), are used for the anticodon. Furthermore, modified bases obtained by modifying these bases may be used. When the codon is specifically recognized by the anticodon, the genetic information on the mRNA is read and translated into a protein. The codon sequence on the mRNA in the 5' to 3' direction and the anticodon sequence on the tRNA in the 5' to 3' direction bind complementarily; therefore, complementary base pairs are formed between the bases for the first, second, and third letters of the codon, and the bases for the third, second, and first letters of the anticodon, respectively. In the present disclosure, "modified bases" refer to bases having structures partially different from those of A, C, G, and U.

[0017]

In the present disclosure, an anticodon in the first tRNA may be represented by "N₁₁N₁₂N₁₃" and an anticodon in the second tRNA may be represented by "N₂₁N₂₂N₂₃". Here, N₁₁, N₁₂, and N₁₃, and N₂₁, N₂₂, and N₂₃ represent the bases at the first letter, second letter, and third letter of the anticodons, respectively. According to the tRNA numbering rule described below, N₁₁, N₁₂, and N₁₃, and N₂₁, N₂₂, and N₂₃ are numbered as positions 34, 35, and 36 of the tRNAs, respectively.

[0018]

In the present disclosure, bases A, C, G, U, and T may be denoted by lowercase letters, but the uppercase letters and lowercase letters are used synonymously; for example, GGG and ggg are used synonymously.

[0019]

In the present disclosure, thermodynamically stable base pairs are referred to as being "complementary" to each other. In addition to Watson-Crick base pairs such as adenine and uracil (A-U) and guanine and cytosine (G-C), a non-Watson-Crick-type wobble base pair formed between guanine and uracil (G-U) is also included in the "complementary" base pairs of the present disclosure. In particular, since there is some spatial fluctuation (wobble) between the third letter of the codon and the first letter of the anticodon, formation of a non-Watson-Crick base pair, as described above, may be permitted (wobble hypothesis).

[0020]

In the present disclosure, a constant relationship between a codon and an anticodon may be referred to as "complementary". A codon-anticodon relationship where Watson-Crick base pairs are formed between the first letter of the codon and the third letter of the anticodon and between the second letter of the codon and the second letter of the anticodon, and a Watson-Crick-type or where a wobble base pair is formed between the third letter of the codon and the first letter of the anticodon is referred to as "complementary". For example, anticodons complementary to the UCU codon are AGA and GGA, and codons complementary to the GCG anticodon are CGC and CGU.

[0021]

“Messenger RNA (mRNA)” refers to an RNA that carries genetic information that can be translated into a protein. Genetic information is coded on mRNA as codons, and each of these codons corresponds to one among all 20 different amino acids. Protein translation begins at the initiation codon and ends at the stop codon. In principle, the initiation codon in eukaryotes is AUG, but in prokaryotes (eubacteria and archaea), GUG and UUG may also be used as initiation codons in addition to AUG. AUG is a codon that encodes methionine (Met), and in eukaryotes and archaea, translation is initiated directly from methionine. On the other hand, in eubacteria, only the initiation codon AUG corresponds to N-formylmethionine (fMet); therefore, translation is initiated from formylmethionine. There are three stop codons: UAA (ochre), UAG (amber), and UGA (opal). When the stop codon is recognized by a protein called a translation termination factor (release factor (RF)), the peptide chain synthesized up to that point is dissociated from the tRNA, and the translation process ends.

[0022]

“Transfer RNA (tRNA)” refers to a short RNA of 100 bases or less that mediates peptide synthesis using mRNA as a template. In terms of secondary structure, it has a cloverleaf-like structure consisting of three stem loops (the D arm, the anticodon arm, and the T arm) and one stem (the acceptor stem). Depending on the tRNA, an additional variable loop may be included. The anticodon arm has a region consisting of three consecutive bases called an anticodon, and the codon is recognized when the anticodon forms a base pair with the codon on the mRNA. Meanwhile, a nucleic acid sequence (CCA sequence) consisting of cytidine-cytidine-adenosine exists at the 3' end of tRNA, and an amino acid is added to the adenosine residue at the end (specifically, the hydroxyl group at position 2 or position 3 of the ribose of the adenosine residue and the carboxyl group of the amino acid form an ester bond). A tRNA to which an amino acid is bound is called “an aminoacyl tRNA”. In the present disclosure, aminoacyl tRNA is also included in the definition of tRNA. Further, as described later, a method is known in which two terminal residues (C and A) are removed from the CCA sequence of tRNA and then this is used for the synthesis of aminoacyl-tRNA. Such a tRNA from which the CA sequence at the 3' end has been removed is also included in the definition of tRNA in the present disclosure. Addition of amino acids to tRNA is carried out by an enzyme called aminoacyl-tRNA synthetase (aaRS or ARS), *in vivo*. Usually, there is one aminoacyl-tRNA synthetase for each amino acid, and each aminoacyl-tRNA synthetase specifically recognizes only a specific tRNA as a substrate from multiple tRNAs; accordingly, correspondence between tRNAs and amino acids is strictly controlled.

[0023]

Each base in a tRNA is numbered according to the tRNA numbering rule (Sprinzl et al.,

Nucleic Acids Res (1998) 26: 148-153). In the present disclosure, bases in the tRNAs are numbered according to this numbering rule. For example, the anticodon is numbered as positions 34 to 36 and the CCA sequence at the 3' end is numbered as positions 74 to 76, respectively.

5 [0024]

In the present disclosure, to indicate a base at a specific position in a tRNA, the tRNA numbering rule (Sprinzl et al., Nucleic Acids Res (1998) 26: 148-153) and the base abbreviations (A, C, G, or U) are used. For example, "32U" means that the base at position 32 according to the tRNA numbering rule is U (uracil). Furthermore, these are used to indicate substitutions of bases at specific positions in tRNA. For example, "C32U" means substitution from C (cytosine) to U (uracil) at position 32 according to the tRNA numbering rule.

10 [0025]

"Anticodon loop" refers to the bases at positions 32 to 38 in tRNA, or more specifically seven consecutive bases containing three consecutive bases of the anticodon and two bases each on the 5' side and 3' side of the anticodon. Four types of bases, adenine (A), guanine (G), cytosine (C), and uracil (U), are used in the anticodon loop. Modified bases obtained by modifying them may also be used.

15 [0026]

A "tRNA body" in the present disclosure refers to the main part (the main structural portion composed of nucleic acids) of the tRNA excluding the anticodon (positions 34 to 36). In some embodiments, the tRNA body of the present disclosure refers to positions 1 to 33 and positions 37 to 76 in a tRNA. In another embodiment, the tRNA body of the present disclosure refers to positions 1 to 33 and positions 37 to 74 in a tRNA. A "chimeric tRNA body" refers to a tRNA body in which a portion of the tRNA body is derived from a specific source or a specific type of tRNA, while the remaining portion is derived from a different source or a different type of tRNA. Chimeric tRNA bodies do not include tRNA bodies derived from only a single type of tRNA. A chimeric tRNA body may be a tRNA body derived from two or more types of tRNAs, or it may be derived from 3 or more types of tRNAs. A chimeric tRNA body of the present disclosure may be a chimeric tRNA body whose combination of bases at positions 32, 33, 37, and 38 and the base sequences of the other portions have different origins. An example of a chimeric tRNA body is a tRNA body whose combination of bases at positions 32, 33, 37, and 38 is derived from tRNA Pro2, and the remaining portions of the nucleic acid sequence are derived from tRNA Glu2. In the present disclosure, to determine the origin of the combination of bases at positions 32, 33, 37, and 38 of a certain tRNA, one can refer to the combination of bases at positions 32, 33, 37, and 38 in the base sequences (SEQ ID NOs: 274 to 319) of tRNAs derived from *Escherichia coli*, and determine that it is derived from a tRNA having the same

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combination of bases at positions 32, 33, 37, and 38. For example, the base combination CUxxxAC of positions 32, 33, 37, and 38, can be determined to be derived from tRNA Glu2 or tRNA Asp1 by referring to SEQ ID NOs: 274 to 319. In this case, tRNA bodies whose base sequences of the portions other than positions 32, 33, 37, and 38 are derived from tRNA Glu2 or tRNA Asp1, are not included in the chimeric tRNA bodies, since these tRNA bodies are tRNA bodies derived from only a single type of tRNA. Based on the base sequence information for positions 32 to 38 in a tRNA, the tRNA origin can be determined according to the following criteria: UUxxxAC = Ala1B; AUxxxAU = Ala2; CUxxxGA = Arg3; CUxxxAC = Glu2 or Asp1; UUxxxGU = Leu2, Leu1, or Leu3; UUxxxAA = Phe, Cys, Gly1, Gly3, Leu4, Leu5, Thr1, Thr2, or Thr3; AUxxxGU = Pro2; UUxxxGA = Pro3 or Pro1; CUxxxAA = Ser5, Arg2, Arg4, Arg5, Asn, Gly2, Ile1, Ile2, Lys, Met, Ser1, Ser2, Ser3, Thr4, Trp, Tyr1, Tyr2, or Val1; and UUxxxAU = Val2 (collective term for Val2A and Val2B), Gln1, Gln2, or His (here, xxx refers to the three bases of the anticodon). For example, the base combination at positions 32, 33, 37, and 38 in tRNAs whose base sequence is UUxxxGU for positions 32 to 38 can be determined to be derived from tRNA Leu2, tRNA Leu1, or tRNA Leu3.

[0027]

A base, combination of bases, or base sequence is “derived from” a certain origin refers to the base, combination of bases, or base sequence or a sequence highly similar to that base, combination of bases, or base sequence being isolated from a certain origin. For example, when a base, combination of bases, or base sequence constituting a tRNA is isolated from a specific type of tRNA, the base, combination of bases, or base sequence is described as being “derived from” the specific type of tRNA.

[0028]

In the present disclosure, a tRNA may be described as follows:

- “tRNA Xxx” or “tRNA(Xxx)”: refers to a tRNA body having a specific base sequence distinguished by Xxx. Examples include tRNA Glu2 or tRNA(Glu2), tRNA Ser5 or tRNA(Ser5), tRNA AsnE2 or tRNA(AsnE2), and tRNA Asp1 or tRNA(Asp1).
- “tRNA(Xxx)nnn”: refers to a full length tRNA whose anticodon sequence is nnn, which is a tRNA carrying a specific tRNA body distinguished by Xxx. Examples include tRNA(Glu2)uga and tRNA(AsnE2)uga.
- “tRNA(Xxx+Yyy)”: refers to a chimeric tRNA body which has a specific base sequence distinguished by Xxx at positions other than positions 32, 33, 37, and 38, and has a specific combination of bases distinguished by Yyy at positions 32, 33, 37, and 38. Examples include tRNA(Glu2+Ser5) and tRNA(AsnE2+Phe).
- “tRNA(Xxx+Yyy)nnn”: refers to a full length tRNA whose anticodon sequence is nnn, which is a tRNA carrying a chimeric tRNA body which has a specific base sequence distinguished by

Xxx at positions other than positions 32, 33, 37, and 38, and has a combination of specific bases distinguished by Yyy at positions 32, 33, 37, and 38. Examples include tRNA(Glu2+Ser5)uga and tRNA(AsnE2+Phe)uga.

· “-CA” may be added at the end of the name of the above tRNA to indicate that the CA sequence at the 3’ end of the tRNA has been removed. Examples include tRNA(Glu2)-CA, tRNA(Glu2)uga-CA, tRNA(Glu2+Ser5)-CA, and tRNA(Glu2+Ser5)uga-CA.

[0029]

In the present disclosure, a specific combination of bases at positions 32, 33, 37, and 38 in a tRNA may be denoted by the name of the tRNA from which it is derived. In the present disclosure, the base sequence of positions 32 to 38 is denoted XXxxxXX, and the combination of bases at positions 32, 33, 37, and 38 in a tRNA is named as follows:

Ala1B sequence = UUxxxAC; Ala2 sequence = AUxxxAU; Arg3 sequence = CUxxxGA; Glu2 sequence = CUxxxAC; Leu2 sequence = UUxxxGU; Phe sequence = UUxxxAA; Pro2 sequence = AUxxxGU; Pro3 sequence = UUxxxGA; Ser5 sequence = CUxxxAA; and Val2 sequence =

[0030]

“Initiator tRNA” is a specific tRNA used at the start of mRNA translation. The initiator tRNA attached to the initiator amino acid is catalyzed by a translation initiation factor (IF), introduced into the ribosome, and binds to the initiation codon on the mRNA, thereby translation is initiated. Since AUG, which is a methionine codon, is generally used as an initiation codon, the initiator tRNA has an anticodon corresponding to AUG, and has methionine (formylmethionine for prokaryotes) attached to it as the initiator amino acid. Examples of the initiator tRNA include tRNA fMet (SEQ ID NOs: 283 and 284).

[0031]

“Elongator tRNA” is a tRNA used in the elongation reaction of a peptide chain in the translation step. In peptide synthesis, amino-acid-attached elongator tRNA is sequentially transported to the ribosome by the GTP bound translation elongation factor (EF) EF-Tu/eEF-1, and this promotes the peptide chain elongation reaction. Examples of the elongator tRNA include tRNAs corresponding to various amino acids (SEQ ID NOs: 274 to 282 and 285 to 319).

[0032]

“Translation system” in the present disclosure is defined as a composition for translating a peptide (it may be called a “composition for translation” in the present disclosure). A typical translation system contains as constituent components, ribosomes, translation factors, tRNAs, amino acids, aminoacyl-tRNA synthetase (aaRS), and factors necessary for peptide translation reactions such as ATP and GTP, but is not limited thereto. The main types of translation systems include translation systems that utilize living cells and translation systems that utilize cell extract

solutions (cell-free translation systems (used synonymously to “compositions for cell-free translation” in the present disclosure)). As the translation system utilizing living cells, a known example is a system in which a desired aminoacyl-tRNA and mRNA are introduced into living cells such as *Xenopus* oocytes and mammalian cells by the microinjection method or the lipofection method to perform peptide translation (Nowak et al., Science (1995) 268: 439-442). Known examples of cell-free translation systems include translation systems that utilize extract solutions from *E. coli* (Chen et al., Methods Enzymol (1983) 101: 674-690), yeast (Gasior et al., J Biol Chem (1979) 254: 3965-3969), wheat germ (Erickson et al., Methods Enzymol (1983) 96: 38-50), rabbit reticulocytes (Jackson et al., Methods Enzymol (1983) 96: 50-74), HeLa cells (Barton et al., Methods Enzymol (1996) 275: 35-57), insect cells (Swerdel et al., Comp Biochem Physiol B (1989) 93: 803-806), and such. Such a translation system can be appropriately prepared by a method known to those skilled in the art or a similar method. The cell-free translation system also includes a translation system constructed by isolating and purifying each of the factors required for peptide translation and reconstituting them (reconstituted cell-free translation system) (Shimizu et al., Nat Biotech (2001) 19: 751-755). Reconstituted cell-free translation systems may usually include ribosomes, amino acids, tRNAs, aminoacyl-tRNA synthetases (aaRS), translation initiation factors (for example, IF1, IF2, and IF3), translation elongation factors (for example, EF-Tu, EF-Ts, and EF-G), translation termination factors (for example, RF1, RF2, and RF3), ribosome recycling factors (RRF), NTPs as energy sources, energy regeneration systems, and other factors required for translation, but are not limited thereto. When the transcription reaction from DNA is also performed, RNA polymerases and the like may be further included. Various factors contained in the cell-free translation system can be isolated and purified by methods well known to those skilled in the art, and a reconstituted cell-free translation system can be appropriately constructed using them. Alternatively, a commercially available reconstituted cell-free translation system such as PUREfrex® from Gene Frontier or PURExpress® from New England BioLabs can be used. For a reconstituted cell-free translation system, a desired translation system can be constructed by reconstituting only the necessary components from the constituent components of the translation system.

[0033]

An aminoacyl-tRNA, synthesized in a translation system by including a specific combination of amino acid, tRNA, and aminoacyl-tRNA synthetase in the translation system, is used for peptide translation. Instead of the above, aminoacyl-tRNA prepared outside the translation system can be directly used as a constituent component of the translation system (this is sometimes called a “pre-charge method” in the present disclosure). Examples of the pre-charge method include the method of attaching an amino acid to a tRNA using aaRS outside a

translation system, the pdCpA method, the pCpA method, and the method using an artificial RNA catalyst (flexizyme). In particular, when amino acids that are difficult to aminoacylate with an aaRS, such as some unnatural amino acids, are used for translation, it is desirable to use the pre-charge method which uses as a constituent component a tRNA aminoacylated in advance with an unnatural amino acid by the pdCpA method, the pCpA method, or the method using a flexizyme.

[0034]

The translation is started by adding an mRNA to the translation system. An mRNA usually contains a sequence that encodes the target peptide, and may further include a sequence for increasing the efficiency of the translation reaction (for example, a Shine-Dalgarno (SD) sequence in prokaryotes, or a Kozac sequence in eukaryotes). Pre-transcribed mRNA may be added directly to the system, or instead of mRNA, a template DNA containing a promoter and an RNA polymerase appropriate for the DNA (for example, T7 promoter and T7 RNA polymerase) can be added to the system so that mRNA will be transcribed from the template DNA.

[0035]

“Misreading of a codon” refers to introduction of an unintended amino acid by translation, due to the recognition of a codon not complementary to a specific anticodon by an aminoacyl tRNA carrying the specific anticodon. An example is unintentional introduction of the amino acid acylated on the tRNA by translation, due to the erroneous recognition of the CCG codon by an aminoacyl tRNA carrying the AGG anticodon complementary to the CCU codon. In the natural genetic code table, Pro is assigned to both the CCG and CCU codons; therefore, the amino acid introduced by translation is not different whether or not such misreading takes place. However, when the genetic code table is reprogrammed such that different amino acids are assigned to the CCG and CCU codons, this codon misreading may become a problem.

[0036]

In the present disclosure, “alkyl” is a monovalent group derived from an aliphatic hydrocarbon by removing one arbitrary hydrogen atom; it does not contain a hetero atom or an unsaturated carbon-carbon bond in the skeleton; and it has a subset of hydrocarbyl or hydrocarbon-group structures containing hydrogen and carbon atoms. The length of the carbon chain length, *n*, is in the range of 1 to 20. The examples of alkyl include C₂-C₁₀ alkyl, C₁-C₆ alkyl, and C₁-C₃ alkyl, and specific examples include methyl, ethyl, propyl, butyl, pentyl, hexyl, isopropyl, t-butyl, sec-butyl, 1-methylpropyl, 1,1-dimethylpropyl, 2,2-dimethylpropyl, 1,2-dimethylpropyl, 1,1,2-trimethylpropyl, 1,2,2-trimethylpropyl, 1,1,2,2-tetramethylpropyl, 1-methylbutyl, 2-methylbutyl, 3-methylbutyl, 1,1-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 2,2-dimethylbutyl, 2,3-dimethylbutyl, 3,3-dimethylbutyl, 1-ethylbutyl, 2-ethylbutyl, isopentyl, and neopentyl.

[0037]

In the present disclosure, “cycloalkyl” means a saturated or partially saturated cyclic monovalent aliphatic hydrocarbon group, and includes a monocyclic ring, a bicyclic ring, and a spiro ring. Examples of cycloalkyl include C₃-C₁₀ cycloalkyl, and specific examples include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, and bicyclo[2.2.1]heptyl.

[0038]

In the present disclosure, “alkenyl” is a monovalent group having at least one double bond (two adjacent SP² carbon atoms). Depending on the arrangement of double bonds and substituents (if present), the geometric configuration of the double bond can be entgegen (E) or zusammen (Z), and cis or trans configurations. It can be a straight chain or branched chain alkenyl, and includes a straight chain alkenyl containing an internal olefin. Examples of the alkenyl include C₂-C₁₀ alkenyl and C₂-C₆ alkenyl, and specific examples include vinyl, allyl, 1-propenyl, 2-propenyl, 1-butenyl, 2-butenyl (including cis and trans), 3-butenyl, pentenyl, and hexenyl.

[0039]

In the present disclosure, “alkynyl” is a monovalent group having at least one triple bond (two adjacent SP carbon atoms). It can be a straight or branched chain alkynyl, and includes an internal alkylene. Examples of the alkynyl include C₂-C₁₀ alkynyl and C₂-C₆ alkynyl, and specific examples include ethynyl, 1-propynyl, propargyl, 3-butylnyl, pentynyl, hexynyl, 3-phenyl-2-propynyl, 3-(2'-fluorophenyl)-2-propynyl, 2-hydroxy-2-propynyl, 3-(3-fluorophenyl)-2-propynyl, and 3-methyl-(5-phenyl)-4-pentynyl.

[0040]

In the present disclosure, “aryl” means a monovalent aromatic hydrocarbon ring. Examples of the aryl include C₆-C₁₀ aryl, and specific examples include phenyl and naphthyl (such as 1-naphthyl and 2-naphthyl).

[0041]

In the present disclosure, “heteroaryl” means a monovalent aromatic ring group containing a hetero atom in the atoms constituting the ring, and may be partially saturated. The ring may be a monocyclic ring or a fused bicyclic ring (for example, a bicyclic heteroaryl formed by fusing with benzene or a monocyclic heteroaryl). The number of atoms constituting the ring is, for example, five to ten (5- to 10-membered heteroaryl). The number of heteroatoms contained in the ring-constituting atoms is, for example, one to five. Specific examples of the heteroaryl include furyl, thienyl, pyrrolyl, imidazolyl, pyrazolyl, thiazolyl, isothiazolyl, oxazolyl, isoxazolyl, oxadiazolyl, thiadiazolyl, triazolyl, tetrazolyl, pyridyl, pyrimidyl, pyridazinyl, pyrazinyl, triazinyl, benzofuranyl, benzothienyl, benzothiadiazolyl, benzothiazolyl,

benzoxazolyl, benzooxadiazolyl, benzimidazolyl, indolyl, isoindolyl, indazolyl, quinolyl, isoquinolyl, cinnolinyl, quinazolinyl, quinoxalinyl, benzodioxolyl, indoliziny, and imidazopyridyl.

[0042]

5 In the present disclosure, “arylalkyl (aralkyl)” is a group containing both aryl and alkyl, and means, for example, a group in which at least one hydrogen atom of the above-mentioned alkyl is substituted with aryl. Examples of the aralkyl include C₅-C₁₀ aryl C₁-C₆ alkyl, and specific examples include benzyl.

[0043]

10 In the present disclosure, “alkylene” means a divalent group derived by further removing one arbitrary hydrogen atom from the above-mentioned “alkyl”, and may be linear or branched. Examples of the straight chain alkylene include C₂-C₆ straight chain alkylene, C₄-C₅ straight chain alkylene and the like. Specific examples include -CH₂-, -(CH₂)₂-, -(CH₂)₃-, -(CH₂)₄-, -(CH₂)₅-, and -(CH₂)₆-. Examples of the branched alkylene include C₂-C₆ branched
15 alkylene and C₄-C₅ branched alkylene. Specific examples include -CH(CH₃)CH₂-, -C(CH₃)₂-, -CH(CH₃)CH₂CH₂-, -C(CH₃)₂CH₂-, -CH₂CH(CH₃)CH₂-, CH₂C(CH₃)₂-, and -CH₂CH₂CH(CH₃)-.
[0044]

In the present disclosure, “alkenylene” means a divalent group derived by further removing one arbitrary hydrogen atom from the above-mentioned “alkenyl”, and may be linear
20 or branched. Depending on the arrangement of double bonds and substituents (if present), it can take the form of entgegen (E) or zusammen (Z), and cis or trans configurations. Examples of the straight chain alkenylene include C₂-C₆ straight chain alkenylene and C₄-C₅ straight chain alkenylene. Specific examples include -CH=CH-, -CH=CHCH₂-, -CH₂CH=CH-, -CH=CHCH₂CH₂-, -CH₂CH=CHCH₂-, -CH₂CH₂CH=CH-, -CH=CHCH₂CH₂CH₂-, -CH₂CH=CHCH₂CH₂-, -CH₂CH₂CH=CHCH₂-, and -CH₂CH₂CH₂CH=CH-.
25 [0045]

In the present disclosure, “arylene” means a divalent group derived by further removing one arbitrary hydrogen atom from the above-mentioned aryl. The ring may be a monocyclic ring or a fused ring. The number of atoms constituting the ring is not particularly limited, but is, for
30 example, six to ten (C₆-C₁₀ arylene). Specific examples of arylene include phenylene and naphthylene.

[0046]

In the present disclosure, “heteroarylene” means a divalent group derived by further removing one arbitrary hydrogen atom from the above-mentioned heteroaryl. The ring may be a
35 monocyclic ring or a fused ring. The number of atoms constituting the ring is not particularly limited, but is, for example, five to ten (5- to 10-membered heteroarylene). As the heteroarylene,

specific examples include pyrrolediyl, imidazoldiyl, pyrazole-diyl, pyridinediyl, pyridazinediyl, pyrimidinediyl, pyrazinediyl, triazole-diyl, triazinediyl, isoxazole-diyl, oxazole-diyl, oxadiazole-diyl, isothiazole-diyl, thiazole-diyl, thiadiazole-diyl, furandiyl, and thiophenediyl.
[0047]

5 <Compositions, kits, and methods>

In one aspect, the present disclosure relates to compositions for translation and kits for translation, comprising a first tRNA to which a first amino acid is attached and a second tRNA to which a second amino acid is attached. In one aspect, the present disclosure relates to methods for translating a nucleic acid using a first tRNA to which a first amino acid is attached and a
10 second tRNA to which a second amino acid is attached. Using these compositions, kits, and methods can reduce or suppress mistranslation into unintended amino acids attributable to misreading of codons by tRNAs. Therefore, in one aspect, the present disclosure relates to methods for producing peptides while reducing or suppressing misreading of codons by tRNAs, and compositions, kits, and such to be used for such methods.

15 [0048]

Compositions in the present disclosure may contain in addition to tRNAs of the present disclosure, buffer, substances, and such generally used for nucleic acid translation. Furthermore, in one aspect, tRNAs of the present disclosure may be provided with various substances generally used for peptide translation by packaging them in advance as kits. In a further aspect,
20 various substances included in the kits of the present disclosure may be in powder form or liquid form depending on the manner of use. Furthermore, these may be stored in appropriate containers, and used when appropriate.

[0049]

<tRNA and tRNA body>

25 In some embodiments, a combination of bases at positions 32, 33, 37, and 38 of the tRNAs of the present disclosure may be

- (1) 32U, 33U, 37G, and 38A;
- (2) 32A, 33U, 37G, and 38U;
- (3) 32A, 33U, 37A, and 38U;
- 30 (4) 32U, 33U, 37G, and 38U;
- (5) 32U, 33U, 37A, and 38U; or
- (6) 32C, 33U, 37G, and 38A.

In some embodiments, a tRNA of the present disclosure may be a tRNA whose positions 32, 33, 37, and 38 are a combination of bases selected from the group consisting of: the
35 above (1) to (4); the above (1) to (3); or the above (1), (3), and (4); or the combination of bases of the above (1); the above (2); the above (3); the above (4); the above (5); or the above (6). In

several embodiments, none of the bases at positions 32, 33, 37, and 38 in a tRNA of the present disclosure are modified bases.

[0050]

In the present disclosure, the anticodon in the first tRNA of the present disclosure may be represented by $N_{11}N_{12}N_{13}$, and the anticodon in the second tRNA of the present disclosure may be represented by $N_{21}N_{22}N_{23}$. The above N_{11} , N_{12} , and N_{13} , and $N_{21}N_{22}N_{23}$ may be each independently A, C, G, or U. The first, second, and third letters of the anticodon in a tRNA of the present disclosure may be each independently A, C, G, or U. The base sequence of the second and third letters of the anticodon in a tRNA of the present disclosure may be CC, GC, AC, GU, CG, GG, AG, or GA; alternatively, it may be GG, AG, or CC. In some embodiments, the base sequence of the second and third letters of the above-mentioned anticodon is not CG. In some embodiments, a tRNA of the present disclosure may not have a modified base in the anticodon, or may have a modified base at the first letter of the anticodon, or may have the later-described modification on a nucleoside at this first letter.

[0051]

In some embodiments, the tRNA of the present disclosure is a prokaryote-derived tRNA or a eukaryote-derived tRNA. A tRNA may be produced by engineering a prokaryote-derived tRNA or a eukaryote-derived tRNA, and the tRNA produced by the engineering may have the highest base sequence identity with the prokaryote-derived tRNA or the eukaryote-derived tRNA. Eukaryotes are further classified into animals, plants, fungi, and protists. The tRNA of the present disclosure may be, for example, a human-derived tRNA. Prokaryotes are further classified into eubacteria and archaea. Examples of eubacteria include *E. coli*, *Bacillus subtilis*, lactic acid bacteria, and *Desulfitobacterium hafniense*. Examples of archaea include extreme halophile, thermophile, or methane bacteria (for example, *Methanosarcina mazei*, *Methanosarcina barkeri*, and *Methanocaldococcus jannaschii*). The tRNA of the present disclosure may be, for example, tRNA derived from *E. coli*, *Desulfitobacterium hafniense*, or *Methanosarcina mazei*.

[0052]

In some embodiments, a tRNA of the present disclosure may differ from the base sequence of the reference tRNA in one or more bases; in 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, or 12 or more bases; in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 bases; or in 15 or fewer, 14 or fewer, 13 or fewer, 12 or fewer, 11 or fewer, 10 or fewer, 9 or fewer, 8 or fewer, 7 or fewer, 6 or fewer, 5 or fewer, 4 or fewer, 3 or fewer, or 2 or fewer bases. In some embodiments, a tRNA of the present disclosure may have a sequence identity of 80% or higher, 85% or higher, 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, or

98% or higher relative to the base sequence of the reference tRNA. In the present disclosure, the “percent (%) sequence identity” relative to a certain base sequence is defined as the percentage of bases in a candidate sequence that are identical with the bases in the reference base sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent base sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, Megalign (DNASTAR) software, or GENETYX® (GENETYX CORPORATION). Those skilled in the art can determine appropriate parameters for achieving sequence alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0053]

In some embodiments, a tRNA of the present disclosure may be different in the base sequence of the anticodon loop (positions 32-38) from the base sequence of a reference tRNA. In a certain embodiment, a tRNA of the present disclosure may have a chimeric anticodon loop. “Chimeric anticodon loop” in the present disclosure means an anticodon loop in which the bases of positions 32, 33, 37, and 38 and the base sequence of the anticodon are derived from different tRNAs. For example, whether an anticodon loop containing the UGA anticodon is a chimeric anticodon loop can be determined as follows. First, since tRNA Ser1 (SEQ ID NO: 306) derived from *E. coli* is identified as a tRNA carrying the UGA anticodon, the UGA anticodon can be determined to be derived from tRNA Ser 1 (when determining the origin of an anticodon, one can refer to the base sequences of tRNAs set forth in SEQ ID NOs: 274 to 319). Here, the base sequence of the anticodon loop of the above-mentioned tRNA Ser1 is CUugaAA. Therefore, when the bases at positions 32, 33, 37, and 38 in the anticodon loop have a combination of bases other than that in the Ser5 sequence (32C, 33U, 37A, and 38A), this anticodon loop is determined to be a chimeric anticodon loop. In a certain embodiment, the anticodon loop in a tRNA of the present disclosure may be different from the anticodon loop (positions 32 to 38 according to the tRNA numbering rule) included in a tRNA having the base sequence of any one of SEQ ID NOs: 274 to 282, 285 to 304, and 306 to 319. In some embodiments, a tRNA body of the present disclosure may be different from the base sequence of a reference tRNA body in one or more bases; in 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, or 12 or more bases; in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 bases; or in 15 or fewer, 14 or fewer, 13 or fewer, 12 or fewer, 11 or fewer, 10 or fewer, 9 or fewer, 8 or fewer, 7 or fewer, 6 or fewer, 5 or fewer, 4 or fewer, 3 or fewer, or 2 or fewer bases. In some embodiments, a tRNA body of the present disclosure may have a sequence identity of 80% or higher, 85% or higher, 90% or higher, 91% or higher, 92% or higher, 93% or higher,

94% or higher, 95% or higher, 96% or higher, 97% or higher, or 98% or higher relative to the base sequence of the reference tRNA body. In some embodiments, a tRNA of the present disclosure may be different from a reference tRNA at least in the base of one position selected from the group consisting of positions 32, 33, 37, and 38. In some embodiments, a tRNA body of the present disclosure may be different from a reference tRNA body at least in the base of one position selected from the group consisting of positions 32, 33, 37, and 38. In some embodiments, a tRNA of the present disclosure is characterized in that it is not a naturally occurring tRNA. In an embodiment, a tRNA body of the present disclosure may be a tRNA body not derived from a tRNA having the base sequence of SEQ ID NO: 275, and/or a tRNA body not derived from any of the tRNAs having the base sequences of SEQ ID NOs: 294, 295, and 296, and/or a tRNA body not derived from any of the tRNAs having the base sequences of SEQ ID NOs: 302, 303, and 304. In an embodiment, when positions 32, 33, 37, and 38 in a tRNA body of the present disclosure have the Leu2 sequence, the tRNA body may not be derived from any of the tRNAs having the base sequences of SEQ ID NOs: 294, 295, and 296; when these positions have the Ala2 sequence, the tRNA body may not be derived from a tRNA having the base sequence of SEQ ID NO: 275; when these positions have the Pro2 sequence or the Pro3 sequence, the tRNA body may not be derived from any of the tRNAs having the base sequences of SEQ ID NOs: 302, 303, and 304.

[0054]

A reference tRNA and a reference tRNA body of the present disclosure may each be a natural tRNA derived from any organism (for example, *E. coli*) or a body thereof; or an unnatural tRNA formed by artificially synthesizing a sequence different from that of a natural tRNA, or a body thereof; or a tRNA formed by artificially synthesizing the sequence of a natural tRNA or such (artificial tRNA), or a body thereof; or a tRNA chimera formed by artificially combining tRNAs of different origins, or a body thereof.

[0055]

A reference tRNA or a reference tRNA body of the present disclosure may each be selected appropriately from tRNAs or tRNA bodies carrying any base sequences. In some embodiments, the reference tRNA or reference tRNA body may be at least one tRNA selected from the group consisting of tRNA Ala, tRNA Arg, tRNA Asn, tRNA Asp, tRNA Cys, tRNA Gln, tRNA Glu, tRNA Gly, tRNA His, tRNA Ile, tRNA Leu, tRNA Lys, tRNA Met, tRNA Phe, tRNA Pro, tRNA Ser, tRNA Thr, tRNA Trp, tRNA Tyr, tRNA Val, and tRNA Sec (selenocysteine) (SEQ ID NOs: 274 to 282, and 285 to 319), and tRNA Glu2, tRNA AsnE2, and tRNA Asp1 (SEQ ID NOs: 322 to 324), or a body thereof. tRNA fMet (SEQ ID NOs: 283 and 284), tRNA Pyl (pyrrolysine), tRNA AsnE2 (see, Ohta, A.; Murakami, H.; Higashimura, E.; Suga, H. Chem. Biol. 2007, 14, 1315-1322) and bodies thereof may also be used as references.

Furthermore, tRNA Pro1E2 (see, WO2019/077887), which is a tRNA chimera formed by transferring the T stem of tRNA Glu2 to tRNA Pro1 and further mutating it, and a body thereof, may also be used as references. In a certain embodiment, the tRNA or tRNA body of the present disclosure may be at least one tRNA selected from the group consisting of tRNA Glu2, tRNA Asp1, and tRNA AsnE2, or a body thereof. Exemplary base sequences for positions 1 to 74 of some tRNA bodies are shown in SEQ ID NOs: 253 to 255, 320, and 321. The reference tRNA bodies presented as examples herein may also be used as sequences from which the portions other than the anticodon loop in a chimeric tRNA body of the present disclosure originate. [0056]

In some embodiments, the difference of the tRNA or tRNA body of the present disclosure from the reference tRNA or the reference tRNA body may be generated by engineering a portion of the sequence of the reference tRNA or the reference tRNA body based on the sequence information thereof (for example, the base sequence information). In this case, once the sequence information of the tRNA or the tRNA body of the present disclosure is obtained, the tRNA or the tRNA body of the present disclosure can be prepared without requiring the reference sequence information thereafter. In the present disclosure, “engineer” means introducing to the base sequence of an existing tRNA or a tRNA body (existing sequence), at least 1, or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 or more of at least one type of alteration selected from the following group: (i) addition (adding any new base to the existing sequence); (ii) deletion (removing any nucleotide from the existing sequence); (iii) substitution (replacing any base in the existing sequence with any other base); (iv) insertion (adding any new nucleotide between any two nucleotides in the existing sequence); and (v) modification (changing a part of the structure of any nucleoside (for example, the base portion or the sugar portion) in the existing sequence to another structure). Engineering can be performed on any structure (for example, D arm, anticodon arm, T arm, acceptor stem, and variable loop) of the tRNA or tRNA body. In a certain embodiment, engineering of the tRNA or tRNA body of the present disclosure is performed on at least 1, 2 or more, 3 or more, or 4 bases selected from the group consisting of positions 32, 33, 37, and 38, or the group consisting of positions 32, 37, and 38 in the tRNA. In some embodiments, the tRNA and tRNA body of the present disclosure may not be those actually engineered based on a reference tRNA or a reference tRNA body. tRNAs or tRNA bodies carrying a base sequence that would be obtained if a reference tRNA or a reference tRNA body were engineered as mentioned above, are also included in the tRNAs and tRNA bodies of the present disclosure.

[0057]

Engineering in the present disclosure includes, for example, substituting bases such that the combination of bases at positions 32, 33, 37, and 38 in a tRNA becomes the same as the

combination of bases at the corresponding positions in the tRNA of the present disclosure. The base prior to substitution is not particularly limited; however, for example, the base at position 32 before substitution may be C or U, the base at position 37 before substitution may be A, the base at position 38 before substitution may be A or C, and/or the base at position 33 before substitution may be U. In some embodiments, when the base at position 32, 33, 37, or 38 is already the desired base, substitution is unnecessary. For example, when the base at position 33 of tRNA before substitution is U, and the desired base at position 33 is also U, substitution of the base at position 33 is unnecessary.

[0058]

In some embodiments, tRNAs of the present disclosure may carry a chimeric tRNA body. Chimeric tRNA bodies include, for example, those whose anticodon loop portion (*i.e.*, positions 32, 33, 37, and 38) and the other portions of the tRNA body are derived from different tRNA bodies. The base sequences of positions 1 to 74 in the chimeric tRNA bodies are exemplified below:

[0059]

[Table 2]

Name of chimeric tRNA body	SEQ ID NO:	Origin of the parts other than the anticodon loop in the tRNA body	Origin of the combination of bases at positions 34,33,37,and 38
tRNA(Glu2+Leu2)-CA	256	tRNA Glu2	tRNA Leu2
tRNA(Glu2+Pro3)-CA	257		tRNA Pro3
tRNA(Glu2+Pro2)-CA	258		tRNA Pro2
tRNA(Glu2+Ala2)-CA	259		tRNA Ala2
tRNA(Glu2+Val2)-CA	260		tRNA Val2
tRNA(Glu2+Arg3)-CA	261		tRNA Arg3
tRNA(Asp1+Leu2)-CA	262	tRNA Asp1	tRNA Leu2
tRNA(Asp1+Pro3)-CA	263		tRNA Pro3
tRNA(Asp1+Pro2)-CA	264		tRNA Pro2
tRNA(Asp1+Ala2)-CA	265		tRNA Ala2
tRNA(Asp1+Val2)-CA	266		tRNA Val2
tRNA(Asp1+Arg3)-CA	267		tRNA Arg3
tRNA(AsnE2+Leu2)-CA	268	tRNA AsnE2	tRNA Leu2
tRNA(AsnE2+Pro3)-CA	269		tRNA Pro3
tRNA(AsnE2+Pro2)-CA	270		tRNA Pro2
tRNA(AsnE2+Ala2)-CA	271		tRNA Ala2
tRNA(AsnE2+Val2)-CA	272		tRNA Val2
tRNA(AsnE2+Arg3)-CA	273		tRNA Arg3

[0060]

In some embodiments, the base sequence of positions 32 to 38 in a tRNA of the present disclosure may be different from the base sequence of positions 32 to 38 in a wildtype tRNA of *E. coli* or a naturally occurring tRNA. Without limitation, in some embodiments, when tRNAs of the present disclosure carry a chimeric tRNA body which has the combination of bases 32U, 33U, 37G, and 38A, tRNAs whose anticodon(s) is/are UGG and/or CGG may be excluded from the tRNAs of the present disclosure. In some embodiments, when tRNAs of the present disclosure carry a chimeric tRNA body which has the combination of bases 32A, 33U, 37G, and 38U, tRNAs whose anticodon(s) is/are AGG and/or GGG may be excluded from the tRNAs of the present disclosure. In some embodiments, when tRNAs of the present disclosure carry a chimeric tRNA body which has the combination of bases 32A, 33U, 37A, and 38U, tRNAs whose anticodon(s) is/are AGC and/or GGC may be excluded from the tRNAs of the present disclosure. In some embodiments, when tRNAs of the present disclosure carry a chimeric tRNA body which has the combination of bases 32C, 33U, 37G, and 38A, tRNAs whose anticodon(s)

is/are UCG and/or CCG may be excluded from the tRNAs of the present disclosure. In some embodiments, when tRNAs of the present disclosure carry a chimeric tRNA body which has the combination of bases 32U, 33U, 37G, and 38U, tRNAs whose anticodon has A as the base at the second letter and G as the base at the third letter may be excluded from the tRNAs of the present disclosure.

[0061]

In some embodiments, although the base sequences other than those of the anticodon loops are not particularly limited in the tRNAs of the present disclosure, they may be selected from sequences other than those of tRNA Ala, tRNA Pro, and tRNA Leu. Here, the base sequences other than those of the anticodon loops may be base sequences at positions 1 to 31 and positions 39 to 76 of tRNA.

[0062]

In some embodiments, the base sequences of positions 1 to 31 and positions 39 to 74 in a tRNA of the present disclosure are not particularly limited, but they may be derived from the base sequences of positions 1 to 31 and positions 39 to 74 in a tRNA having the base sequence of at least one selected from the group consisting of (a) SEQ ID NO: 253, (b) SEQ ID NO: 255, and (c) SEQ ID NO: 254. In a particular embodiment, the base sequences of positions 1 to 31 and positions 39 to 74 in a tRNA of the present disclosure may be the base sequences of positions 1 to 31 and positions 39 to 74 in a tRNA having the base sequence of at least one selected from the group consisting of the above (a) to (c). In some embodiments, the base sequences of positions 1 to 31 and positions 39 to 74 in a tRNA of the present disclosure may have a sequence identity of 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, or 98% or more to the base sequences of positions 1 to 31 and positions 39 to 74 in a tRNA having the base sequence of any one of the above (a) to (c).

[0063]

In some embodiments, a tRNA of the present disclosure may have the base C at position 75 and the base A at position 76. Positions 75 and 76 in a tRNA of the present disclosure may be pCpA (a dinucleotide containing cytidine and adenosine) or pdCpA (a dinucleotide containing deoxycytidine and adenosine). In some embodiments, a tRNA of the present disclosure may be a tRNA which has an amino acid attached to its 3' end, or more specifically, to the adenosine residue at its 3' end, or even more specifically, to the adenosine residue at position 76 which is its 3' end.

[0064]

Modification in the present disclosure includes, for example, a modification performed on the base or nucleoside of the first letter of the anticodon in tRNA (for example, replacement

with lysidine, a lysidine derivative, agmatidine, or an agmatidine derivative). Here, a lysidine derivative is a molecule produced by modifying a part of the structure of lysidine (for example, the base portion), and which has the same codon discrimination ability (ability to form complementary base pairs) as that of lysidine when used as a part of an anticodon. Furthermore, an agmatidine derivative is a molecule produced by modifying a part of the structure of agmatidine (for example, the base portion) and which has the same codon discrimination ability (ability to form complementary base pairs) as that of agmatidine when used as a part of an anticodon. In the present disclosure, bases that have undergone modifications presented as examples herein or other modifications may be called “modified bases”. In the present disclosure, “L” in the base sequence or the nucleic acid sequence means lysidine.

[0065]

Lysidine in natural tRNA is synthesized by the action of an enzyme called tRNA Ile-lysine synthetase (TilS). TilS has the activity of specifically recognizing tRNA corresponding to isoleucine (tRNA Ile2) as a substrate, and altering (converting) cytidine (C) at the first letter (N₁) of its anticodon to lysidine (k2C). The lysidine in a tRNA of the present disclosure may be synthesized with or without the mediation of TilS.

[0066]

In some embodiments, tRNAs of the present disclosure may not contain modified bases. In the present disclosure, a tRNA prepared by *in vitro* transcription may be called “transcribed tRNA”. The tRNAs of the present disclosure may be transcribed tRNAs, and they may be transcribed tRNAs not containing modified bases. In the present disclosure, the term “artificial tRNA” may be used to distinguish these from naturally-occurring tRNAs. The tRNAs of the present disclosure may be artificial tRNAs, and they may be artificial tRNAs not containing modified bases or modified nucleosides. Methods for producing tRNAs not containing modified bases, tRNAs not containing modified nucleosides, transcribed tRNAs, and artificial tRNAs are not particularly limited; however, they may be prepared, for example, by synthesizing tRNAs from template DNAs by *in vitro* transcription reaction using RNA polymerases such as T7 RNA polymerase, and purifying the RNAs when necessary. RNeasy kit (Qiagen) and such can be used for RNA purification. In some embodiments, the bases of the tRNAs of the present disclosure are composed of A, C, G, and U. In some embodiments, tRNAs of the present disclosure may have bases consisting of A, C, G, and U.

[0067]

In some embodiments, an amino acid may be attached to a tRNA of the present disclosure. The amino acid is normally attached to the 3' end of a tRNA, or more specifically to the adenosine residue of the CCA sequence at the 3' end. In a certain embodiment, the above 3' end adenosine residue may be at position 76 according to the tRNA numbering rule. The

specific types of amino acids attached to tRNA can each be appropriately selected from the amino acids described below, and examples include unnatural amino acids.

[0068]

The amino acids in the present disclosure include α -amino acids, β -amino acids, and γ -amino acids. Regarding three-dimensional structures, both L-type amino acids and D-type amino acids are included. Furthermore, amino acids in the present disclosure include natural and unnatural amino acids. The natural amino acids consist of the following 20 α -amino acids: glycine (Gly), alanine (Ala), serine (Ser), threonine (Thr), valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), histidine (His), glutamic acid (Glu), aspartic acid (Asp), glutamine (Gln), asparagine (Asn), cysteine (Cys), methionine (Met), lysine (Lys), arginine (Arg), and proline (Pro). Natural amino acids are usually L-type amino acids.

[0069]

In the present disclosure, unnatural amino acids refer to all amino acids excluding the above-mentioned natural amino acids consisting of 20 α -amino acids. Examples of unnatural amino acids include β -amino acids, γ -amino acids, D-type amino acids, α -amino acids whose side chains differ from natural amino acids, α,α -disubstituted amino acids, amino acids whose main chain amino group has a substituent (also referred to as “N-substituted amino acids” in this disclosure), and hydroxycarboxylic acid (hydroxy acid). Examples of N-substituted amino acids include, N-methyl amino acid, N-ethyl amino acid, N-propyl amino acid, N-butyl amino acid, but is not limited thereto. The side chain of the unnatural amino acid is not particularly limited, but may have, for example, alkyl, alkenyl, alkynyl, aryl, heteroaryl, aralkyl, and cycloalkyl, in addition to the hydrogen atom. Further, in the case of an α,α -disubstituted amino acid, two side chains may form a ring. Furthermore, these side chains may have one or more substituents. In a particular embodiment, the substituents can be selected from any functional group containing a halogen atom, O atom, S atom, N atom, B atom, Si atom, or P atom. For example, in the present disclosure, “C₁-C₆ alkyl having halogen as a substituent” means a “C₁-C₆ alkyl” in which at least one hydrogen atom in an alkyl is substituted with a halogen atom, and specific examples include, trifluoromethyl, difluoromethyl, fluoromethyl, pentafluoroethyl, tetrafluoroethyl, trifluoroethyl, difluoroethyl, fluoroethyl, trichloromethyl, dichloromethyl, chloromethyl, pentachloroethyl, tetrachloroethyl, trichloroethyl, dichloroethyl, and chloroethyl. In addition, for example, “C₅-C₁₀ aryl C₁-C₆ alkyl having a substituent” means “C₅-C₁₀ aryl C₁-C₆ alkyl” in which at least one hydrogen atom in aryl and/or alkyl is substituted with a substituent. Furthermore, the meaning of the phrase “having two or more substituents” includes having a certain functional group (for example, a functional group containing an S atom) as a substituent, and the functional group has another substituent (for example, a substituent such as amino or halogen). For specific examples of unnatural amino acids, one can refer to WO2013/100132, WO2018/143145, and such.

[0070]

The amino group of the main chain of the unnatural amino acid may be an unsubstituted amino group (-NH_2 group) or a substituted amino group (-NHR group). Here, R indicates an alkyl, alkenyl, alkynyl, aryl, heteroaryl, aralkyl, or cycloalkyl which optionally has a substituent.

Further, like proline, the carbon chain attached to the N atom of the main chain amino group and the α -position carbon atom may form a ring. The substituent can be selected from any functional group containing a halogen atom, O atom, S atom, N atom, B atom, Si atom, or P atom.

Examples of alkyl substitution of an amino group include N-methylation, N-ethylation, N-propylation, and N-butylation, and example of aralkyl substitution of an amino group include N-benzylation. Specific examples of an N-methylamino acid include N-methylalanine, N-methylglycine, N-methylphenylalanine, N-methyltyrosine, N-methyl-3-chlorophenylalanine, N-methyl-4-chlorophenylalanine, N-methyl-4-methoxyphenylalanine, N-methyl-4-thiazolealanine, N-methylhistidine, N-methylserine and N-methylaspartic acid.

[0071]

Examples of a substituent containing a halogen atom include fluoro (-F), chloro (-Cl), bromo (-Br), and iodo (-I).

[0072]

Examples of a substituent containing an O atom include hydroxyl (-OH), oxy (-OR), carbonyl (-C=O-R), carboxyl ($\text{-CO}_2\text{H}$), oxycarbonyl (-C=O-OR), carbonyloxy (-O-C=O-R), thiocarbonyl (-C=O-SR), carbonylthio (-S-C=O-R), aminocarbonyl (-C=O-NHR), carbonyl amino (-NH-C=O-R), oxycarbonyl amino (-NH-C=O-OR), sulfonyl amino ($\text{-NH-SO}_2\text{-R}$), aminosulfonyl ($\text{-SO}_2\text{-NHR}$), sulfamoyl amino ($\text{-NH-SO}_2\text{-NHR}$), thiocarboxyl (-C(=O)-SH), carboxyl carbonyl ($\text{-C(=O)-CO}_2\text{H}$).

[0073]

Examples of oxy (-OR) include alkoxy, cycloalkoxy, alkenyloxy, alkynyloxy, aryloxy, heteroaryloxy, and aralkyloxy.

[0074]

Examples of carbonyl (-C=O-R) include formyl (-C=O-H), alkylcarbonyl, cycloalkylcarbonyl, alkenylcarbonyl, alkynylcarbonyl, arylcarbonyl, heteroarylcarbonyl, and aralkylcarbonyl.

[0075]

Examples of oxycarbonyl (-C=O-OR) include alkyloxycarbonyl, cycloalkyloxycarbonyl, alkenyloxycarbonyl, alkynyloxycarbonyl, aryloxycarbonyl, heteroaryloxycarbonyl, and aralkyloxycarbonyl.

[0076]

Examples of carbonyloxy (-O-C=O-R) include alkylcarbonyloxy,

cycloalkylcarbonyloxy, alkenylcarbonyloxy, alkynylcarbonyloxy, arylcarbonyloxy, heteroarylcarbonyloxy, and aralkylcarbonyloxy.

[0077]

Examples of thiocarbonyl ($-\text{C}=\text{O}-\text{SR}$) include alkylthiocarbonyl, cycloalkylthiocarbonyl, alkenylthiocarbonyl, alkynylthiocarbonyl, arylthiocarbonyl, heteroarylthiocarbonyl, and aralkylthiocarbonyl.

[0078]

Examples of carbonylthio ($-\text{S}-\text{C}=\text{O}-\text{R}$) include alkylcarbonylthio, cycloalkylcarbonylthio, alkenylcarbonylthio, alkynylcarbonylthio, arylcarbonylthio, heteroarylcarbonylthio, and aralkylcarbonylthio.

[0079]

Examples of aminocarbonyl ($-\text{C}=\text{O}-\text{NHR}$) include alkylaminocarbonyl, cycloalkylaminocarbonyl, alkenylaminocarbonyl, alkynylaminocarbonyl, arylaminocarbonyl, heteroarylaminocarbonyl, and aralkylaminocarbonyl. Furthermore, the H atom attached to the N atom in $-\text{C}=\text{O}-\text{NHR}$ may be substituted with a substituent selected from the group consisting of alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl.

[0080]

Examples of carbonylamino ($-\text{NH}-\text{C}=\text{O}-\text{R}$) include alkylcarbonylamino, cycloalkylcarbonylamino, alkenylcarbonylamino, alkynylcarbonylamino, arylcarbonylamino, heteroarylcarbonylamino, and aralkylcarbonylamino. Furthermore, the H atom attached to the N atom in $-\text{NH}-\text{C}=\text{O}-\text{R}$ may be substituted with a substituent selected from the group consisting of alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl.

[0081]

Examples of oxycarbonylamino ($-\text{NH}-\text{C}=\text{O}-\text{OR}$) include alkoxycarbonylamino, cycloalkoxycarbonylamino, alkenyloxycarbonylamino, alkynyloxycarbonylamino, aryloxycarbonylamino, heteroaryloxycarbonylamino, and aralkyloxycarbonylamino. Furthermore, the H atom attached to the N atom in $-\text{NH}-\text{C}=\text{O}-\text{OR}$ may be substituted with a substituent selected from the group consisting of alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl.

[0082]

Examples of sulfonylamino ($-\text{NH}-\text{SO}_2-\text{R}$) include alkylsulfonylamino, cycloalkylsulfonylamino, alkenylsulfonylamino, alkynylsulfonylamino, arylsulfonylamino, heteroarylsulfonylamino, and aralkylsulfonylamino. Furthermore, the H atom attached to the N atom in $-\text{NH}-\text{SO}_2-\text{R}$ may be substituted with a substituent selected from the group consisting of alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl.

[0083]

Examples of aminosulfonyl ($-\text{SO}_2\text{--NHR}$) include alkylaminosulfonyl, cycloalkylaminosulfonyl, alkenylaminosulfonyl, alkynylaminosulfonyl, arylaminosulfonyl, heteroarylaminosulfonyl, and aralkylaminosulfonyl. Furthermore, the H atom attached to the N atom in $-\text{SO}_2\text{--NHR}$ may be substituted with a substituent selected from the group consisting of alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl.

[0084]

Examples of sulfamoylamino ($-\text{NH--SO}_2\text{--NHR}$) include alkylsulfamoylamino, cycloalkylsulfamoylamino, alkenylsulfamoylamino, alkynylsulfamoylamino, arylsulfamoylamino, heteroarylaminosulfonyl, and aralkylsulfamoylamino. Furthermore, at least one of the two H atoms attached to the N atoms in $-\text{NH--SO}_2\text{--NHR}$ may be substituted with a substituent selected from the group consisting of alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl. When the two H atoms are both substituted, a substituent may each be independently selected, or these two substituents may form a ring.

[0085]

Examples of a substituent containing an S atom include thiol ($-\text{SH}$), thio ($-\text{S--R}$), sulfinyl ($-\text{S=O--R}$), sulfonyl ($-\text{S(O)}_2\text{--R}$), and sulfo ($-\text{SO}_3\text{H}$).

[0086]

Examples of thio ($-\text{S--R}$) include alkylthio, cycloalkylthio, alkenylthio, alkynylthio, arylthiol, heteroarylthio, and aralkylthio.

[0087]

Examples of sulfinyl ($-\text{S=O--R}$) include alkylsulfinyl, cycloalkylsulfinyl, alkenylsulfinyl, alkynylsulfinyl, arylsulfinyl, heteroarylsulfinyl, and aralkylsulfinyl.

[0088]

Examples of sulfonyl ($-\text{S(O)}_2\text{--R}$) include alkylsulfonyl, cycloalkylsulfonyl, alkenylsulfonyl, alkynylsulfonyl, arylsulfonyl, heteroarylsulfonyl, and aralkylsulfonyl.

[0089]

Examples of a substituent containing an N atom include azide ($-\text{N}_3$), cyano ($-\text{CN}$), primary amino ($-\text{NH}_2$), secondary amino ($-\text{NH--R}$), tertiary amino ($-\text{NR(R')}$), amidino ($-\text{C(=NH)--NH}_2$), substituted amidino ($-\text{C(=NR)--NR'R''}$), guanidino ($-\text{NH=C(=NH)--NH}_2$), substituted guanidino ($-\text{NR--C(=NR''')--NR'R''}$), and aminocarbonylamino ($-\text{NR--CO--NR'R''}$).

[0090]

Examples of the secondary amino ($-\text{NH--R}$) include alkylamino, cycloalkylamino, alkenylamino, alkynylamino, arylamino, heteroarylaminosulfonyl, and aralkylamino.

[0091]

The two substituents R and R' on the N atom in the tertiary amino ($-\text{NR(R')}$) can each be independently selected from the group consisting of alkyl, cycloalkyl, alkenyl, alkynyl, aryl,

heteroaryl, and aralkyl. Examples of the tertiary amino include, for example, alkyl(aralkyl)amino. These two substituents may form a ring.

[0092]

The three substituents R, R', and R'' on the N atom in the substituted amidino ($\text{C}(=\text{NR})\text{--NR}'\text{R}''$) can each be independently selected from the group consisting of a hydrogen atom, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl. Examples of the substituted amidino include alkyl(aralkyl)(aryl)amidino. These substituents may together form a ring.

[0093]

The four substituents R, R', R'', and R''' on the N atom in the substituted guanidino ($\text{NR--C}(=\text{NR}''')\text{--NR}'\text{R}''$) can each be independently selected from the group consisting of a hydrogen atom, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl. These substituents may together form a ring.

[0094]

The three substituents R, R', and R'' on the N atom in the aminocarbonylamino ($\text{--NR--CO--NR}'\text{R}''$) can each be independently selected from the group consisting of a hydrogen atom, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl. These substituents may together form a ring.

[0095]

Examples of a substituent containing a B atom include boryl ($\text{--BR}(\text{R}')$) and dioxyboryl (--B(OR)(OR')). The two substituents R and R' on the B atom can each be independently selected from the group consisting of a hydrogen atom, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, and aralkyl. These substituents may together form a ring.

[0096]

The hydroxycarboxylic acid in the present disclosure includes α -hydroxycarboxylic acid, β -hydroxycarboxylic acid, and γ -hydroxycarboxylic acid. A side chain other than a hydrogen atom may be attached to the carbon at the α -position in the hydroxycarboxylic acid, as with amino acids. Regarding three-dimensional structures, both the L-type and D-type can be included. The structure of the side chain can be defined similarly to the side chain of the above-mentioned natural amino acid or unnatural amino acid. Examples of hydroxycarboxylic acids include hydroxyacetic acid, lactic acid, and phenyllactic acid.

[0097]

The amino acid in the present disclosure may be a translatable amino acid. As used in the present disclosure, a "translatable" amino acid means amino acids that can be incorporated into a peptide by translational synthesis (for example, using the translation system described in this disclosure). Whether a certain amino acid is translatable can be confirmed by a translation

synthesis experiment using a tRNA to which the amino acid is attached. A reconstituted cell-free translation system may be used in the translation synthesis experiment (see for example, WO2013100132).

[0098]

5 In some embodiments, examples of the amino acid in the present disclosure include Pic2 ((2S)-piperidine-2-carboxylic acid), dA ((2R)-2-aminopropanoic acid), MeHph ((2S)-2-(methylamino)-4-phenyl-butanoic acid), SPh2Cl ((2S)-2-amino-3-(2-chlorophenoxy)propanoic acid), MeG (2-(methylamino)acetic acid), nBuG (2-(butylamino)acetic acid), and the like.

[0099]

10 The unnatural amino acid according to the present disclosure can be prepared by a conventionally known chemical synthesis method, a synthesis method described in the later-discussed Examples, or a synthesis method similar thereto.

[0100]

<Preparation of tRNA>

15 A tRNA can be synthesized, for example, by preparing a DNA encoding a desired tRNA gene, then placing an appropriate promoter such as T7, T3, or SP6 upstream of the DNA, and performing a transcription reaction with the DNA as a template using an RNA polymerase adapted to each promoter. Furthermore, tRNA can also be prepared by purification from biological materials. For example, tRNA can be recovered by preparing an extract solution from
20 a material containing tRNA such as cells, and adding thereto a probe containing a sequence complementary to the base sequence of tRNA. In this case, the material for the preparation may be cells transformed with an expression vector capable of expressing a desired tRNA. Usually, tRNAs synthesized by *in vitro* transcription only contain four typical bases: adenine, guanine, cytosine, and uracil. On the other hand, tRNAs synthesized in cells may contain modified bases
25 resulting from modification of the typical nucleosides. It is considered that a modified base (for example, lysidine) in a natural tRNA is specifically introduced into that tRNA by the action of an enzyme for that modification (for example, Tils) after the tRNA is synthesized by transcription.

[0101]

Aminoacyl-tRNAs can also be prepared by chemical and/or biological synthesis
30 methods. For example, an aminoacyl-tRNA can be synthesized using an aminoacyl-tRNA synthetase (ARS) to attach an amino acid to a tRNA. The amino acid may be either natural amino acid or unnatural amino acid as long as it can serve as a substrate for ARS. Alternatively, a natural amino acid may be attached to a tRNA and then chemically modified. Furthermore, as there are many reports that introducing an amino acid mutation into ARSs enhanced their action
35 on unnatural amino acids (see for example, WO2006/135096, WO2007/061136, WO2007/103307, WO2008/001947, WO2010/141851, and WO2015/120287), such mutated

ARSs may be used to attach an amino acid to tRNA. In addition to the method using ARSs, aminoacyl-tRNAs can be synthesized by, for example, removing the CA sequence from the 3' end of tRNA, and ligating an aminoacylated pdCpA (a dinucleotide comprising as nucleosides deoxycytidine and adenosine) to it using RNA ligase (pdCpA method; Hecht et al., J Biol Chem (1978) 253: 4517-4520). A method using pCpA (a dinucleotide comprising as nucleosides cytidine and adenosine) instead of pdCpA is also known (pCpA method; Wang et al., ACS Chem Biol (2015)10: 2187-2192). Furthermore, aminoacyl-tRNAs can also be synthesized by attaching an unnatural amino acid previously activated by esterification to a tRNA, using flexizyme, an artificial RNA catalyst (WO2007/066627, WO2012/026566, H. Murakami et al., Chemistry & Biology, Vol. 10, 2003, 655-662; H. Murakami et al., Chemistry & Biology, Vol. 10, 2003, 1077-1084; H. Murakami et al., Nature Methods 3, 2006, 357-359; N. Niwa et al., Bioorganic & Medicinal Chemistry Letters 19, 2009, 3892-3894). A flexizyme is an artificial RNA catalyst that can conjugate an amino acid or a hydroxyl acid to a tRNA. Flexizymes in the present disclosure include flexizyme (Fx) in its original form, and dinitrobenzyl flexizyme (dFx), enhanced flexizyme (eFx), and aminoflexizyme (aFx) that are engineered therefrom.

[0102]

In one aspect, the present disclosure provides sets of tRNAs suitable for peptide translation. A set of tRNAs contains a plurality of different types of tRNAs, and a plurality of different types of amino acids can be translated from those tRNAs. In one aspect, the present disclosure provides a composition for translation, which contains a plurality of different types of tRNAs suitable for peptide translation. In another aspect, the present disclosure provides a method for producing the above composition for translation. In another aspect, the present disclosure provides a method for producing peptides, comprising providing a plurality of different types of tRNAs suitable for peptide translation. In one aspect, tRNAs of the present disclosure are included in the plurality of different types of tRNAs described above. The following description relates to such tRNAs suitable for peptide translation, compositions for translation, methods for producing compositions for translation, methods for reducing codon misreading, and methods for producing peptides.

[0103]

In some embodiments, a set of tRNAs in the present disclosure may include the tRNA of the present disclosure described above (it may be referred to as "first tRNA" in the present disclosure) and a second tRNA. While any tRNA can be used as the second tRNA, it may be the above tRNA of the present disclosure, independently of the first tRNA.

[0104]

In some embodiments, the bases at the first letter of the anticodons in the first tRNA and the second tRNA included in a set of tRNAs in the present disclosure may be different from each

other. The bases at the second letter of the anticodons in the above-mentioned first tRNA and second tRNA may be the same, and the bases at the third letter of the anticodons in the above-mentioned first tRNA and second tRNA may be the same. In some embodiments, a codon complementary to the anticodon in the first tRNA and a codon complementary to the anticodon in the second tRNA may be present in the same codon box. In some embodiments, at least two types of amino acids can be translated from a single codon box by using the first tRNA of the present disclosure and the second tRNA of the present disclosure. In some embodiments, the base at the first letter of the anticodon in the first tRNA may be A or G, and the base at the first letter of the anticodon in the second tRNA may be C or U. In some embodiments, the respective anticodons in the first tRNA and the second tRNA may have the following combinations for the bases at the first letter: (A, C); (C, A); (G, C); (C, G); (A, U); (U, A); (G, U); and (U, G). Here, (A, C) indicates that the base at the first letter of the anticodon in the first tRNA is A, and the base at the first letter of the anticodon in the second tRNA is C.

[0105]

In some embodiments, each tRNA bodies of the first tRNA and the second tRNA included in a set of tRNAs of the present disclosure, may be different from each other or the same. In some embodiments, the base sequences of positions 1 to 31 and positions 39 to 74 in the first tRNA and in the second tRNA may be the same, or they may have sequence identity of 80% or higher, 85% or higher, 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, or 98% or higher.

[0106]

In some embodiments, mutually different amino acids may be attached to the first tRNA and the second tRNA included in the sets of tRNAs in the present disclosure. In the present disclosure, sometimes the amino acid attached to the first tRNA is called the first amino acid, and the amino acid attached to the second tRNA is called the second amino acid. In a certain embodiment, at least one selected from the first amino acid and second amino acid in the present disclosure may be an unnatural amino acid. More specifically, at least one or both of the set of tRNAs in the present disclosure may have an unnatural amino acid attached thereto. In some embodiments, either one or both of the first tRNA and the second tRNA may be a tRNA to which an unnatural amino acid is attached outside a translation system.

[0107]

Without being limiting, in some embodiments, when either one or both of the first tRNA the second tRNA have chimeric tRNA bodies carrying the combination of bases 32U, 33U, 37G, and 38U, sets of tRNAs containing the first tRNA and the second tRNA which have at least one anticodon combination selected from the group consisting of (L1) to (L4) below may be excluded from the sets of tRNAs of the present disclosure:

(L1) GCG and CCG, and CCG and GCG;

(L2) AAG and UAG, AAG and CAG, and UAG and CAG;

(L3) bases at the second letter are both C, and bases at the third letter are both G in the anticodons; and

5 (L4) bases at the second letter are both A, and bases at the third letter are both G in the anticodons.

[0108]

The compositions for translation in the present disclosure are not limited as long as they contain tRNAs of the present disclosure, and they may contain constituent components necessary
10 for translation, and contain the same constituent components as the translation systems of the present disclosure. Compositions for translation in the present disclosure may be cell-free translation systems or reconstituted cell-free translation systems. Without intending to be limiting, in some embodiments, a composition for translation of the present disclosure may be a cell-free translation system reconstituted by an *E. coli*-derived factor, and may contain
15 ribosomes, translation initiation factors, translation termination factors, translation elongation factors, amino acids, aminoacyl-tRNA synthetase (aaRS), and such. In some embodiments, compositions for translation of the present disclosure may comprise *E. coli*-derived ribosomes. In some embodiments, tRNAs of the present disclosure may be *E. coli*-derived tRNAs.

[0109]

20 In some embodiments, compositions for translation of the present disclosure may contain sets of tRNAs of the present disclosure. A composition for translation in the present disclosure may contain the following number of sets of tRNAs of the present disclosure: 1 set, 2 sets, 3 sets, 4 sets, 5 sets, 6 sets, 7 sets, or 8 sets; or 1 or more sets, 2 or more sets, 3 or more sets, 4 or more sets, 5 or more sets, or 6 or more sets; or not more than 8 sets, not more than 7 sets,
25 not more than 6 sets, not more than 5 sets, not more than 4 sets, not more than 3 sets, or not more than 2 sets. In further embodiments, compositions for translation of the present disclosure may contain tRNAs other than those mentioned above.

[0110]

In some embodiments, when a composition for translation of the present disclosure
30 contains an aaRS, the set of tRNAs of the present disclosure contained in the composition for translation may be tRNAs having orthogonal relationship to the aaRS. The tRNAs having orthogonal relationship to aaRS means tRNAs that are not aminoacylated by the aaRS present in the composition for translation, but can be taken up into a ribosome for translational incorporation of an amino acid. Examples of such tRNAs include tRNA Glu2, tRNA AsnE2,
35 tRNA Asp1, and tRNA Pro1E2, or tRNAs derived from them. When necessary, the aaRSs that recognize these tRNAs are removed from the compositions for translation. In some

embodiments, a composition for translation of the present disclosure may not contain an aaRS that can attach an amino acid to either one of the set of tRNAs.

[0111]

In some embodiments, compositions for translation and kits for translation of the present disclosure can reduce misreading of codons. Compositions for translation and kits for translation of the present disclosure can reduce the rate of translational incorporation of unintended amino acids caused by codon misreading. Compositions for translation and kits for translation of the present disclosure can reduce the rate of translation of a codon contained in a template mRNA by a tRNA carrying an anticodon not complementary to this codon. In some embodiments, compositions for translation and kits for translation of the present disclosure can reduce misreading of a codon by a tRNA in which the second and third letters of its anticodon are complementary to the second and first letters of the codon, respectively, the anticodon and the codon are in a relationship where Watson-Crick base pairs may be formed, and the first letter of the anticodon is not complementary to the third letter of the anticodon. In some embodiments, because of this reduction in misreading, use of a composition for translation and a kit for translation of the present disclosure will enable two or more amino acids to be assigned within the same codon box. In a specific embodiment, two or more unnatural amino acids may be assigned within the same codon box. For example, reducing misreading of the CCG codon by a tRNA carrying the AGG anticodon enables, while assigning an amino acid to the CCG codon, assigning a different amino acid to a codon complementary to the AGG anticodon (for example, CCU) , or more specifically, it enables assigning two different amino acids to the same codon box (the CCM box in this case).

This way, reducing the frequency of translational incorporation of a wrong amino acid caused by codon misreading, which may take place when two or more amino acids are assigned to the same codon box, enables increasing the number of amino acids assigned in the genetic code table and enables accurate peptide translation.

[0112]

In some embodiments, since the properties of the tRNA itself can be changed according to the present disclosure, the compositions and kits are more convenient than methods involving adjusting the amount of aminoacyl tRNA contained in the composition for translation or constituting the kit for translation, or interrupting the translation reaction. Although not intended to be bound by specific theory, for example, when the amount of aminoacyl tRNA in a translation system is increased to prevent misreading of codons due to depletion of aminoacyl tRNAs, misreading may take place from the opposite direction. The compositions for translation and kits for translation of the present disclosure are also useful in that they can accomplish accurate peptide translation without increasing the amount of aminoacyl tRNA in the translation

system. In certain embodiments, use of a composition for translation and a kit for translation of the present disclosure in combination with the above-mentioned methods for adjusting the amount of aminoacyl tRNA and interrupting the translation reaction may result in better effects of reducing codon misreading. In one embodiment, in compositions for translation and kits for translation of the present disclosure, multiple types of tRNAs attached with different amino acids assigned to different codons in the same codon box, can be said to be in an independent relationship in which they do not misread each other, or more specifically, in an orthogonal relationship. The naturally occurring biological translation system essentially has a strict correspondence established between a codon and an amino acid; therefore, addition of a tRNA having no orthogonality may cause the correspondence to collapse, and lead to a catastrophic effect on the function of the translation system. Therefore, in one embodiment of the present disclosure, establishment of orthogonality among the multiple types of tRNAs may be one of the important features.

[0113]

In some embodiments, compositions for translation and kits for translation of the present disclosure reduces codon misreading, while enabling assigning multiple types of amino acids, particularly multiple types of amino acids including unnatural amino acids, to each of codon boxes, such as UCM, CUM, CCM, CGM, ACM, GUM, GCM, and GGM, to which only one type of amino acid is assigned in the natural genetic code table. How to assign the amino acids is not particularly limited, and for example, the first amino acid may be assigned to the M_1M_2U codon or the M_1M_2C codon, and the second amino acid may be assigned to the M_1M_2A codon or the M_1M_2G codon; or the first amino acid may be assigned to the M_1M_2U codon, and the second amino acid may be assigned to the M_1M_2G codon.

[0114]

In some embodiments, compositions for translation and kits for translation of the present disclosure may contain at least one type of mRNA carrying a codon complementary to the anticodon in the second tRNA, and/or at least one type of mRNA carrying a codon complementary to the anticodon in the first tRNA. In a certain embodiment, the two codons may exist on the same mRNA or on different mRNAs. In some embodiments, a composition for translation of the present disclosure may contain multiple types of mRNAs having sequences different from each other, and may contain an mRNA library.

[0115]

In some embodiments, a method for producing a composition for translation and a kit for translation of the present disclosure may comprise a step of preparing the first and/or second tRNA by *in vitro* transcription. In some embodiments, a method for producing a composition for translation and a kit for translation of the present disclosure may comprise preparing a tRNA of

the present disclosure by attaching an amino acid to the tRNA outside a translation system. The method may also comprise preparing a first tRNA and a second tRNA by attaching an amino acid to the tRNAs outside a translation system. In some embodiments, a method for producing a composition for translation and a kit for translation of the present disclosure may comprise the above method for preparing tRNAs of the present disclosure. In some embodiments, the above-mentioned amino acid may be an unnatural amino acid.

[0116]

In some embodiments, a method for producing peptides of the present disclosure may comprise using a composition for translation or a kit for translation of the present disclosure to translate a nucleic acid that serves as a template. While the method for translation is not limited, examples include cell-free translation (*in vitro* translation), such as translation using a reconstituted cell-free translation system, or more specifically, translation using an *E. coli*-derived reconstituted cell-free translation system.

[0117]

In some embodiments, methods for producing peptides of the present disclosure may comprise using a composition for translation or a kit for translation of the present disclosure to translate an mRNA carrying a codon complementary to an anticodon in a second tRNA and/or an mRNA carrying a codon complementary to an anticodon in a first tRNA. In a certain embodiment, the two codons may be included in the same mRNA or in different mRNAs. In some embodiments, methods for producing peptides of the present disclosure may comprise using a composition for translation of the present disclosure to translate multiple types of nucleic acids with sequences different from each other, translate a nucleic acid library, or translate an mRNA library. The mRNAs may encode peptides having the desired or random amino acid sequences. Adding the mRNAs to a translation system of the present disclosure will allow translation of the mRNAs into peptides. On the other hand, when RNA polymerases for transcribing DNAs into mRNAs are contained in the translation system, adding DNAs to the translation system of the present disclosure will allow transcription of the DNAs into mRNAs and translation of the mRNAs to peptides to be performed together.

[0118]

In some embodiments, a codon complementary to the above-mentioned anticodon may be a codon that forms Watson-Crick base pairs with all three bases of the anticodon, or a codon whose third letter forms a wobble base pair with the first letter of the anticodon.

[0119]

In some embodiments, a method for producing peptides of the present disclosure may comprise assigning multiple types of amino acids to at least 1, 2 or more, 3 or more, 4 or more, 5 or more, or 6 or more codon boxes selected from the group consisting of UCM, CUM, CCM,

CGM, ACM, GUM, GCM, and GGM. Even in such cases, accurate peptide translation can be possible.

[0120]

In some embodiments, according to a method for producing peptides of the present disclosure, codon misreading may be reduced, while assigning different types of amino acids to codons which are in the same codon box and in which the third letters are at least one combination selected from the group consisting of the following (i) to (iv): (i) U and G, (ii) C and G, (iii) U and A, and (iv) C and A. In some embodiments, according to a translation method of the present disclosure, codon misreading may be reduced, while assigning different types of amino acids to the UCM₃, CUM₃, CCM₃, CGM₃, ACM₃, GUM₃, GCM₃, or GGM₃ codons in which the M₃'s are at least one combination selected from the group consisting of the above (i) to (iv).

[0121]

In the present disclosure, reduction in codon misreading by tRNAs may be evaluated by using a plurality of tRNAs carrying different anticodons, to which different amino acids have been attached, to translate a single type of template mRNA. For the plurality of tRNAs, one can select tRNAs carrying an anticodon complementary to a particular codon that is to be translated, and tRNAs carrying an anticodon that is different from the above-mentioned anticodon and is complementary to a codon present in the same codon box as the above-mentioned codon.

[0122]

For example, to evaluate misreading of the CCG codon by a tRNA carrying the AGG anticodon, a translation system containing a tRNA carrying the AGG anticodon to which an amino acid AA₁ is attached, and a tRNA carrying the CGG anticodon to which an amino acid AA₂ is attached, is used to translate a template mRNA containing the CCG codon. In this case, the targeted translated amino acid is AA₂ which is introduced through translation by the tRNA carrying the CGG anticodon complementary to the CCG codon, and the translated amino acid that occurred through misreading is AA₁ which is introduced through translation by the tRNA carrying the AGG anticodon not complementary to the CCG codon. Percentages of such translated products can be used as indices to evaluate reduction in misreading.

[0123]

As the template mRNA for use in the above-mentioned evaluation, an mRNA selected from MR-1 to MR-7 described in the Examples may be used, or other mRNAs may be used according to the codon to be evaluated. As the translation system for translating the template mRNA, a prokaryote-derived reconstituted cell-free protein synthesis system (for example, the PURE system) may be used. "Translation condition 1" of the present disclosure may be used as the condition for translation. Translation methods and evaluation methods are described in more

detail in the Examples.

[0124]

In the present disclosure, reduction of codon misreading by tRNA can be evaluated by the percentage (%) of misread peptides relative to an intended product. This percentage is calculated using the following equation. When the percentage is low in comparison to control tRNA in which the combination of bases at positions 32, 33, 37 and 38 is un-engineered, the evaluated tRNA is determined to have effects of reducing codon misreading. The rate of reduction in percentage is not particularly limited, but a tRNA of the present disclosure may show 5% or higher, 10% or higher, 20% or higher, 30% or higher, 40% or higher, or 50% or higher reduction in the percentage as compared to the control. In the present disclosure, the peptide obtained when the codon is correctly read may be referred to as the correctly read translation product or intended product.

[Equation 1]

$$\text{Percentage of misread peptides relative to the intended product (\%)} = \frac{\text{Amount of translated peptide obtained when misreading takes place (\mu M)}}{\text{Amount of translated peptide obtained when correctly read (\mu M)}} \times 100$$

[0125]

The amount of translated peptide may be determined by the following method. More specifically, the translation product solution obtained after completion of the translation reaction is diluted and analyzed using a LC-FLR-MS setup. An exemplary degree of dilution is 10-fold. By determining the retention time of the translated peptide of interest from the obtained MS data, and quantifying the fluorescence peak at the retention time, the amount of translated peptide is evaluated. The quantification is carried out by producing a calibration curve using a standard, and calculating the content by relative quantification. As the standard, LCT-67 or LCT-12 may be used.

The sequence of LCT-67 is BdpF:Thr:Phe:Ile:Ile:Gly:Phe:Ile:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 237), and the sequence of LCT-12 is BdpF:Thr:Ile:Phe:Pro:Gly:Phe:Ile:Ile:Thr:Thr:Gly:Thr:Gly:Thr:Gly:Ala (SEQ ID NO: 238).

[0126]

In one aspect, the present disclosure provides peptides and peptide libraries produced by using the compositions for translation of the present disclosure. The peptides of the present disclosure include peptides obtained by performing chemical modification or such after translation, and peptide-nucleic acid complexes formed by linking nucleic acids.

[0127]

Examples of post-translational modification include cyclization of a linear peptide. As the bond for forming the cyclic portion, for example, a peptide bond formed from an amino group and a carboxyl group can be used. In addition, an amide bond, disulfide bond, ether bond, thioether bond, ester bond, thioester bond, carbon-carbon bond, alkyl bond, alkenyl bond, phosphonate ether bond, azo bond, amine bond, C=N-C bond, lactam bridge, carbamoyl bond, urea bond, thiourea bond, thioamide bond, sulfinyl bond, sulfonyl bond, triazole bond, benzoxazole bond, and such formed from a combination of appropriate functional groups can be used. The carbon-carbon bond can be formed by a transition metal-catalyzed reaction such as a Suzuki reaction, a Heck reaction, and a Sonogashira reaction. In one embodiment, the peptides of the present disclosure contain at least one set of functional groups capable of forming the above-mentioned bond in the molecule. The formation of the cyclic portion may be performed by producing a linear peptide using the translation system of the present disclosure and then separately performing a reaction for linking the above-mentioned functional groups with each other. Regarding the synthesis of the peptide having a cyclic portion, one can refer to WO2013/100132, WO2012/026566, WO2012/033154, WO2012/074130, WO2015/030014, WO2018/052002, Comb. Chem. High Throughput Screen (2010)13: 75-87, Nat. Chem. Biol. (2009) 5: 502-507, Nat. Chem. Biol. (2009) 5: 888-90, Bioconjug. Chem. (2007) 18: 469-476, ChemBioChem (2009) 10: 787-798, Chem. Commun. (Camb) (2011) 47: 9946-9958, and such. [0128]

There are known methods for identifying peptides that may bond to a targeted molecule through a peptide library (display library) that uses peptide-nucleic acid complexes. A display library is a library in which a phenotype and a genotype are associated with each other as a result of formation of a single complex by linking a peptide to a nucleic acid encoding that peptide. Examples of major display libraries include libraries prepared by the mRNA display method (Roberts and Szostak, Proc. Natl. Acad. Sci. USA (1997) 94: 12297-12302), *in vitro* virus method (Nemoto et al., FEBS Lett. (1997) 414: 405-408), cDNA display method (Yamaguchi et al., Nucleic Acids Res. (2009) 37: e108), ribosome display method (Mattheakis et al, Proc. Natl. Acad. Sci. USA (1994) 91: 9022-9026), covalent display method (Reiersen et.al., Nucleic Acids Res. (2005) 33: e10), CIS display method (Odegrip et.al., Proc. Natl. Acad. Sci. USA (2004) 101: 2806-2810), and such. Alternatively, a library prepared by using the *in vitro* compartmentalization method (Tawfik and Griffiths, Nat. Biotechnol. (1998) 16: 652-656) can be mentioned as one embodiment of the display library. [0129]

In one aspect, the present disclosure provides a method for identifying a peptide having binding activity to a target molecule, which comprises contacting the target molecule with a peptide library described in the present disclosure. The target molecule is not particularly

limited and can be appropriately selected from, for example, low molecular weight compounds, high molecular weight compounds, nucleic acids, peptides, proteins, sugars, and lipids. The target molecule may be a molecule existing outside the cell or a molecule existing inside the cell. Alternatively, it may be a molecule existing in the cell membrane, in which case any of the extracellular domain, the transmembrane domain, and the intracellular domain may be the target. In the step of contacting the target molecule with the peptide library, the target molecule is usually immobilized on some kind of solid-phase carrier (for example, a microtiter plate or microbeads). Then, by removing the peptides not binding to the target molecule and recovering only the peptides binding to the target molecule, the peptides having binding activity to the target molecule can be selectively concentrated (panning method). When the peptide library used is a nucleic acid display library, the recovered peptides have the nucleic acid encoding their respective genetic information attached to them; therefore, the nucleic acid sequence encoding the recovered peptide and the amino acid sequence can be readily identified by isolating and analyzing them. Furthermore, based on the obtained nucleic acid sequence or amino acid sequence, the identified peptides can be individually produced by chemical synthesis or gene recombination techniques.

[0130]

In one aspect, the present disclosure provides methods for reducing misreading of codons by tRNAs, and compositions and kits for reducing misreading of codons by tRNAs. Such compositions and kits may contain tRNAs of the present disclosure. Furthermore, such a method may involve obtaining tRNAs of the present disclosure by engineering tRNAs. Specifically, it is a method for reducing misreading of a second codon by a tRNA carrying an anticodon complementary to a first codon, comprising substituting at least one base at a position selected from the group consisting of positions 32, 33, 37, and 38 in the tRNA; wherein, the substituted tRNA is a tRNA of the present disclosure, the bases at the first letters of the first codon and the second codon are the same, the bases at the second letters of the first codon and the second codon are the same, and the bases at the third letters of the first codon and the second codon are different from each other.

[0131]

In some embodiments, in the method for reducing codon misreading of the present disclosure, the first codon is M_1M_2X and the second codon is M_1M_2Y , wherein the above M_1 and M_2 are each independently A, C, G, or U, and the above X and Y are bases different from each other, each selected from A, C, G, and U. The combination of the bases for the above X and Y may be any one selected from the group consisting of (a1) to (a8) below: (a1) U and G; (a2) G and U; (a3) U and A; (a4) A and U; (a5) C and A; (a6) A and C; (a7) C and G; and (a8) G and C. In the present disclosure, the combination of the bases for the above X and Y is M_{31} and M_{32}

means that the above X is base M₃₁ and the above Y is base M₃₂. In some embodiments, the combination of the bases for the above X and Y may be any one selected from the group consisting of the above (a1) to (a3), (a5), and (a7), or the group consisting of the above (a1) and (a2). In some embodiments, the above M₁M₂ may be a base sequence of any one selected from the group consisting of (b1) to (b8) below: (b1) CC; (b2) CU; (b3) GG; (b4) GU; (b5) GC; (b6) UC; (b7) CG; and (b8) AC. In certain embodiments, the above M₁M₂ may be any one base sequence selected from the group consisting of the above (b1) to (b3).

[0132]

In some embodiments, in a method for reducing codon misreading of the present disclosure, the first codon and the anticodon in the tRNA may form Watson-Crick base pairs at all three bases, or the base at the third letter of the first codon and the base at the first letter of the anticodon in the tRNA may form a wobble base pair.

[0133]

In some embodiments, methods for reducing codon misreading of the present disclosure may be methods for reducing misreading of a codon complementary to the anticodon in the second tRNA of the present disclosure by the first tRNA of the present disclosure. In some embodiments, methods for reducing codon misreading of the present disclosure may include the above methods for preparing tRNAs of the present disclosure.

[Examples]

[0134]

The present invention is further illustrated by the following examples, but is not limited thereto.

The following abbreviations were used in the Examples.

AA: ammonium acetate

CH₂CN: cyanomethyl group

DBU: 1,8-diazabicyclo[5.4.0]-7-undecene

DCM: dichloromethane

DIC: N,N-diisopropylcarbodiimide

DIPEA: N,N-diisopropylethylamine

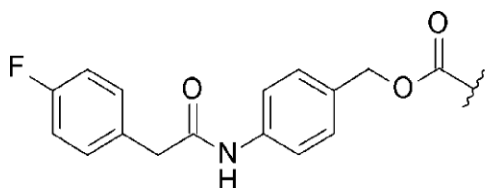
DMF: dimethylformamide

DMSO: dimethyl sulfoxide

FA: formic acid

Fmoc: 9-fluorenylmethyloxycarbonyl group

F-Pnaz: 4-(2-(4-fluorophenyl)acetamido)benzyloxycarbonyl group



HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol

MeCN: acetonitrile

NMP: N-methyl-2-pyrrolidone

5 TEA: triethylamine

TFA: trifluoroacetic acid

TFE: 2,2,2-trifluoroethanol

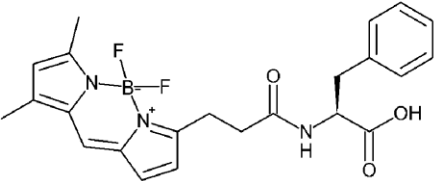
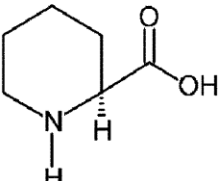
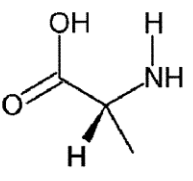
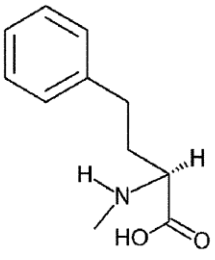
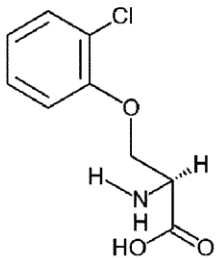
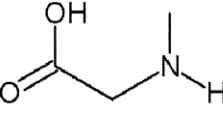
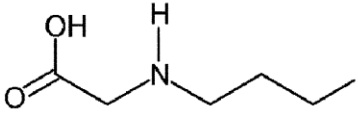
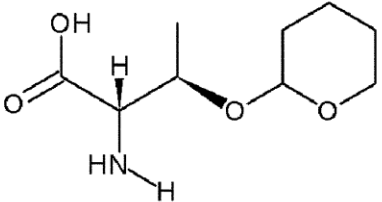
THF: tetrahydrofuran

[0135]

10 The following abbreviations were used in this Example: Gly or G (glycine), Ile or I (isoleucine), Leu or L (leucine), Phe or F (phenylalanine), Pro or P (proline), Thr or T (threonine). In addition to these, the abbreviations shown in Table 3 were used in the present disclosure. For the amino acid sequences in the present disclosure, BdpFL-Phe may be written as “BdpF”.

15 [0136]

[Table 3]

Abbreviation	Structural formula	Abbreviation	Structural formula
BdpFL -Phe		Pic2	
dA		MeHph	
SPh2Cl		MeG	
nBuG		Thr (THP)	

[0137]

The LCMS analysis conditions are shown below in Table 4.

[0138]

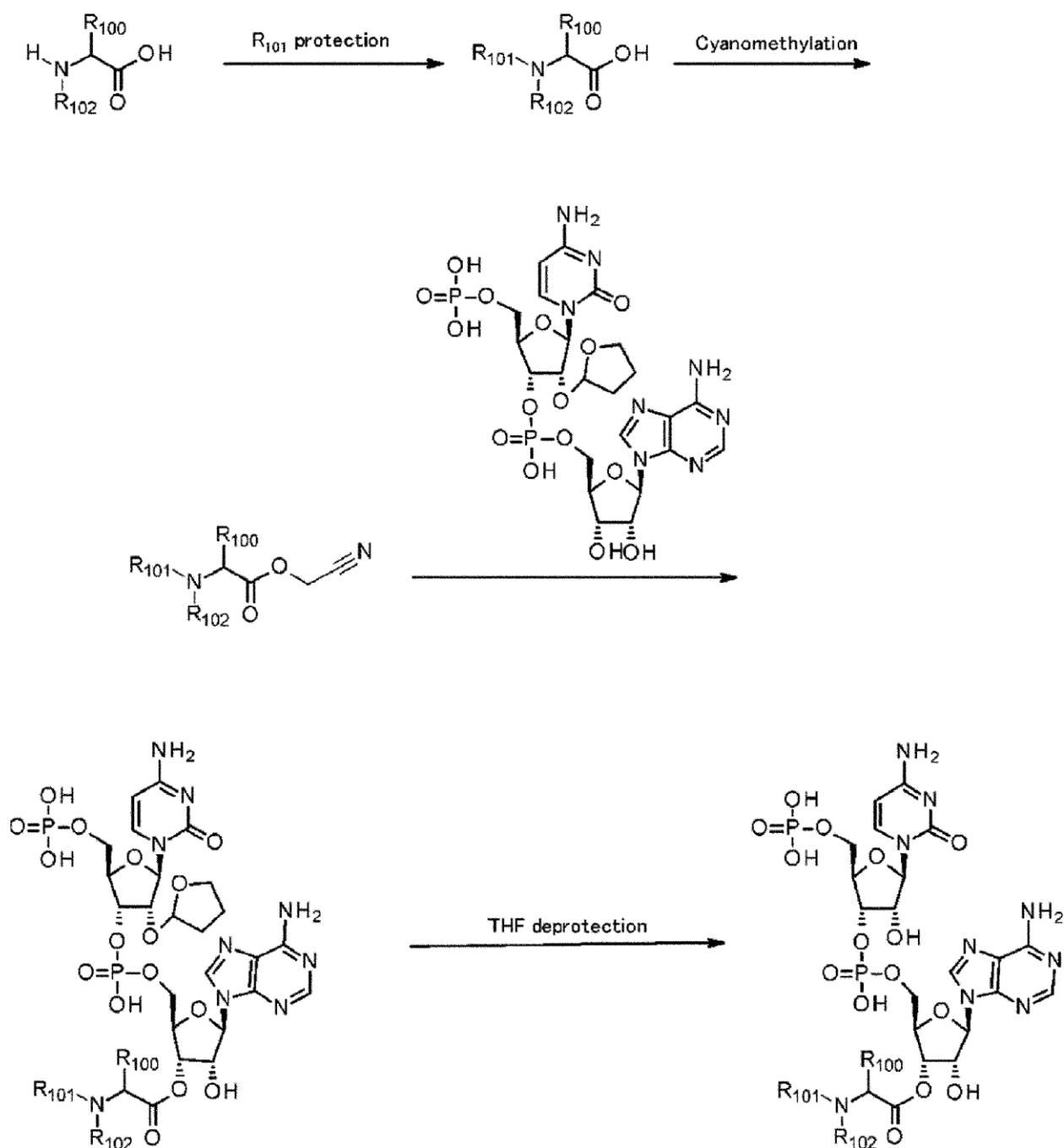
5 [Table 4]

Preparative condition	System	Column (I.D. x length (mm))	Mobile phase	Gradient (A/B)	Flow rate (ml/min)	Column temperature (°C)	Wavelength
SOD FA05_01	Acquity UPLC/SQD	Aldrich Ascentis Express C18 1.7 µm (2.1 x 50)	A) 0.1% FA, H2O B) 0.1% FA CH3CN	95/5 => 0/100 (1.0 min) => 0/100 (0.4 min)	1.0	35	210-400nm PDA total
SOD FA05_02	Acquity UPLC/SQD2	Aldrich Ascentis Express C18 2.7 µm (2.1 x 50)	A) 0.1% FA, H2O B) 0.1% FA CH3CN	95/5 => 0/100 (1.0 min) => 0/100 (0.4 min)	0.9	35	210-400nm PDA total
SMD method 1	Shimadzu LCMS-2020 LC-20AD	Shim-Pack XR-QDS 1.7 µm (2.1 x 50)	A) 0.1% FA, H2O B) 0.1% FA CH3CN	90/10 => 0/100 (1.1 min) => 0/100 (0.6 min)	1.2	40	190-400nm PDA total
SMD method 2	Shimadzu LCMS-2020 LC-20ADXR	CORTECS C18 2.7 µm (2.1 x 50)	A) 0.1% FA, H2O B) 0.1% FA CH3CN	90/10 => 0/100 (1.2 min) => 0/100 (0.5 min)	1.0	40	190-400nm PDA total
SMD method 3	Shimadzu LCMS-2020 LC-20ADXR	CORTECS C18 2.7 µm (2.1 x 50)	A) 0.1% FA, H2O B) 0.1% FA CH3CN	95/5 => 0/100 (1.0 min) => 0/100 (0.5 min)	1.0	40	190-400nm PDA total
SMD method 4	Shimadzu LCMS-2020 LC-30AD	Halo C18 2.0 µm (2.1 x 30)	A) 0.05% TFA, H2O B) 0.05% TFA CH3CN	95/5 => 0/100 (0.7 min) => 0/100 (0.25 min)	0.8	40	190-400nm PDA total
SOD AA50 long	Acquity UPLC/SQD2	Aldrich Ascentis Express C18 5.0 µm (2.1 x 50)	A) 10mM AcONH4, H2O B) MeOH	50/50 => 0/100 (4.5 min) => 0/100 (0.5 min)	0.9	35	210-400nm PDA total
LTQ TEA/HFIP05_01	Acquity UPLC/LTQ Orbitrap XL	Waters ACQUITY UPLC BEH C18 1.7 µm (2.1 x 50)	A) 15mM TEA, 400mM HFIP H2O B) 15mM TEA, 400mM HFIP MeOH	95/5 => 10/90 (9.0 min) => 10/90 (1.0 min)	0.2	30	190-400nm PDA total
LTQ TEA/HFIP05_02	Acquity UPLC/LTQ Orbitrap XL	Waters ACQUITY UPLC BEH C18 1.7 µm (2.1 x 50)	A) 15mM TEA, 400mM HFIP H2O B) 15mM TEA, 400mM HFIP MeOH	95/5 => 95/5 (8.0 min) => 10/90 (2.0 min)	0.2	30	190-400nm PDA total
LTQ TEA/HFIP05_03	Acquity UPLC/LTQ Orbitrap XL	Waters ACQUITY UPLC BEH C18 1.7 µm (2.1 x 50)	A) 15mM TEA, 400mM HFIP H2O B) 15mM TEA, 400mM HFIP MeOH	95/5 => 70/30 (9.0 min) => 10/90 (1.0 min)	0.2	30	190-400nm PDA total

[0139]

Example 1. Synthesis of aminoacyl pCpAs

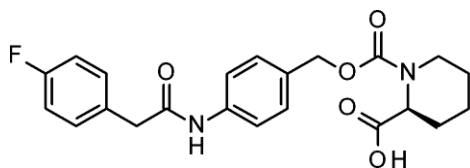
Aminoacyl pCpAs (SS14, SS15, SS16, and SS45) were synthesized according to the following scheme.



5

[0140]

Synthesis of (S)-1-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)piperidine-2-carboxylic acid (Compound SS17, F-Pnaz-Pic2-OH)



[0141]

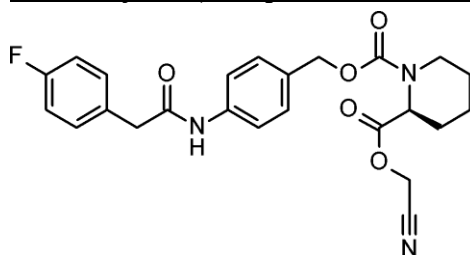
Under nitrogen atmosphere, DMF (330 μ L) was added to a mixture of (S)-piperidine-2-carboxylic acid (42.6 mg, 0.33 mmol) and (4-nitrophenyl)-4-(2-(4-fluorophenyl)acetamido)benzyl carbonate (Compound ts11) (140 mg, 0.44 mmol) synthesized by the method of a patent literature (WO2018143145A1) at room temperature. After stirring this mixture at room temperature for 5 minutes, triethylamine (105.6 μ L, 2.25 mmol) was added at 0°C. The reaction mixture was stirred at room temperature for 30 minutes, and then purified by reverse-phase silica gel column chromatography (0.1% aqueous formic acid solution/0.1% formic acid-acetonitrile solution) to obtain (S)-1-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)piperidine-2-carboxylic acid (Compound SS17, F-Pnaz-Pic2-OH) (92 mg, 67%).

LCMS (ESI) m/z = 413 ($M-H$)⁻

Retention time: 0.70 minutes (analysis condition SQDFA05_01)

[0142]

Synthesis of 1-(4-(2-(4-fluorophenyl)acetamido)benzyl) 2-(cyanomethyl) (S)-piperidine-1,2-dicarboxylate (Compound SS18, F-Pnaz-Pic2-OCH₂CN)



[0143]

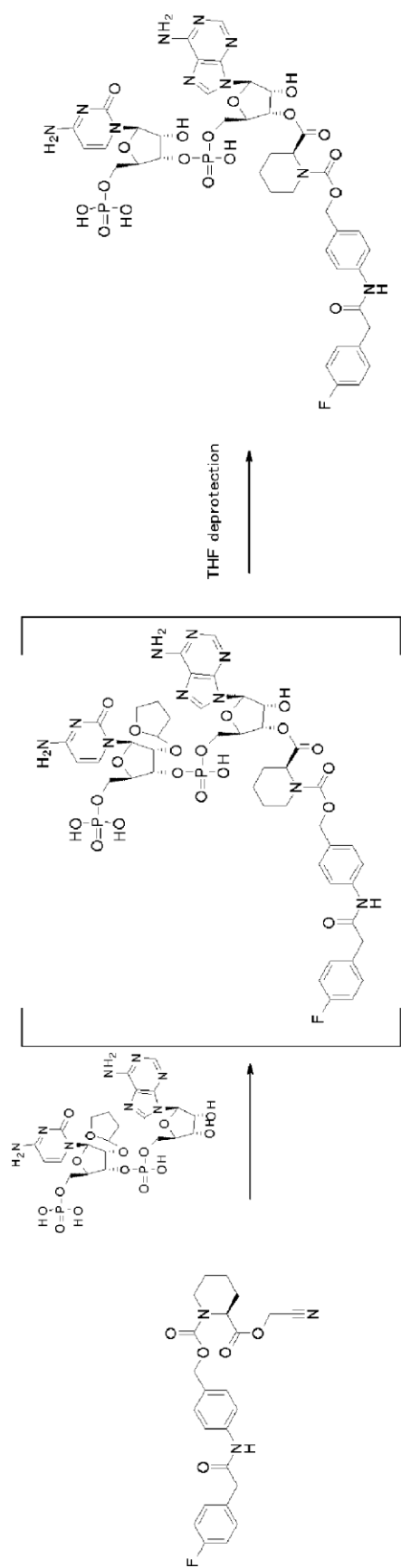
Under nitrogen atmosphere, (S)-1-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)piperidine-2-carboxylic acid (Compound SS17, F-Pnaz-Pic2-OH) (30 mg, 0.072 mmol) and N-ethyl-isopropylpropan-2-amine (DIPEA) (20.23 μ L, 0.116 mmol) were dissolved in acetonitrile (90 μ L), added with 2-bromoacetonitrile (5.34 μ L, 0.080 mmol) at 0°C, and the mixture was stirred at room temperature for 2 hours. The reaction solution was concentrated to obtain a crude product, 1-(4-(2-(4-fluorophenyl)acetamido)benzyl) 2-(cyanomethyl) (S)-piperidine-1,2-dicarboxylate (Compound SS18, F-Pnaz-Pic2-OCH₂CN). The obtained crude product was dissolved in acetonitrile (2.00 mL), and was directly used in the next step.

LCMS (ESI) m/z = 452 ($M-H$)⁻

Retention time: 0.79 minutes (analysis condition SQDFA05_01)

[0144]

5 Synthesis of 1-(4-(2-(4-fluorophenyl)acetamido)benzyl) 2-((2R,3S,4R,5R)-2-((((((2R,3S,4R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-4-hydroxy-2-((phosphonoxy)methyl)tetrahydrofuran-3-yl)oxy)(hydroxy)phosphoryl)oxy)methyl)-5-(6-amino-9H-purin-9-yl)-4-hydroxytetrahydrofuran-3-yl) (2S)-piperidine-1,2-dicarboxylate (Compound SS14, F-Pnaz-Pic2-pCpA)



[0145]

((2R,3R,4R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)oxy)-4-(((tetrahydrofuran-2-yl)oxy)tetrahydrofuran-2-yl)methyl dihydrogen phosphate (Compound pc01) (113 mg, 0.156 mmol) synthesized by a method described in a literature (Helv. Chim. Acta, 90, 297-310) was dissolved in Buffer A (40 mL), a solution of 1-(4-(2-(4-fluorophenyl)acetamido)benzyl) 2-(cyanomethyl) (S)-piperidine-1,2-dicarboxylate (Compound SS18, F-Pnaz-Pic2-OCH₂CN) (35.4 mg, 0.078 mmol) in acetonitrile (2.00 mL) was added, and the mixture was stirred at room temperature for 150 minutes. The reaction solution was cooled to 0°C, and then trifluoroacetic acid (2.00 mL) was added. The reaction solution was stirred at 0°C for 45 minutes, and then purified by reverse-phase silica gel column chromatography (0.05% aqueous trifluoroacetic acid solution/ 0.05% trifluoroacetic acid-acetonitrile) to obtain the title compound (Compound SS14, F-Pnaz-Pic2-pCpA) (6.0 mg, 7.3%).

LCMS (ESI) m/z = 1047.5 (M-H)⁻

Retention time: 0.50 minutes (analysis condition SQDFA05_01)

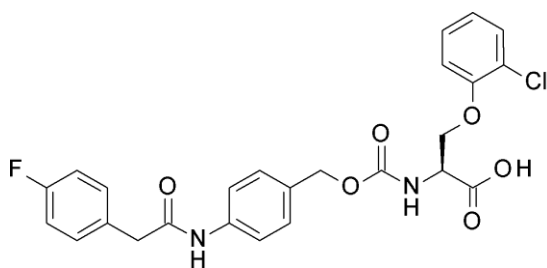
[0146]

Buffer A was prepared as follows.

Acetic acid was added to an aqueous solution of N,N,N-trimethylhexadecan-1-aminium chloride (6.40 g, 20 mmol) and imidazole (6.81 g, 100 mmol) to give Buffer A (1L) of 20 mM N,N,N-trimethylhexadecan-1-aminium and 100 mM imidazole at pH 8.

[0147]

Synthesis of O-(2-chlorophenyl)-N-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)-L-serine (Compound SS19, F-Pnaz-SPh2Cl-OH)



[0148]

Under nitrogen atmosphere, DMSO (15 mL) and triethylamine (0.95 g, 9.42 mmol) were added to a mixture of O-(2-chlorophenyl)-L-serine (Compound aa63) (1.25 g, 5.80 mmol) synthesized by a method described in a patent literature (WO2018225864) and (4-nitrophenyl)-4-(2-(4-fluorophenyl)acetamido)benzyl carbonate (Compound ts11) (2 g, 4.71 mmol) synthesized by a method described in a patent literature (WO2018143145A1) at room temperature. The reaction mixture was stirred at room temperature for 16 hours and then purified by reverse-phase silica gel column chromatography (0.1% aqueous formic acid

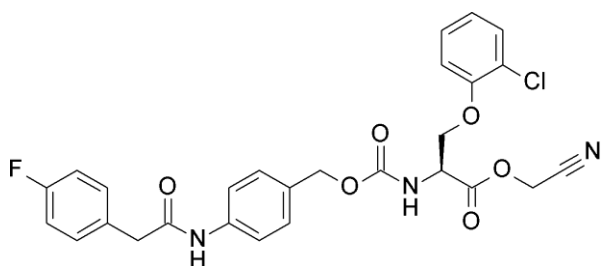
solution/0.1% formic acid-acetonitrile solution) to obtain O-(2-chlorophenyl)-N-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)-L-serine (Compound SS19, F-Pnaz-SPh2Cl-OH) (1.8 g, 73%).

LCMS (ESI) m/z = 523 (M+Na)+

5 Retention time: 1.26 minutes (analysis condition SMD method 1)

[0149]

Synthesis of cyanomethyl O-(2-chlorophenyl)-N-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)-L-serinate (Compound SS20, F-Pnaz-SPh2Cl-OCH₂CN)



10

[0150]

Under nitrogen atmosphere, O-(2-chlorophenyl)-N-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)-L-serine (Compound SS19, F-Pnaz-SPh2Cl-OH) (800 mg, 1.60 mmol) and N-ethyl-isopropylpropan-2-amine (DIPEA) (0.412 g, 3.19 mmol) were dissolved in DCM (15 mL), 2-bromoacetonitrile (760 mg, 6.34 mmol) was added at room temperature, and the mixture was stirred at room temperature for 16 hours. The reaction solution was concentrated and purified by reverse-phase silica gel column chromatography (0.1% aqueous formic acid solution/0.1% formic acid-acetonitrile solution) to obtain cyanomethyl O-(2-chlorophenyl)-N-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)-L-serinate (Compound SS20, F-Pnaz-SPh2Cl-OCH₂CN) (220 mg, 26%). The obtained product was dissolved in acetonitrile (5 mL), and used in the next step.

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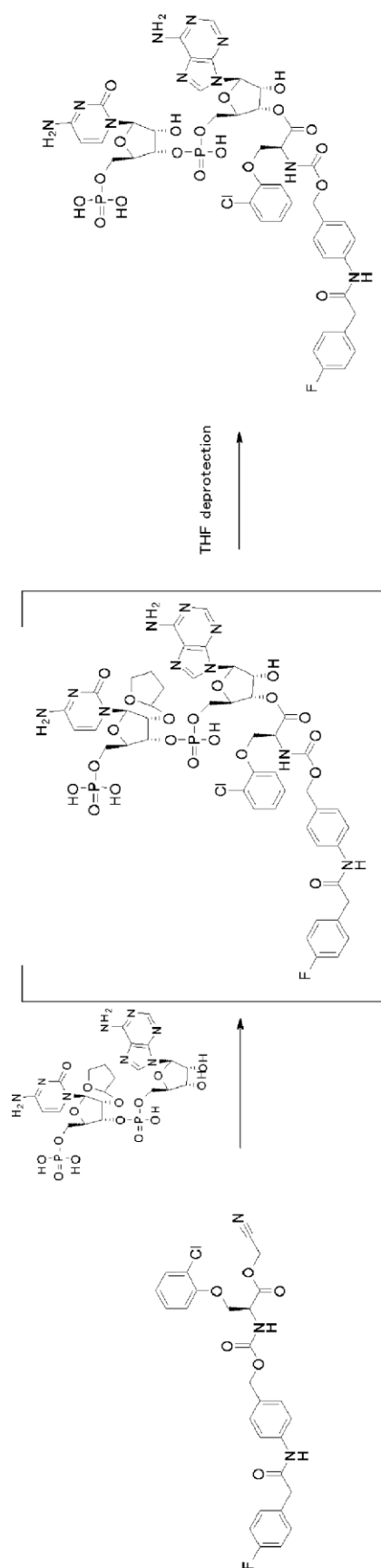
LCMS (ESI) m/z = 562 (M+Na)+

Retention time: 1.15 minutes (analysis condition SMD method 2)

[0151]

25

Synthesis of (2R,3S,4R,5R)-2-((((((2R,3S,4R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-4-hydroxy-2-((phosphonoxy)methyl)tetrahydrofuran-3-yl)oxy)(hydroxy)phosphoryl)oxy)methyl)-5-(6-amino-9H-purin-9-yl)-4-hydroxytetrahydrofuran-3-yl O-(2-chlorophenyl)-N-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)-L-serinate (Compound SS15, F-Pnaz-SPh2Cl-pCpA)



[0152]

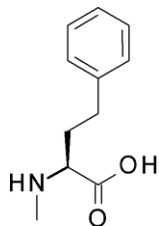
((2R,3R,4R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)oxy)-4-(((tetrahydrofuran-2-yl)oxy)tetrahydrofuran-2-yl)methyl dihydrogen phosphate (Compound pc01) (400 mg, 0.55 mmol) was dissolved in Buffer A (100 mL), a solution of cyanomethyl O-(2-chlorophenyl)-N-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)-L-serinate (Compound SS20, F-Pnaz-SPh2Cl-OCH₂CN) (220 mg, 0.41 mmol) in acetonitrile (5 mL) was added to it dropwise over 15 minutes or longer using a syringe pump, and the mixture was stirred at room temperature for 5 minutes. Next, trifluoroacetic acid (2.3 mL) was added to the reaction solution. The reaction solution was freeze-dried, and then purified by reverse-phase silica gel column chromatography (0.05% aqueous trifluoroacetic acid solution/ 0.05% trifluoroacetic acid-acetonitrile) to obtain the title compound (Compound SS15, F-Pnaz-SPh2Cl-pCpA) (20.7 mg, 2%).

LCMS (ESI) m/z = 1133.4 (M-H)⁻

Retention time: 0.55 minutes (analysis condition SQDFA05_01)

[0153]

Synthesis of ((S)-2-(methylamino)-4-phenylbutanoic acid (Compound SS21, MeHph-OH)



[0154]

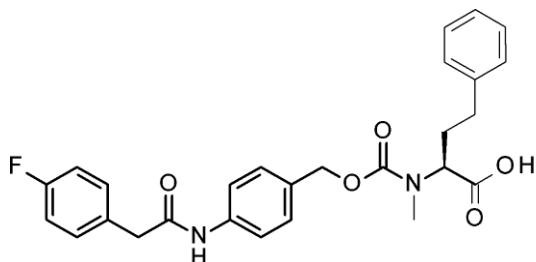
DCM (903 μ L), water (903 μ L), and piperidine (178 μ L, 1.805 mmol) were added to (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)(methyl)amino)-4-phenylbutanoic acid (Compound aa11) (150 mg, 0.361 mmol) synthesized by a method described in a patent literature (WO2018225864) at room temperature. The reaction mixture was stirred at room temperature for 30 minutes and then purified by reverse-phase silica gel column chromatography (0.1% aqueous formic acid solution/ 0.1% formic acid-acetonitrile solution) to obtain ((S)-2-(methylamino)-4-phenylbutanoic acid (Compound SS21, MeHph-OH) (55 mg, 79%).

LCMS (ESI) m/z = 192 (M-H)⁻

Retention time: 0.15 minutes (analysis condition SQDFA05_02)

[0155]

Synthesis of (S)-2-((((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)(methyl)amino)-4-phenylbutanoic acid (Compound SS22, F-Pnaz-MeHph-OH)



[0156]

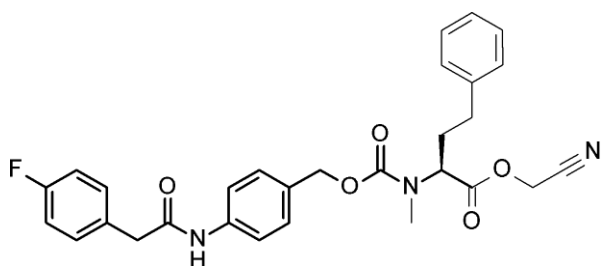
Under nitrogen atmosphere, DMSO (727 μ L) was added to a mixture of ((S)-2-(methylamino)-4-phenylbutanoic acid (Compound SS21, MeHph-OH) (35.1 mg, 0.182 mmol) and (4-nitrophenyl)-4-(2-(4-fluorophenyl)acetamido)benzyl carbonate (Compound ts11) (85 mg, 0.20 mmol) synthesized a method described in by a patent literature (WO2018143145A1) at room temperature. Triethylamine (76 μ L, 0.545 mmol) was added at 50°C. The reaction mixture was stirred at 40°C for 16 hours, and then purified by reverse-phase silica gel column chromatography (0.1% aqueous formic acid solution/ 0.1% formic acid-acetonitrile solution) to obtain (S)-2-((((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)(methyl)amino)-4-phenylbutanoic acid (Compound SS22, F-Pnaz-MeHph-OH) (80 mg, 92%).

LCMS (ESI) m/z = 477 (M-H)⁻

Retention time: 0.85 minutes (analysis condition SQDFA05_02)

[0157]

Synthesis of cyanomethyl (S)-2-((((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)(methyl)amino)-4-phenylbutanoate (Compound SS23, F-Pnaz-MeHph-OCH₂CN)



[0158]

Under nitrogen atmosphere, acetonitrile (533 μ L) was added to a mixture of (S)-2-((((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)(methyl)amino)-4-phenylbutanoic acid (Compound SS22, F-Pnaz-MeHph-OH) (77 mg, 0.16 mmol) and N-ethyl-isopropylpropan-2-amine (DIPEA) (31 μ L, 0.176 mmol) at room temperature. Then, 2-bromoacetonitrile (86 μ L, 1.280 mmol) was added at room temperature, and the reaction mixture was stirred at 40°C for 1 hour. The reaction solution was concentrated to obtain a crude product, cyanomethyl (S)-2-((((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)(methyl)amino)-4-phenylbutanoate

(Compound SS23, F-Pnaz-MeHph-OCH₂CN). The obtained crude product was dissolved in acetonitrile (5.00 mL) and was directly used in the next step.

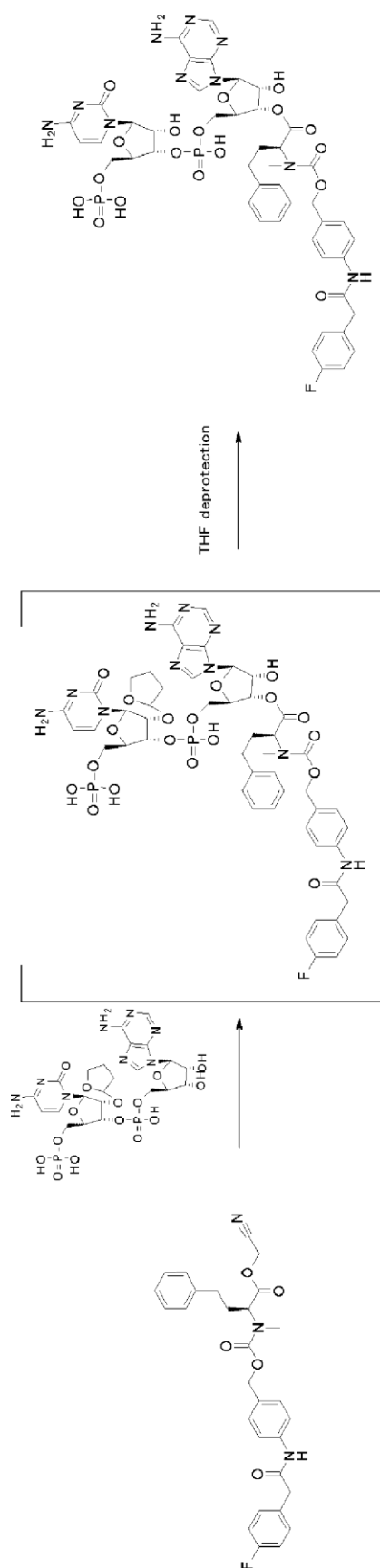
LCMS (ESI) m/z = 516 (M-H)⁻

Retention time: 0.92 minutes (analysis condition SQDFA05_02)

5 [0159]

Synthesis of (2R,3S,4R,5R)-2-((((((2R,3S,4R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-4-hydroxy-2-((phosphonooxy)methyl)tetrahydrofuran-3-yl)oxy)(hydroxy)phosphoryl)oxy)methyl)-5-(6-amino-9H-purin-9-yl)-4-hydroxytetrahydrofuran-3-yl (2S)-2-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)(methyl)amino)-4-phenylbutanoate (Compound

10 SS16, F-Pnaz-MeHph-pCpA)



[0160]

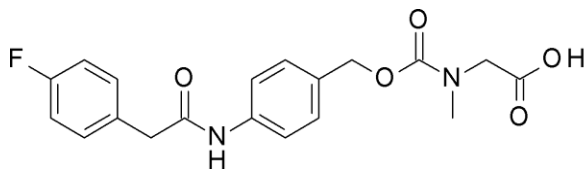
((2R,3R,4R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)hydroxy)phosphoryl)oxy)-4-(((tetrahydrofuran-2-yl)oxy)tetrahydrofuran-2-yl)methyl dihydrogen phosphate (Compound pc01) (127 mg, 0.176 mmol) was dissolved in Buffer A (100 mL), a solution of cyanomethyl (S)-2-(((4-(2-(4-fluorophenyl)acetamido)benzyl)oxy)carbonyl)(methyl)amino)-4-phenylbutanoate (Compound SS23, F-Pnaz-MeHph-OCH₂CN) (83 mg, 0.16 mmol) in acetonitrile (5.00 mL) was added, and the mixture was stirred at room temperature for 1 hour. The reaction solution was cooled to 0°C, and then trifluoroacetic acid (5.00 mL) was added. The reaction solution was stirred at 0°C for one hour, and then purified by reverse-phase silica gel column chromatography (0.05% aqueous trifluoroacetic acid solution/ 0.05% trifluoroacetic acid-acetonitrile), and then further purified by reverse-phase silica gel column chromatography (0.1% aqueous formic acid solution/ 0.1% formic acid acetonitrile solution) to obtain the title compound (Compound SS16, F-Pnaz-MeHph-pCpA) (26 mg, 14.6%).

LCMS (ESI) m/z = 1111.5 (M-H)⁻

Retention time: 0.64 minutes (analysis condition SQDFA05_02)

[0161]

Synthesis of N-(((4-(2-(4-fluorophenyl)acetamide)benzyl)oxy)carbonyl)-N-methylglycine (Compound SS46, F-Pnaz-MeG-OH)



[0162]

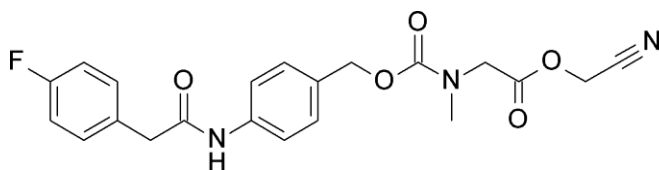
Under a nitrogen atmosphere, DMSO (15 mL) and triethylamine (953.4 mg, 9.42 mmol) were added at room temperature to a mixture of sarcosine (483 mg, 5.42 mmol) and (4-nitrophenyl)-4-(2-(4-fluorophenyl)acetamide)benzyl carbonate (Compound ts11) (2.0 g, 4.71 mmol) synthesized by a method described in a patent literature (WO2018143145A1). The reaction mixture was stirred at room temperature for 16 hours, and then purified by reverse-phase silica gel column chromatography (0.1% aqueous formic acid solution/0.1% formic acid-acetonitrile solution), to obtain N-(((4-(2-(4-fluorophenyl)acetamide)benzyl)oxy)carbonyl)-N-methylglycine (Compound SS46, F-Pnaz-MeG-OH) (1.4 g, 79%).

LCMS (ESI) m/z=397 (M+Na)⁺

Retention time: 0.88 minutes (analysis condition SMD method 3)

[0163]

Synthesis of cyanomethyl N-(((4-(2-(4-fluorophenyl)acetamide)benzyl)oxy)carbonyl)-N-methylglycinate (Compound SS47, F-Pnaz-MeG-OCH₂CN)



[0164]

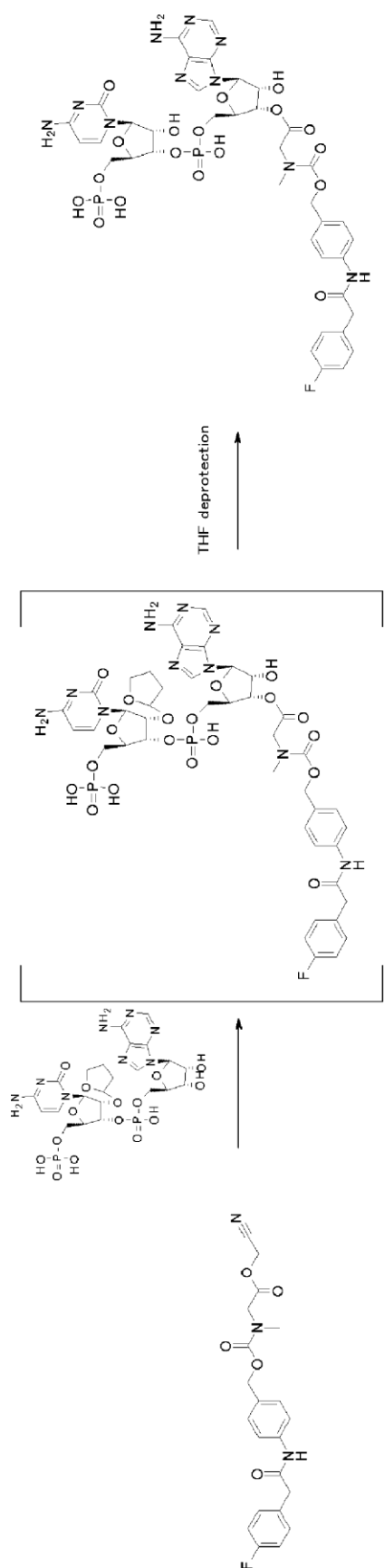
Under a nitrogen atmosphere, N-(((4-(2-(4-fluorophenyl)acetamide)benzyl)oxy)carbonyl)-N-methylglycine (Compound SS46, F-Pnaz-MeG-OH) (1.38 g, 3.69 mmol) and N-ethyl-isopropylpropan-2-amine (DIPEA) (0.95 g, 7.38 mmol) were dissolved in DMF (28 mL), 2-bromoacetonitrile (1.74 g, 14.75 mmol) was added at room temperature, and the mixture was stirred at room temperature for 16 hours. The reaction solution was concentrated, and purified by normal-phase silica gel column chromatography (ethyl acetate/petroleum ether) to obtain cyanomethyl N-(((4-(2-(4-fluorophenyl)acetamide)benzyl)oxy)carbonyl)-N-methylglycinate (Compound SS47, F-Pnaz-MeG-OCH₂CN) (1.2 g, 79%).

LCMS (ESI) m/z=436 (M+Na)⁺

Retention time: 0.70 minutes (analysis condition SMD method 4)

[0165]

Synthesis of (2R,3S,4R,5R)-2-((((((2R,3S,4R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-4-hydroxy-2-((phosphonooxy)methyl)tetrahydrofuran-3-yl)oxy)(hydroxy)phosphoryl)oxy)methyl)-5-(6-amino-9H-purin-9-yl)-4-hydroxytetrahydrofuran-3-yl N-(((4-(2-(4-fluorophenyl)acetamide)benzyl)oxy)carbonyl)-N-methylglycinate(Compound SS45, F-Pnaz-MeG-pCpA)



[0166]

((2R,3R,4R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-3-((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)oxy)-4-(((tetrahydrofuran-2-yl)oxy)tetrahydrofuran-2-yl)methyl dihydrogenphosphate (Compound pc01) (422 mg, 0.58 mmol) synthesized by a method described in literature (Helv. Chim. Acta, 90, 297-310) was dissolved in Buffer A (100 mL), a solution of cyanomethyl N-(((4-(2-(4-fluorophenyl)acetamide)benzyl)oxy)carbonyl)-N-methylglycinate (Compound SS47, F-Pnaz-MeG-OCH₂CN) (120.7 mg, 0.29 mmol) in acetonitrile (5 mL) was added dropwise over 15 minutes or more using a syringe pump, and the mixture was stirred at room temperature for 5 hours. Trifluoroacetic acid (2.3 mL) was added to the reaction solution, and this reaction solution was freeze-dried and then purified by reverse-phase silica gel column chromatography (0.05% aqueous trifluoroacetic acid solution/0.05% trifluoroacetic acid-acetonitrile) to obtain the title compound (Compound SS45, F-Pnaz-MeG-pCpA) (76.7 mg, 26%).

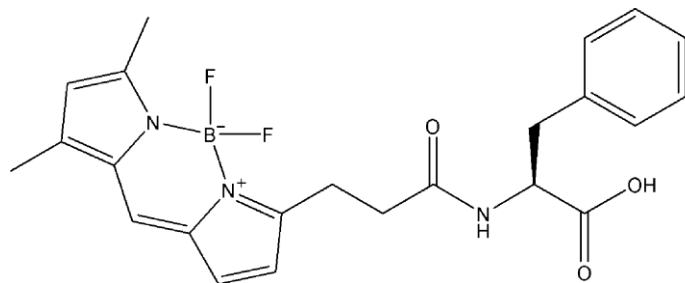
LCMS (ESI) m/z=1007.5 (M-H)⁻

Retention time: 0.48 minutes (analysis condition SQDFA05_02)

[0167]

Example 2. Synthesis of BdpFL-Phe-pCpA(MT01)

Synthesis of (3-(5, 5-difluoro-7,9-dimethyl-5H-4λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanoyl)-L-phenylalanine (Compound MT02 BdpFL-Phe-OH)



[0168]

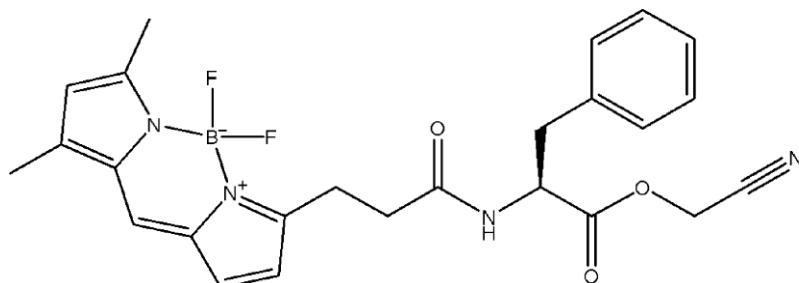
Under nitrogen atmosphere, DIC (0.128 mL, 0.822 mmol) was added to a solution of 3-(2-carboxyethyl)-5,5-difluoro-7,9-dimethyl-5H-5λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium (200 mg, 0.685 mmol) and 1-hydroxypyrrolidine-2,5-dione (87 mg, 0.753 mmol) in NMP (4.5 mL) at room temperature, and then the mixture was stirred at 40°C overnight. After returning to room temperature, L-phenylalanine (113 mg, 0.685 mmol) and TEA (0.191 mL, 1.369 mmol) were added to the reaction solution, and stirred at 40°C overnight. The reaction solution was purified by reverse-phase column chromatography (0.1% FA-MeCN/H₂O) to obtain (3-(5, 5-difluoro-7,9-dimethyl-5H-4λ⁴,5λ⁴-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanoyl)-L-phenylalanine (Compound MT02, BdpFL-Phe-OH) (102 mg, 34% yield).

LCMS (ESI) m/z = 438.3 (M-H)⁻

Retention time: 0.78 minutes (analysis condition SQDFA05_02)

[0169]

Synthesis of (3-(5,5-difluoro-7,9-dimethyl-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanoyl)-L-phenylalanine cyanomethyl ester (Compound MT03, BdpFL-Phe-OCH₂CN)



[0170]

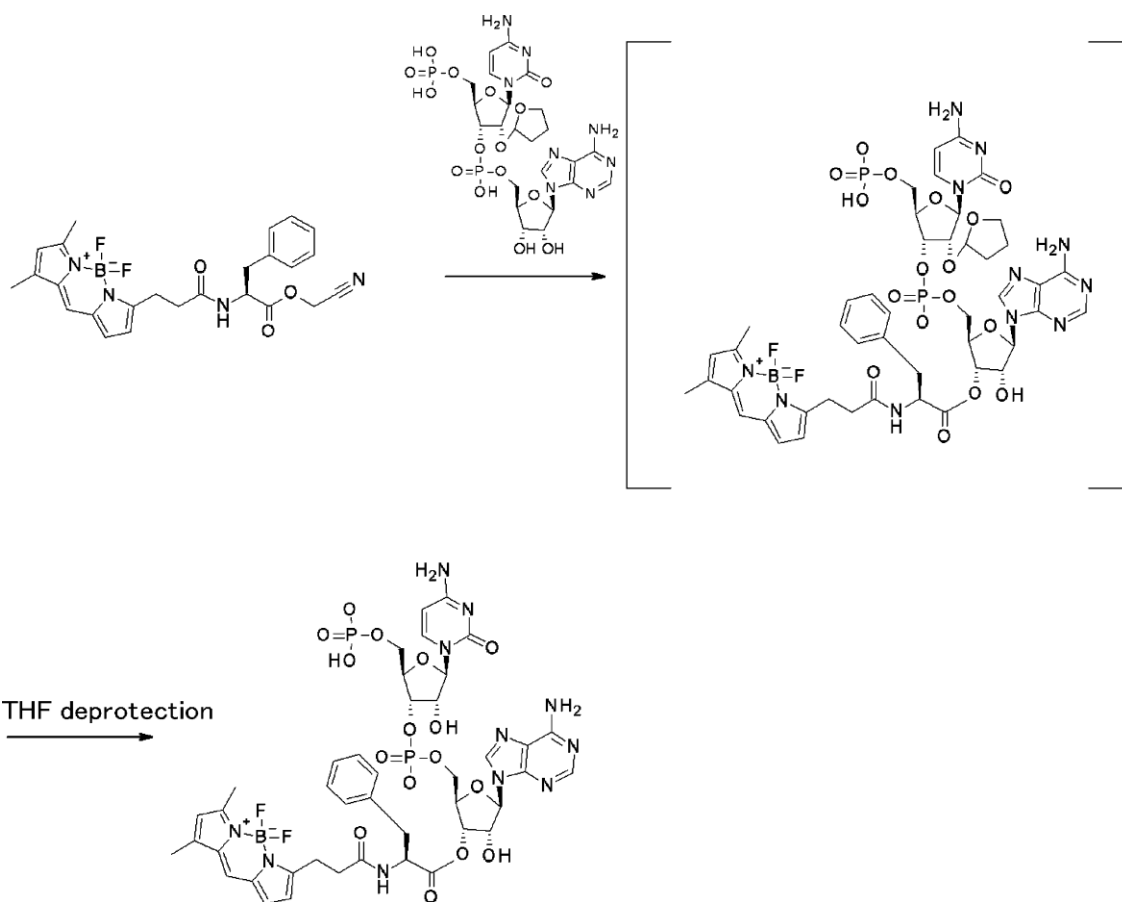
Under nitrogen atmosphere, (3-(5,5-difluoro-7,9-dimethyl-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanoyl)-L-phenylalanine (50 mg, 0.114 mmol) and N-ethylisopropylpropan-2-amine (DIPEA) (31.0 μ L, 0.177 mmol) were dissolved in acetonitrile (500 μ L), 2-bromoacetonitrile (12 μ L, 0.177 mmol) was added at 0°C, and then the mixture was stirred at 40°C for 3 hours. The reaction solution was concentrated to obtain (3-(5,5-difluoro-7,9-dimethyl-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanoyl)-L-phenylalanine cyanomethyl ester (Compound MT03, BdpFL-Phe-OCH₂CN) as a crude product. The obtained crude product was directly used in the next step.

LCMS (ESI) m/z = 477.3 (M-H)–

Retention time: 0.86 minutes (analysis condition SQDFA05_01)

[0171]

Synthesis of 3-(3-(((2S)-1-(((2R,3S,4R,5R)-2-((((2R,3S,4R,5R)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-4-hydroxy-2-((phosphonoxy)methyl)tetrahydrofuran-3-yl)oxy)(hydroxy)phosphoryl)oxy)methyl)-5-(6-amino-9H-purin-9-yl)-4-hydroxytetrahydrofuran-3-yl)oxy)-1-oxo-3-phenylpropan-2-yl)amino)-3-oxopropyl)-5,5-difluoro-7,9-dimethyl-5H-5 λ^4 -dipyrrolo [1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium (Compound MT01, BdpFL-Phe-pCpA)



[0172]

5 ((2R,3R,4R,5R)-5-(4-amino-2-oxypyrimidin-1(2H)-yl)-3-((((((2R,3S,4R,5R)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methoxy)(hydroxy)phosphoryl)oxy)-4-
 10 ((tetrahydrofuran-2-yl)oxy)tetrahydrofuran-2-yl)methyl dihydrogen phosphate (Compound pc01) (33.2 mg, 0.046 mmol) was dissolved in Buffer A (11.3 mL), a solution of (3-(5,5-difluoro-7,9-dimethyl-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-3-yl)propanoyl)-L-phenylalanine cyanomethyl ester (Compound MT03, BdpFL-Phe-OCH₂CN) (11 mg, 0.023 mmol) in acetonitrile (0.13 mL) was added, and then the mixture was stirred at room temperature
 15 for 45 minutes. TFA (0.56 mL) was added to the reaction solution at 0°C and stirred for 5 minutes, and then stirred at room temperature for 10 minutes. The reaction solution was purified by reverse-phase silica gel column chromatography (0.05% TFA-MeCN/H₂O) to obtain the title compound (Compound MT01, BdpFL-Phe-pCpA) (2.1 mg, 8.5% yield).

LCMS (ESI) m/z = 1072.5 (M-H)⁻

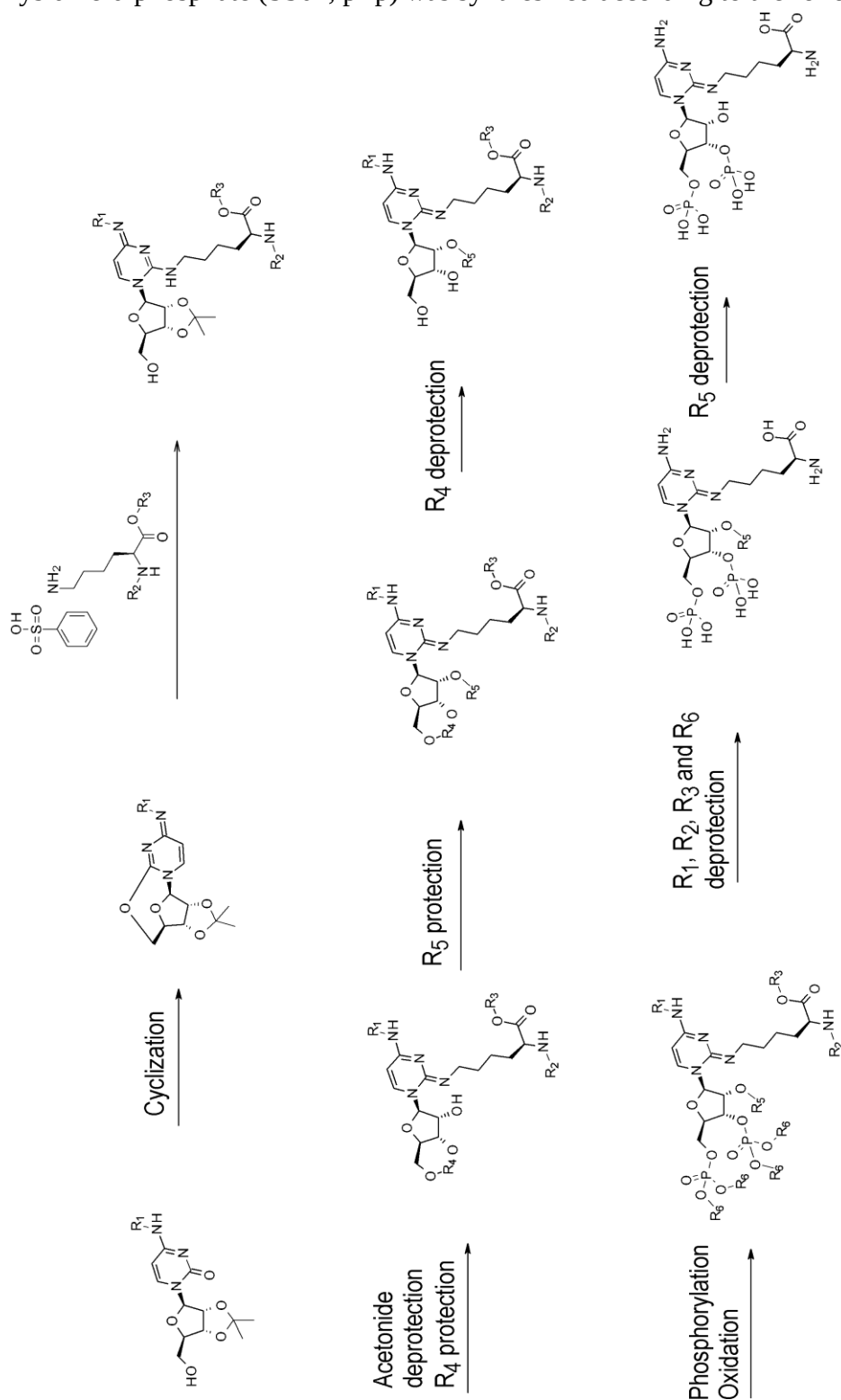
15 Retention time: 0.56 minutes (analysis condition SQDFA05_02)

[0173]

Synthesis of lysidine-diphosphate for introducing a lysidine unit at the 3' end of a tRNA fragment by a ligation method – an alternative method

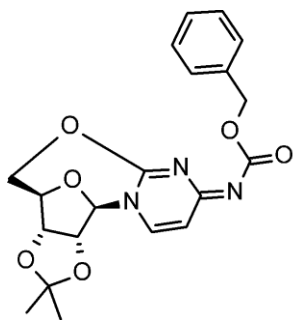
The method for synthesizing the diphosphate of lysidine used for introducing a lysidine

unit at the 3' end of a tRNA fragment by a ligation method was improved. More specifically, lysidine-diphosphate (SS04, pLp) was synthesized according to the following scheme.



[0174]

- 5 Synthesis of benzyl ((3aR,4R,12R,12aR)-2,2-dimethyl-3a,4,12,12a-tetrahydro-5H,8H-4,12-epoxy[1,3]dioxolo[4,5-e]pyrimido[2,1-b][1,3]oxazocin-8-ylidene)carbamate (Compound SS24)



[0175]

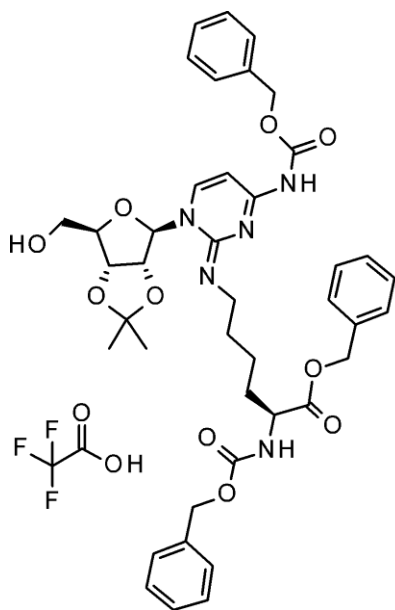
Under nitrogen atmosphere, DCM (17.2 mL) was added to a mixture of 2',3'-O-isopropylidene-4-N-(benzyl-oxy-carbonyl)-cytidine (718.2 mg, 1.72 mmol), which is a literature
 5 (Antiviral Chemistry & Chemotherapy, 2003, 14(4), 183-194)-known compound, and triphenylphosphine (474 mg, 1.81 mmol), at room temperature. After cooling the mixture in an ice bath, diisopropyl azodicarbonate (385 μ L, 1.98 mmol) was added, then the mixture was warmed to room temperature, and stirred at room temperature for 1.5 hours. The reaction solution was concentrated, toluene (20 mL) was added, and then the produced precipitates were
 10 recovered by filtration. The obtained solid was washed three times using toluene to obtain benzyl ((3aR,4R,12R,12aR)-2,2-dimethyl-3a,4,12,12a-tetrahydro-5H,8H-4,12-epoxy[1,3]dioxolo[4,5-e]pyrimido[2,1-b][1,3]oxazocin-8-ylidene)carbamate (Compound SS24) (525.7 mg, 76%).

LCMS (ESI) m/z = 400.3 (M+H)⁺

15 Retention time: 0.48 minutes (analysis condition SQDFA05_02)

[0176]

Synthesis of benzyl (2S)-6-[[1-[(3aR,4R,6R,6aR)-6-(hydroxymethyl)-2,2-dimethyl-3a,4,6,6a-tetrahydrofuro[3,4-d][1,3]dioxol-4-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS25)



[0177]

Under nitrogen atmosphere, THF (7.5 mL) was added to a mixture of benzyl ((3aR,4R,12R,12aR)-2,2-dimethyl-3a,4,12,12a-tetrahydro-5H,8H-4,12-epoxy[1,3]dioxolo[4,5-e]pyrimido[2,1-b][1,3]oxazocin-8-ylidene)carbamate (Compound SS24) (300 mg, 0.75 mmol) and lithium chloride (159 mg, 3.76 mmol), at room temperature, and the mixture was cooled in an ice bath. To this mixture, a mixture of benzyl ((benzyloxy)carbonyl)-L-lysinate benzenesulfonate (813 mg, 2.01 mmol) and DBU (673 μ L, 4.51 mmol) added with THF (7.5 mL) was added in an ice bath, and the reaction mixture was stirred at 0°C for 30 minutes. DMSO was added to the reaction solution in an ice bath, the mixture was warmed to room temperature, and then the reaction solution was concentrated to remove THF. The residue was purified by reverse-phase silica gel column chromatography (0.05% aqueous TFA solution/0.05% TFA-acetonitrile solution) to obtain benzyl (2S)-6-[[1-[(3aR,4R,6R,6aR)-6-(hydroxymethyl)-2,2-dimethyl-3a,4,6,6a-tetrahydrofuro[3,4-d][1,3]dioxol-4-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS25) (726.6 mg) quantitatively.

LCMS (ESI) m/z = 768.6 (M-H)⁻

Retention time: 0.74 minutes (analysis condition SQDFA05_02)

[0178]

Synthesis of benzyl (2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoate; 2,2,2-trifluoroacetic acid (Compound SS26)

[0179]

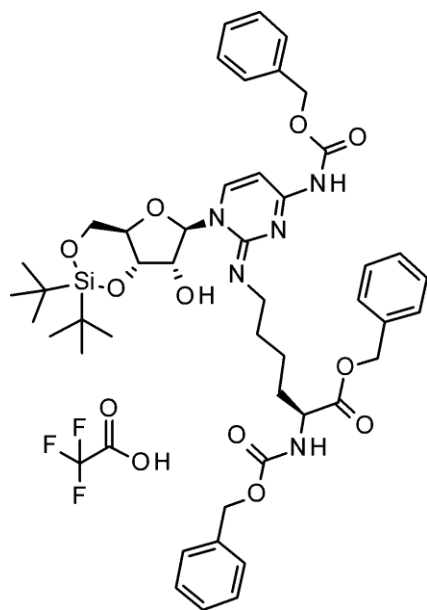
Benzyl (2S)-6-[[1-[(3aR,4R,6R,6aR)-6-(hydroxymethyl)-2,2-dimethyl-3a,4,6,6a-tetrahydrofuro[3,4-d][1,3]dioxol-4-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS25) (281.1 mg, 0.318 mmol) was dissolved in a mixed solvent of TFA (4.24 mL) and ultrapure water (2.12 mL) while cooling in an ice bath, and the mixture was stirred at room temperature for 50 minutes. Toluene and acetonitrile were added, and the reaction solution was concentrated. This operation was repeated several times to distill off water and TFA, and benzyl (2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino] hexanoate; 2,2,2-trifluoroacetic acid (Compound SS26) (272.6 mg) was obtained as a crude product. The obtained crude product, benzyl (2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoate; 2,2,2-trifluoroacetic acid (Compound SS26), was used directly in the next step.

LCMS (ESI) m/z =728.5 (M-H)⁻

Retention time: 0.69 minutes (analysis condition SQDFA05_02)

[0180]

Synthesis of benzyl (2S)-6-[[1-[(4aR,6R,7R,7aS)-2,2-ditert-butyl-7-hydroxy-4a,6,7,7a-tetrahydro-4H-furo[3,2-d][1,3,2]dioxasilin-6-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS27)



[0181]

Under nitrogen atmosphere, the crude product obtained in the previous step, benzyl

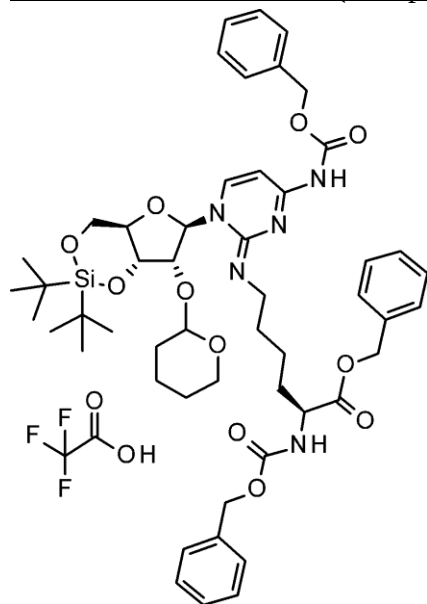
(2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoate; 2,2,2-trifluoroacetic acid (Compound SS26) (258 mg, 0.306 mmol), was dissolved in DMF (3.06 mL). After the mixture was cooled in an ice bath, di-tert-butylsilyl bis(trifluoromethanesulfonate) (396 μ L, 1.22 mmol) was added, and the mixture was stirred in an ice bath for 2 hours. In an ice bath, saturated aqueous sodium bicarbonate solution was added to the reaction solution, and the obtained mixture was purified by reverse-phase silica gel column chromatography (0.05% aqueous TFA solution/0.05% TFA-acetonitrile solution) to obtain benzyl (2S)-6-[[1-[(4aR,6R,7R,7aS)-2,2-ditert-butyl-7-hydroxy-4a,6,7,7a-tetrahydro-4H-furo[3,2-d][1,3,2]dioxasilin-6-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS27) (234.0 mg, 78%, two steps).

LCMS (ESI) m/z = 868.8 (M-H)⁻

Retention time: 0.88 minutes (analysis condition SQDFA05_02)

[0182]

Synthesis of benzyl (2S)-6-[[1-[(4aR,6R,7R,7aR)-2,2-ditert-butyl-7-tetrahydropyran-2-yloxy-4a,6,7,7a-tetrahydro-4H-furo[3,2-d][1,3,2]dioxacillin-6-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS28)



[0183]

Under nitrogen atmosphere, benzyl (2S)-6-[[1-[(4aR,6R,7R,7aS)-2,2-ditert-butyl-7-hydroxy-4a,6,7,7a-tetrahydro-4H-furo[3,2-d][1,3,2]dioxasilin-6-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS27) (30 mg, 0.03 mmol) and TFA (6.98 μ L, 0.09 mmol)

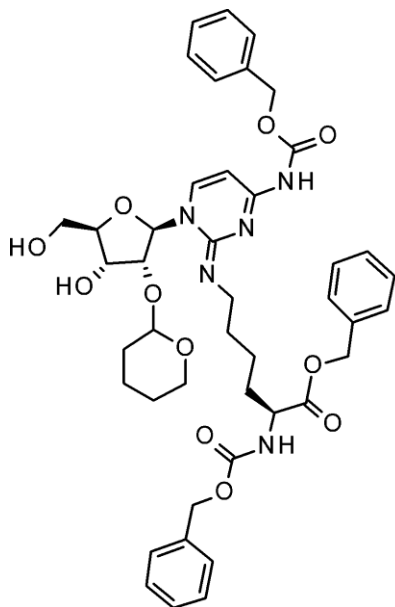
were dissolved in DCM (610 μ L) at room temperature, and 3,4-dihydro-2H-pyran (83 μ L, 0.915 mmol) was added. After stirring the reaction mixture at room temperature for 13 hours, toluene was added, and the reaction solution was concentrated to obtain a crude product, benzyl (2S)-6-[[1-[(4aR,6R,7R,7aR)-2,2-ditert-butyl-7-tetrahydropyran-2-yloxy-4a,6,7,7a-tetrahydro-4H-furo[3,2-d][1,3,2]dioxacillin-6-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS28), as a mixture of diastereomers derived from the asymmetric carbon on the THP protecting group. The obtained crude product, benzyl (2S)-6-[[1-[(4aR,6R,7R,7aR)-2,2-ditert-butyl-7-tetrahydropyran-2-yloxy-4a,6,7,7a-tetrahydro-4H-furo[3,2-d][1,3,2]dioxacillin-6-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS28), was directly used in the next step.

LCMS (ESI) m/z = 952.8 (M-H)-

Retention time: 3.17 minutes, 3.38 minutes (analysis condition SQDAA05long)

[0184]

Synthesis of benzyl (2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4R,5R)-4-hydroxy-5-(hydroxymethyl)-3-tetrahydropyran-2-yloxy-tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoate (Compound SS29)



[0185]

Under nitrogen atmosphere, the crude product obtained in the previous step, benzyl (2S)-6-[[1-[(4aR,6R,7R,7aR)-2,2-ditert-butyl-7-tetrahydropyran-2-yloxy-4a,6,7,7a-tetrahydro-4H-furo[3,2-d][1,3,2]dioxacillin-6-yl]-4-(benzyloxycarbonylamino)pyrimidin-2-ylidene]amino]-2-(benzyloxycarbonylamino)hexanoate; 2,2,2-trifluoroacetic acid (Compound SS28), was dissolved in THF (610 μ L) at room temperature, then tetrabutylammonium fluoride

(tetrahydrofuran solution of approximately 1 mol/L) (305 μ L, approximately 0.305 mmol) was added at room temperature, and the reaction mixture was stirred at room temperature for 30 minutes. The reaction solution was added with DMSO, and then concentrated to distill off THF. The residue was purified by reverse-phase silica gel column chromatography (10 mM aqueous

5 AA solution/ 10 mM AA-acetonitrile solution) to obtain benzyl (2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4R,5R)-4-hydroxy-5-(hydroxymethyl)-3-tetrahydropyran-2-yloxy-tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoate (Compound SS29) (21.51 mg, 87%, two steps) as a mixture of diastereomers derived from the asymmetric carbon on the THP protecting group.

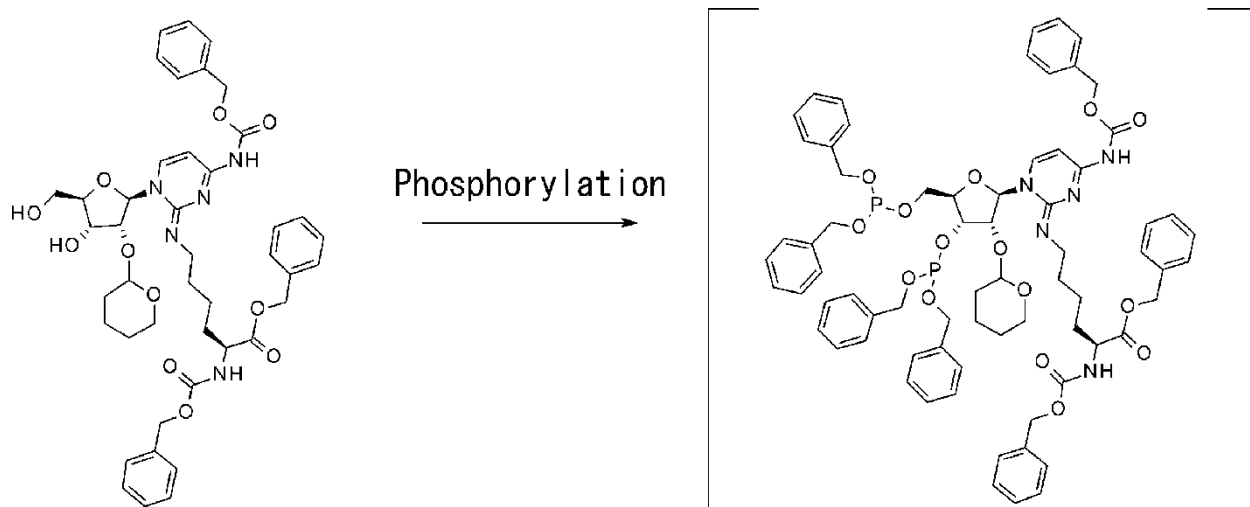
10 LCMS (ESI) m/z = 812.7 (M-H)–

Retention time: 1.74 minutes (analysis condition SQDAA05long)

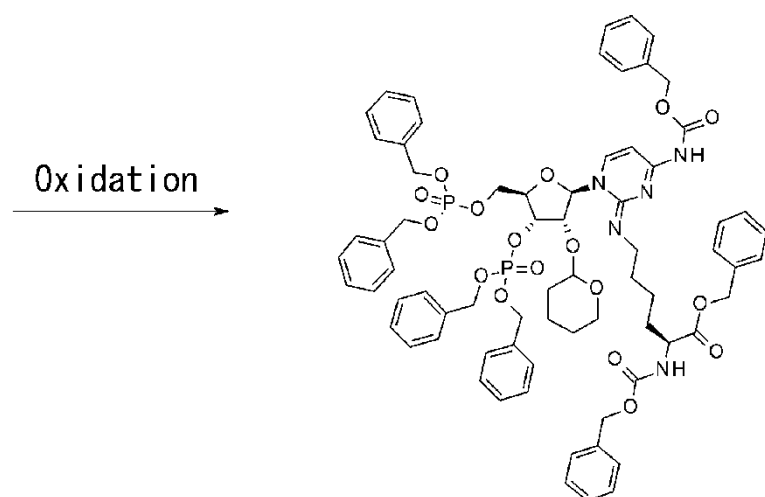
[0186]

Synthesis of benzyl (2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4R,5R)-4-dibenzyloxyphosphoryloxy-5-(dibenzyloxyphosphoryloxymethyl)-3-

15 tetrahydropyran-2-yloxy-tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoate (Compound SS30)



Compound SS29



Compound SS30

[0187]

Under nitrogen atmosphere, benzyl (2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4R,5R)-4-hydroxy-5-(hydroxymethyl)-3-tetrahydropyran-2-yloxy-tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoate (Compound SS29) (21.51 mg, 0.026 mmol) and 1H-tetrazole (22.22 mg, 0.317 mmol) were dissolved in acetonitrile (1.06 mL) at room temperature, dibenzyl N,N-diisopropylphosphoroamidite (53.2 μ L, 0.159 mmol) was added, and the mixture was stirred at room temperature for 1 hour. The mixture was added with Dess-Martin Periodinane (135 mg, 0.317 mmol) and stirred at room temperature for 15 minutes, then the reaction solution was purified by reverse-phase silica gel column chromatography (10 mM aqueous AA solution / 10 mM AA solution in acetonitrile) to obtain benzyl (2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4R,5R)-4-

dibenzyloxyphosphoryloxy-5-(dibenzyloxyphosphoryloxymethyl)-3-tetrahydropyran-2-yloxy-tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoate (Compound SS30) (36.59 mg, two steps) quantitatively, as a mixture of diastereomers derived from the asymmetric carbon on the THP protecting group.

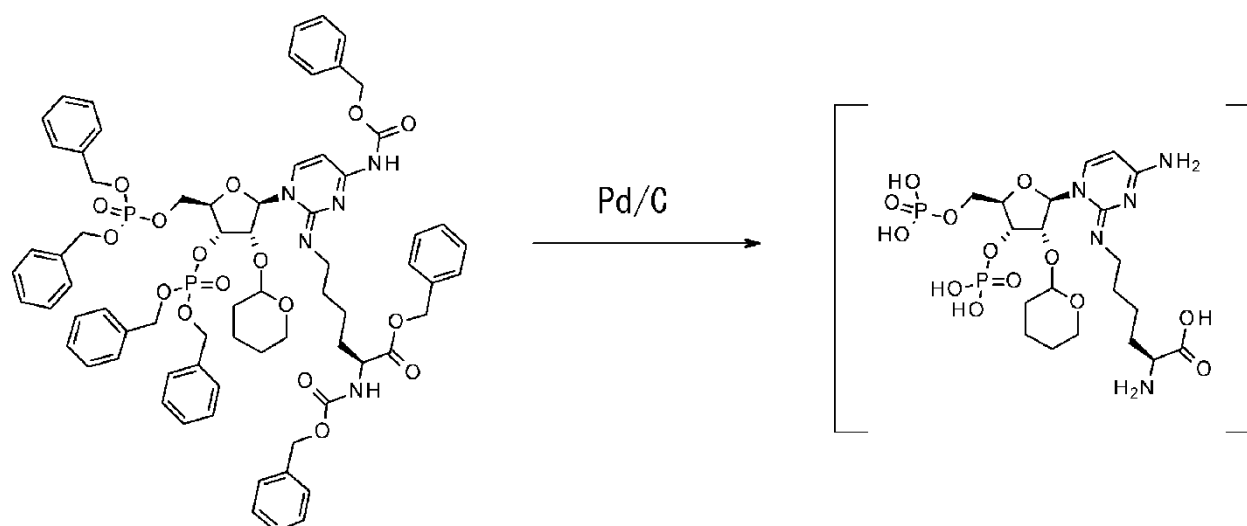
5 LCMS (ESI) m/z = 1332.8 (M-H)⁻

Retention time: 3.08 minutes, 3.11 minutes (analysis condition SQDAA05long)

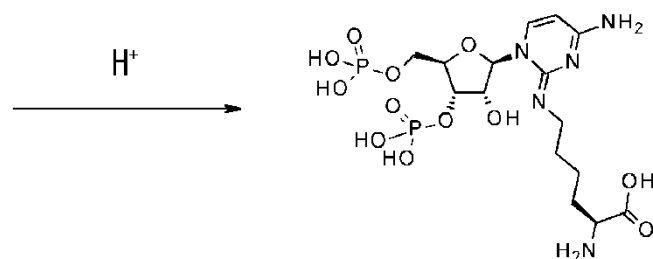
[0188]

Synthesis of (2S)-2-amino-6-[[4-amino-1-[(2R,3R,4S,5R)-3-hydroxy-4-phosphonooxy-5-(phosphonooxymethyl)tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoic acid

10 (Compound SS04, pLp)



Compound SS30



Compound SS04

[0189]

Benzyl (2S)-2-(benzyloxycarbonylamino)-6-[[4-(benzyloxycarbonylamino)-1-[(2R,3R,4R,5R)-4-dibenzyloxyphosphoryloxy-5-(dibenzyloxyphosphoryloxymethyl)-3-

tetrahydropyran-2-yloxy-tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoate (Compound SS30) (36.59 mg, 0.027 mmol) was dissolved in a mixed solvent of methanol (649 μ L) and ultrapure water (152 μ L) at room temperature, and palladium on carbon (10% Pd) (5.84 mg, 5.48 μ mol) was added under nitrogen atmosphere. Under hydrogen atmosphere, this mixture was stirred at room temperature for 18 hours. The reaction solution was filtered through Celite, and washed several times using ultrapure water. To the obtained filtrate (24.66 mL), 1 mol/L hydrogen chloride (2.74 mL, 2.74 mmol) was added, and was left to stand at room temperature for one hour. The reaction solution was filtered through Celite, and washed several times using ultrapure water. After freeze-drying the filtrate, the obtained powder was redissolved using ultrapure water (1.52 mL), and then centrifugation was performed and the supernatant was recovered to obtain an aqueous solution of (2S)-2-amino-6-[[4-amino-1-[(2R,3R,4S,5R)-3-hydroxy-4-phosphonooxy-5-(phosphonooxymethyl)tetrahydrofuran-2-yl]pyrimidin-2-ylidene]amino]hexanoic acid (Compound SS04, pLp) (1.37mL, 17.47 mM, 87%, two steps). LCMS (ESI) m/z = 530.1 (M-H)-

Retention time: 1.60 minutes (analysis condition LTQTEA/HFIP05_02)

Column exchange was performed during the time after analyzing Compound SS04 synthesized in Example 2 and before analyzing Compound SS04 synthesized in Example 3. Compound SS04 synthesized in Example 2 was analyzed again after column exchange, and was confirmed to be the same as Compound SS04 synthesized in Example 3. The results are shown below.

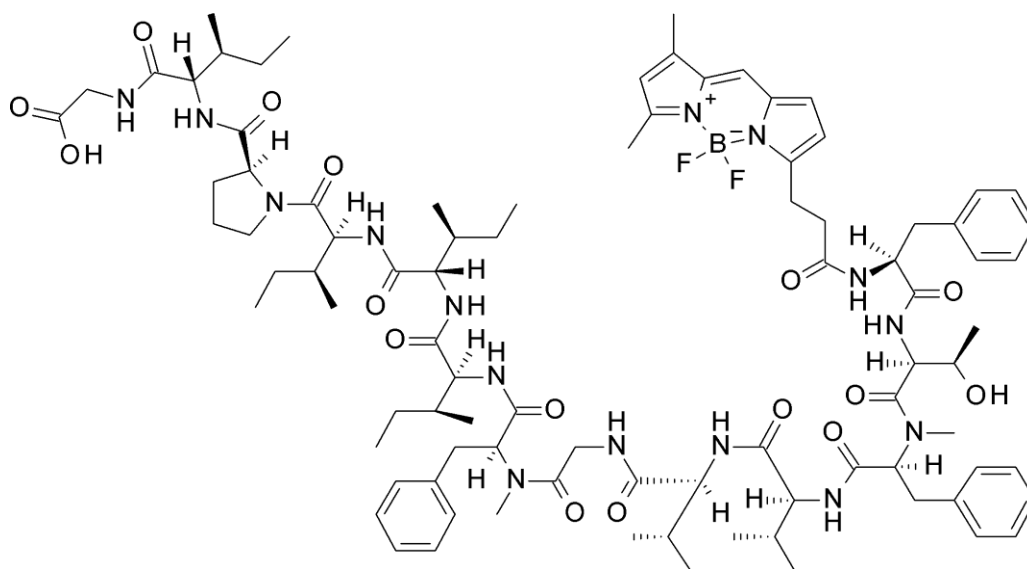
LCMS (ESI) m/z = 530.1 (M-H)-

Retention time: 1.60 minutes (analysis condition LTQTEA/HFIP05_02)

[0190]

Example 3

Example 3-1. Synthesis of a peptide bearing BdpFL at its N terminus (LCT-67)



[0191]

Using 2-chlorotrityl resin bearing Fmoc-Gly-OH (100 mg), and using Fmoc-Gly-OH, Fmoc-Thr(THP)-OH (aa01) synthesized by a method described in patent literature (WO2018225864), Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-MePhe-OH, and Fmoc-Pro-OH as Fmoc amino acids, peptide elongation was performed on a peptide synthesizer (abbreviations of amino acids are described separately in this specification). Peptide elongation was performed according to a peptide synthesis method using the Fmoc method (WO2013100132B2). After the peptide elongation, N-terminal Fmoc group was removed on the peptide synthesizer, and then the resin was washed with DCM.

TFE/DCM (1:1, v/v, 2 mL) was added to the resin, this was shaken for 1 hour, and the peptides were cleaved off from the resin. After completion of the reaction, the resin was removed by filtering the solution inside the tube through a synthesis column, and the resin was washed twice with TFE/DCM (1:1, v/v, 1 mL). All of the extracts were mixed, DMF (2 mL) was added, and then the mixture was concentrated under reduced pressure. The obtained residue was dissolved in NMP (1 mL), 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid N-succinimidyl ester (5 mg, 0.013 mmol) was added at room temperature, the mixture was stirred for 19 hours, and then the reaction solution was subjected to reverse-phase silica gel column chromatography (0.1% FA MeCN/H₂O), and the fraction containing the intermediate was concentrated under reduced pressure. The obtained residue was dissolved in 5% TFA in DCM (2 mL) and stirred at room temperature for 2 hours. The reaction solution was concentrated under reduced pressure, and then the obtained residue was purified by reverse-phase silica gel column chromatography (0.1% FA MeCN/H₂O) to obtain the title compound (LCT-67) (13 mg). The amino acid sequence of LCT-67 is shown in SEQ ID NO: 237.

LCMS (ESI) m/z = 1751.2 (M-H)⁻

[0192]

[illegible]

[0193]

Using 2-chlorotrityl resin bearing Fmoc-Ala-OH (100 mg), and using Fmoc-Gly-OH, Fmoc-Thr(THP)-OH (aa01) synthesized by a method described in patent literature (WO2018225864), Fmoc-Ile-OH, Fmoc-Phe-OH, and Fmoc-Pro-OH as Fmoc amino acids, peptide elongation was performed on a peptide synthesizer (abbreviations of amino acids are described separately in this specification). Peptide elongation was performed according to a peptide synthesis method using the Fmoc method (WO2013100132B2). After the peptide elongation, removal of the N-terminal Fmoc group was performed on the peptide synthesizer, and then the resin was washed with DCM.

TFE/DCM (1:1, v/v, 2 mL) was added to the resin, this was shaken for 1 hour, and the peptides were cleaved off from the resin. After completion of the reaction, the resin was removed by filtering the solution inside the tube through a synthesis column, and the resin was washed twice with TFE/DCM (1:1, v/v, 1 mL). All of the extract solutions were mixed, DMF (2 mL) was added, and then the mixture was concentrated under reduced pressure. The obtained residue was dissolved in NMP (0.5 mL), and one-fourth (125 μ L) of it was used in the next reaction. To the peptide solution in NMP, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid N-succinimide ester (140 μ L) adjusted to 76.5 mM was added at room temperature, the mixture was stirred overnight at 40°C, and then concentrated under reduced pressure. The obtained residue was dissolved in 0.05 M tetramethylammonium hydrogen sulfate in HFIP (1.2 mL, 0.060 mmol) and stirred at room temperature for 2 hours. The reaction solution was purified by reverse-phase silica gel column chromatography (0.1% FA MeCN/H₂O) to obtain the title compound (LCT-12) (0.3 mg). The amino acid sequence of LCT-12 is shown in SEQ ID NO: 238.

LCMS (ESI) m/z = 1972.9 (M-H)⁻

Retention time: 0.74 minutes (analysis condition SQDFA05_01)

[0194]

Example 4 Synthesis of aminoacyl tRNAs

Example 4-1. Preparation of tRNAs

From template DNAs (TD-1 to TD-107), tRNAs (TR-1 to TR-107) were synthesized by *in vitro* transcription reaction using T7 RNA polymerase, and were purified by RNeasy kit (Qiagen).

[0195]

Template DNA SEQ ID NO: 1 (TD-1)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCCTaag
AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0196]

Template DNA SEQ ID NO: 2 (TD-2)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTaag

5 ACGGCGGTAACAGGGGTTTGAATCCCCTAGGGGACGC

[0197]

Template DNA SEQ ID NO: 3 (TD-3)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTaag

10 AAGGCGGTAACAGGGGTTTGAATCCCCTAGGGGACGC

[0198]

Template DNA SEQ ID NO: 4 (TD-4)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTaag

15 ACGGCGGTAACAGGGGTTTGAATCCCCTAGGGGACGC

[0199]

Template DNA SEQ ID NO: 5 (TD-5)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTaag

20 GAGGCGGTAACAGGGGTTTGAATCCCCTAGGGGACGC

[0200]

Template DNA SEQ ID NO: 6 (TD-6)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTaag

25 ATGGCGGTAACAGGGGTTTGAATCCCCTAGGGGACGC

[0201]

Template DNA SEQ ID NO: 7 (TD-7)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTaag

30 GTGGCGGTAACAGGGGTTTGAATCCCCTAGGGGACGC

[0202]

Template DNA SEQ ID NO: 8 (TD-8)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTaag

35 GAGGCGGTAACAGGGGTTTGAATCCCCTAGGGGACGC

[0203]

Template DNA SEQ ID NO: 9 (TD-9)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCATaag
GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

5 [0204]

Template DNA SEQ ID NO: 10 (TD-10)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCATaag
ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

10 [0205]

Template DNA SEQ ID NO: 11 (TD-11)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCCTcag
AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

15 [0206]

Template DNA SEQ ID NO: 12 (TD-12)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTcag
ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

20 [0207]

Template DNA SEQ ID NO: 13 (TD-13)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTcag
AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

25 [0208]

Template DNA SEQ ID NO: 14 (TD-14)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCCTcag
ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

30 [0209]

Template DNA SEQ ID NO: 15 (TD-15)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCCTcag
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

35 [0210]

Template DNA SEQ ID NO: 16 (TD-16)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTcag
ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0211]

5 Template DNA SEQ ID NO: 17 (TD-17)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTcag
GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0212]

10 Template DNA SEQ ID NO: 18 (TD-18)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTcag
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0213]

15 Template DNA SEQ ID NO: 19 (TD-19)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCATcag
GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0214]

20 Template DNA SEQ ID NO: 20 (TD-20)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCATcag
ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0215]

25 Template DNA SEQ ID NO: 21 (TD-21)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCCTagg
AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0216]

30 Template DNA SEQ ID NO: 22 (TD-22)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTagg
ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0217]

35 Template DNA SEQ ID NO: 23 (TD-23)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTagg
AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0218]

Template DNA SEQ ID NO: 24 (TD-24)

5 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTagg
ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0219]

Template DNA SEQ ID NO: 25 (TD-25)

10 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTagg
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0220]

Template DNA SEQ ID NO: 26 (TD-26)

15 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTagg
ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0221]

Template DNA SEQ ID NO: 27 (TD-27)

20 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTagg
GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0222]

Template DNA SEQ ID NO: 28 (TD-28)

25 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTagg
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0223]

Template DNA SEQ ID NO: 29 (TD-29)

30 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCATagg
GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0224]

Template DNA SEQ ID NO: 30 (TD-30)

35 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCATagg

ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0225]

Template DNA SEQ ID NO: 31 (TD-31)

DNA sequence:

5 GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTcgg
AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0226]

Template DNA SEQ ID NO: 32 (TD-32)

DNA sequence:

10 GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTcgg
ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0227]

Template DNA SEQ ID NO: 33 (TD-33)

DNA sequence:

15 GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTcgg
AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0228]

Template DNA SEQ ID NO: 34 (TD-34)

DNA sequence:

20 GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTcgg
ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0229]

Template DNA SEQ ID NO: 35 (TD-35)

DNA sequence:

25 GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTcgg
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0230]

Template DNA SEQ ID NO: 36 (TD-36)

DNA sequence:

30 GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTcgg
ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0231]

Template DNA SEQ ID NO: 37 (TD-37)

DNA sequence:

35 GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTcgg
GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0232]

Template DNA SEQ ID NO: 38 (TD-38)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTcgg

5 GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0233]

Template DNA SEQ ID NO: 39 (TD-39)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCATcgg

10 GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0234]

Template DNA SEQ ID NO: 40 (TD-40)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCATcgg

15 ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0235]

Template DNA SEQ ID NO: 41 (TD-41)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTacc

20 AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0236]

Template DNA SEQ ID NO: 42 (TD-42)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTacc

25 ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0237]

Template DNA SEQ ID NO: 43 (TD-43)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTacc

30 AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0238]

Template DNA SEQ ID NO: 44 (TD-44)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCCTacc

35 ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0239]

Template DNA SEQ ID NO: 45 (TD-45)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCCTacc
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

5 [0240]

Template DNA SEQ ID NO: 46 (TD-46)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTacc
ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

10 [0241]

Template DNA SEQ ID NO: 47 (TD-47)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTacc
GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

15 [0242]

Template DNA SEQ ID NO: 48 (TD-48)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTacc
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

20 [0243]

Template DNA SEQ ID NO: 49 (TD-49)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCATacc
GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

25 [0244]

Template DNA SEQ ID NO: 50 (TD-50)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCATacc
ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

30 [0245]

Template DNA SEQ ID NO: 51 (TD-51)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCCTccc
AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

35 [0246]

Template DNA SEQ ID NO: 52 (TD-52)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTccc
ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0247]

5 Template DNA SEQ ID NO: 53 (TD-53)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTccc
AAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0248]

10 Template DNA SEQ ID NO: 54 (TD-54)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTccc
ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0249]

15 Template DNA SEQ ID NO: 55 (TD-55)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTccc
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0250]

20 Template DNA SEQ ID NO: 56 (TD-56)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTccc
ATGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0251]

25 Template DNA SEQ ID NO: 57 (TD-57)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTccc
GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0252]

30 Template DNA SEQ ID NO: 58 (TD-58)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCCAGGACACCGCCTTccc
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0253]

35 Template DNA SEQ ID NO: 59 (TD-59)

DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCATccc
GTGGCGGTAACAGGGGTTTGAATCCCCTAGGGGACGC

[0254]

Template DNA SEQ ID NO: 60 (TD-60)

5 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCATccc
ATGGCGGTAACAGGGGTTTGAATCCCCTAGGGGACGC

[0255]

Template DNA SEQ ID NO: 61 (TD-61)

10 DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTaag
AAGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTCCGC

[0256]

Template DNA SEQ ID NO: 62 (TD-62)

15 DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTaag
ACGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTCCGC

[0257]

Template DNA SEQ ID NO: 63 (TD-63)

20 DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTaag
AAGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTCCGC

[0258]

Template DNA SEQ ID NO: 64 (TD-64)

25 DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTaag
ACGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTCCGC

[0259]

Template DNA SEQ ID NO: 65 (TD-65)

30 DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTaag
GAGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTCCGC

[0260]

Template DNA SEQ ID NO: 66 (TD-66)

35 DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTaag

ATGCAGGGGGTTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0261]

Template DNA SEQ ID NO: 67 (TD-67)

DNA sequence:

5 GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCTTaag
GTGCAGGGGGTTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0262]

Template DNA SEQ ID NO: 68 (TD-68)

DNA sequence:

10 GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCTTaag
GAGCAGGGGGTTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0263]

Template DNA SEQ ID NO: 69 (TD-69)

DNA sequence:

15 GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCATaag
GTGCAGGGGGTTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0264]

Template DNA SEQ ID NO: 70 (TD-70)

DNA sequence:

20 GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCATaag
ATGCAGGGGGTTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0265]

Template DNA SEQ ID NO: 71 (TD-71)

DNA sequence:

25 GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTcag
AAGCAGGGGGTTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0266]

Template DNA SEQ ID NO: 72 (TD-72)

DNA sequence:

30 GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCTTcag
ACGCAGGGGGTTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0267]

Template DNA SEQ ID NO: 73 (TD-73)

DNA sequence:

35 GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCTTcag
AAGCAGGGGGTTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0268]

Template DNA SEQ ID NO: 74 (TD-74)

DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTcag

5 ACGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0269]

Template DNA SEQ ID NO: 75 (TD-75)

DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCCTcag

10 GAGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0270]

Template DNA SEQ ID NO: 76 (TD-76)

DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCTTcag

15 ATGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0271]

Template DNA SEQ ID NO: 77 (TD-77)

DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCTTcag

20 GTGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0272]

Template DNA SEQ ID NO: 78 (TD-78)

DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCTTcag

25 GAGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0273]

Template DNA SEQ ID NO: 79 (TD-79)

DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCATcag

30 GTGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0274]

Template DNA SEQ ID NO: 80 (TD-80)

DNA sequence:

GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCATcag

35 ATGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0275]

Template DNA SEQ ID NO: 81 (TD-81)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGACTaag
AATCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

5 [0276]

Template DNA SEQ ID NO: 82 (TD-82)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTaagA
CTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

10 [0277]

Template DNA SEQ ID NO: 83 (TD-83)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTaagA
ATCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

15 [0278]

Template DNA SEQ ID NO: 84 (TD-84)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGACTaag
ACTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

20 [0279]

Template DNA SEQ ID NO: 85 (TD-85)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGACTaag
GATCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

25 [0280]

Template DNA SEQ ID NO: 86 (TD-86)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTaagA
TTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

30 [0281]

Template DNA SEQ ID NO: 87 (TD-87)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTaagG
TTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

35 [0282]

Template DNA SEQ ID NO: 88 (TD-88)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTaagG
ATCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0283]

5 Template DNA SEQ ID NO: 89 (TD-89)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGAATaag
GTTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0284]

10 Template DNA SEQ ID NO: 90 (TD-90)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGAATaag
ATTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0285]

15 Template DNA SEQ ID NO: 91 (TD-91)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGACTcag
AATCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0286]

20 Template DNA SEQ ID NO: 92 (TD-92)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTcagA
CTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0287]

25 Template DNA SEQ ID NO: 93 (TD-93)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTcagA
ATCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0288]

30 Template DNA SEQ ID NO: 94 (TD-94)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGACTcag
ACTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0289]

35 Template DNA SEQ ID NO: 95 (TD-95)

DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGACTcag
GATCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0290]

Template DNA SEQ ID NO: 96 (TD-96)

5 DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTcagA
TTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0291]

Template DNA SEQ ID NO: 97 (TD-97)

10 DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTcagG
TTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0292]

Template DNA SEQ ID NO: 98 (TD-98)

15 DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTcagG
ATCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0293]

Template DNA SEQ ID NO: 99 (TD-99)

20 DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGAATcag
GTTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0294]

Template DNA SEQ ID NO: 100 (TD-100)

25 DNA sequence:

GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGAATcag
ATTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0295]

Template DNA SEQ ID NO: 101 (TD-101)

30 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCCTgcc
ACGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0296]

Template DNA SEQ ID NO: 102 (TD-102)

35 DNA sequence:

GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTgcc

GTGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0297]

Template DNA SEQ ID NO: 103 (TD-103)

DNA sequence:

5 GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCTTgcc
GAGGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0298]

Template DNA SEQ ID NO: 104 (TD-104)

DNA sequence:

10 GGCGTAATACGACTCACTATAGTCCCCTTCGTCTAGAGGCCAGGACACCGCCCTtcc
ACGCGGTAACAGGGGTTCGAATCCCCTAGGGGACGC

[0299]

Template DNA SEQ ID NO: 105 (TD-105)

DNA sequence:

15 GGCGTAATACGACTCACTATAGGCGGGGTGGAGCAGCCTGGTAGCTCGTCGGGCTcat
AACCCGAAGATCGTCGGTTCAAATCCGGCCCCCGCAAC

[0300]

Template DNA SEQ ID NO: 106 (TD-106)

DNA sequence:

20 GGCGTAATACGACTCACTATAGGAGCGGTAGTTCAGTCGGTTAGAATACCTGCTTtag
GTGCAGGGGGTCGCGGGTTCGAGTCCCGTCCGTTCCGC

[0301]

Template DNA SEQ ID NO: 107 (TD-107)

DNA sequence:

25 GGCGTAATACGACTCACTATAGGCTCTGTAGTTCAGTCGGTAGAACGGCGGATTtagG
TTCCGTATGTCACTGGTTCGAGTCCAGTCAGAGCCGC

[0302]

tRNA SEQ ID NO: 108 (TR-1)

tRNA(Glu2+Ser5)aag-CA RNA sequence:

30 GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaagAAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0303]

tRNA SEQ ID NO: 109 (TR-2)

tRNA(Glu2+Ala1B)aag-CA RNA sequence:

35 GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaagACGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0304]

tRNA SEQ ID NO: 110 (TR-3)

tRNA(Glu2+Phe)aag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCUaagAAGGCGGUAACAGGGGUUC

5 GAAUCCCCUAGGGGACGC

[0305]

tRNA SEQ ID NO: 111 (TR-4)

tRNA(Glu2)aag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCUaagACGGCGGUAACAGGGGUUC

10 GAAUCCCCUAGGGGACGC

[0306]

tRNA SEQ ID NO: 112 (TR-5)

tRNA(Glu2+Arg3)aag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCUaagGAGGCGGUAACAGGGGUUC

15 GAAUCCCCUAGGGGACGC

[0307]

tRNA SEQ ID NO: 113 (TR-6)

tRNA(Glu2+Val2)aag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCUaagAUGGCGGUAACAGGGGUUC

20 GAAUCCCCUAGGGGACGC

[0308]

tRNA SEQ ID NO: 114 (TR-7)

tRNA(Glu2+Leu2)aag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCUaagGUGGCGGUAACAGGGGUUC

25 GAAUCCCCUAGGGGACGC

[0309]

tRNA SEQ ID NO: 115 (TR-8)

tRNA(Glu2+Pro3)aag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCUaagGAGGCGGUAACAGGGGUUC

30 GAAUCCCCUAGGGGACGC

[0310]

tRNA SEQ ID NO: 116 (TR-9)

tRNA(Glu2+Pro2)aag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCAaagGUGGCGGUAACAGGGGUUC

35 GAAUCCCCUAGGGGACGC

[0311]

tRNA SEQ ID NO: 117 (TR-10)

tRNA(Glu2+Ala2)aag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCCAGGACACCGCCAUaagAUGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

5 [0312]

tRNA SEQ ID NO: 118 (TR-11)

tRNA(Glu2+Ser5)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCCAGGACACCGCCCUcagAAGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

10 [0313]

tRNA SEQ ID NO:119 (TR-12)

tRNA(Glu2+Ala1B)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCCAGGACACCGCCUcagACGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

15 [0314]

tRNA SEQ ID NO:120 (TR-13)

tRNA(Glu2+Phe)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCCAGGACACCGCCUcagAAGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

20 [0315]

tRNA SEQ ID NO:121 (TR-14)

tRNA(Glu2)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCCAGGACACCGCCUcagACGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

25 [0316]

tRNA SEQ ID NO:122 (TR-15)

tRNA(Glu2+Arg3)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCCAGGACACCGCCUcagGAGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

30 [0317]

tRNA SEQ ID NO:123 (TR-16)

tRNA(Glu2+Val2)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCCAGGACACCGCCUcagAUGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

35 [0318]

tRNA SEQ ID NO:124 (TR-17)

tRNA(Glu2+Leu2)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUucagGUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0319]

5 tRNA SEQ ID NO:125 (TR-18)

tRNA(Glu2+Pro3)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUucagGAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0320]

10 tRNA SEQ ID NO:126 (TR-19)

tRNA(Glu2+Pro2)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCAucagGUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0321]

15 tRNA SEQ ID NO:127 (TR-20)

tRNA(Glu2+Ala2)cag-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCAucagAUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0322]

20 tRNA SEQ ID NO:128 (TR-21)

tRNA(Glu2+Ser5)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUaggAAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0323]

25 tRNA SEQ ID NO:129 (TR-22)

tRNA(Glu2+Ala1B)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUaggACGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0324]

30 tRNA SEQ ID NO:130 (TR-23)

tRNA(Glu2+Phe)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUaggAAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0325]

35 tRNA SEQ ID NO:131 (TR-24)

tRNA(Glu2)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaggACGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0326]

tRNA SEQ ID NO:132 (TR-25)

5 tRNA(Glu2+Arg3)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaggGAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0327]

tRNA SEQ ID NO:133 (TR-26)

10 tRNA(Glu2+Val2)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaggAUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0328]

tRNA SEQ ID NO:134 (TR-27)

15 tRNA(Glu2+Leu2)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaggGUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0329]

tRNA SEQ ID NO:135 (TR-28)

20 tRNA(Glu2+Pro3)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaggGAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0330]

tRNA SEQ ID NO:136 (TR-29)

25 tRNA(Glu2+Pro2)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCAUaggGUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0331]

tRNA SEQ ID NO:137 (TR-30)

30 tRNA(Glu2+Ala2)agg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCAUaggAUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0332]

tRNA SEQ ID NO:138 (TR-31)

35 tRNA(Glu2+Ser5)cgg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUcggAAGGCGGUAACAGGGGUUC

GAAUCCCCUAGGGGACGC

[0333]

tRNA SEQ ID NO:139 (TR-32)

tRNA(Glu2+Ala1B)cgg-CA RNA sequence:

5 GUCCCCUUCGUCUAGAGGGCCAGGACACCGCCUucggACGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0334]

tRNA SEQ ID NO:140 (TR-33)

tRNA(Glu2+Phe)cgg-CA RNA sequence:

10 GUCCCCUUCGUCUAGAGGGCCAGGACACCGCCUucggAAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0335]

tRNA SEQ ID NO:141 (TR-34)

tRNA(Glu2)cgg-CA RNA sequence:

15 GUCCCCUUCGUCUAGAGGGCCAGGACACCGCCUucggACGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0336]

tRNA SEQ ID NO:142 (TR-35)

tRNA(Glu2+Arg3)cgg-CA RNA sequence:

20 GUCCCCUUCGUCUAGAGGGCCAGGACACCGCCUucggGAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0337]

tRNA SEQ ID NO:143 (TR-36)

tRNA(Glu2+Val2)cgg-CA RNA sequence:

25 GUCCCCUUCGUCUAGAGGGCCAGGACACCGCCUucggAUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0338]

tRNA SEQ ID NO:144 (TR-37)

tRNA(Glu2+Leu2)cgg-CA RNA sequence:

30 GUCCCCUUCGUCUAGAGGGCCAGGACACCGCCUucggGUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0339]

tRNA SEQ ID NO:145 (TR-38)

tRNA(Glu2+Pro3)cgg-CA RNA sequence:

35 GUCCCCUUCGUCUAGAGGGCCAGGACACCGCCUucggGAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0340]

tRNA SEQ ID NO:146 (TR-39)

tRNA(Glu2+Pro2)cgg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCAUcggGUGGCGGUAACAGGGGUUC

5 GAAUCCCCUAGGGGACGC

[0341]

tRNA SEQ ID NO:147 (TR-40)

tRNA(Glu2+Ala2)cgg-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCAUcggAUGGCGGUAACAGGGGUUC

10 GAAUCCCCUAGGGGACGC

[0342]

tRNA SEQ ID NO:148 (TR-41)

tRNA(Glu2+Ser5)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCCUaccAAGGCGGUAACAGGGGUUC

15 GAAUCCCCUAGGGGACGC

[0343]

tRNA SEQ ID NO:149 (TR-42)

tRNA(Glu2+Ala1B)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCCUaccACGGCGGUAACAGGGGUUC

20 GAAUCCCCUAGGGGACGC

[0344]

tRNA SEQ ID NO:150 (TR-43)

tRNA(Glu2+Phe)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCCUaccAAGGCGGUAACAGGGGUUC

25 GAAUCCCCUAGGGGACGC

[0345]

tRNA SEQ ID NO:151 (TR-44)

tRNA(Glu2)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCCUaccACGGCGGUAACAGGGGUUC

30 GAAUCCCCUAGGGGACGC

[0346]

tRNA SEQ ID NO:152 (TR-45)

tRNA(Glu2+Arg3)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCCAGGACACCGCCCUaccGAGGCGGUAACAGGGGUUC

35 GAAUCCCCUAGGGGACGC

[0347]

tRNA SEQ ID NO:153 (TR-46)

tRNA(Glu2+Val2)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCU^{Uacc}AUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

5 [0348]

tRNA SEQ ID NO:154 (TR-47)

tRNA(Glu2+Leu2)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCU^{Uacc}GUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

10 [0349]

tRNA SEQ ID NO:155 (TR-48)

tRNA(Glu2+Pro3)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCU^{Uacc}GAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

15 [0350]

tRNA SEQ ID NO:156 (TR-49)

tRNA(Glu2+Pro2)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCA^{Uacc}GUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

20 [0351]

tRNA SEQ ID NO:157 (TR-50)

tRNA(Glu2+Ala2)acc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCA^{Uacc}AUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

25 [0352]

tRNA SEQ ID NO:158 (TR-51)

tRNA(Glu2+Ser5)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCU^{Uccc}AAGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

30 [0353]

tRNA SEQ ID NO:159 (TR-52)

tRNA(Glu2+Ala1B)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCU^{Uccc}ACGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

35 [0354]

tRNA SEQ ID NO:160 (TR-53)

tRNA(Glu2+Phe)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUuccAAGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0355]

5 tRNA SEQ ID NO:161 (TR-54)

tRNA(Glu2)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUuccACGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0356]

10 tRNA SEQ ID NO:162 (TR-55)

tRNA(Glu2+Arg3)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUuccGAGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0357]

15 tRNA SEQ ID NO:163 (TR-56)

tRNA(Glu2+Val2)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUuccAUGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0358]

20 tRNA SEQ ID NO:164 (TR-57)

tRNA(Glu2+Leu2)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUuccGUGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0359]

25 tRNA SEQ ID NO:165 (TR-58)

tRNA(Glu2+Pro3)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCUuccGAGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0360]

30 tRNA SEQ ID NO:166 (TR-59)

tRNA(Glu2+Pro2)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCAUuccGUGGCGGUAAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0361]

35 tRNA SEQ ID NO:167 (TR-60)

tRNA(Glu2+Ala2)ccc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCAUcccAUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0362]

tRNA SEQ ID NO:168 (TR-61)

5 tRNA(Asp1+Ser5)aag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCCUaagAAGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0363]

tRNA SEQ ID NO:169 (TR-62)

10 tRNA(Asp1+Ala1B)aag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUaagACGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0364]

tRNA SEQ ID NO:170 (TR-63)

15 tRNA(Asp1+Phe)aag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUaagAAGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0365]

tRNA SEQ ID NO:171 (TR-64)

20 tRNA(Asp1)aag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCCUaagACGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0366]

tRNA SEQ ID NO:172 (TR-65)

25 tRNA(Asp1+Arg3)aag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCCUaagGAGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0367]

tRNA SEQ ID NO:173 (TR-66)

30 tRNA(Asp1+Val2)aag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUaagAUGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0368]

tRNA SEQ ID NO:174 (TR-67)

35 tRNA(Asp1+Leu2)aag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUaagGUGCAGGGGGUCGCGGGU

UCGAGUCCCGUCCGUUCCGC

[0369]

tRNA SEQ ID NO:175 (TR-68)

tRNA(Asp1+Pro3)aag-CA RNA sequence:

5 GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUaagGAGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0370]

tRNA SEQ ID NO:176 (TR-69)

tRNA(Asp1+Pro2)aag-CA RNA sequence:

10 GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCAUaagGUGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0371]

tRNA SEQ ID NO:177 (TR-70)

tRNA(Asp1+Ala2)aag-CA RNA sequence:

15 GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCAUaagAUGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0372]

tRNA SEQ ID NO:178 (TR-71)

tRNA(Asp1+Ser5)cag-CA RNA sequence:

20 GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCCUcagAAGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0373]

tRNA SEQ ID NO:179 (TR-72)

tRNA(Asp1+Ala1B)cag-CA RNA sequence:

25 GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUcagACGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0374]

tRNA SEQ ID NO:180 (TR-73)

tRNA(Asp1+Ph2)cag-CA RNA sequence:

30 GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUcagAAGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0375]

tRNA SEQ ID NO:181 (TR-74)

tRNA(Asp1)cag-CA RNA sequence:

35 GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCCUcagACGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0376]

tRNA SEQ ID NO:182 (TR-75)

tRNA(Asp1+Arg3)cag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCCUcagGAGCAGGGGGUCGCGGGU

5 UCGAGUCCCGUCCGUUCCGC

[0377]

tRNA SEQ ID NO:183 (TR-76)

tRNA(Asp1+Val2)cag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUcagAUGCAGGGGGUCGCGGGU

10 UCGAGUCCCGUCCGUUCCGC

[0378]

tRNA SEQ ID NO:184 (TR-77)

tRNA(Asp1+Leu2)cag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUcagGUGCAGGGGGUCGCGGGU

15 UCGAGUCCCGUCCGUUCCGC

[0379]

tRNA SEQ ID NO:185 (TR-78)

tRNA(Asp1+Pro3)cag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUcagGAGCAGGGGGUCGCGGGU

20 UCGAGUCCCGUCCGUUCCGC

[0380]

tRNA SEQ ID NO:186 (TR-79)

tRNA(Asp1+Pro2)cag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCAUcagGUGCAGGGGGUCGCGGGU

25 UCGAGUCCCGUCCGUUCCGC

[0381]

tRNA SEQ ID NO:187 (TR-80)

tRNA(Asp1+Ala2)cag-CA RNA sequence:

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCAUcagAUGCAGGGGGUCGCGGGU

30 UCGAGUCCCGUCCGUUCCGC

[0382]

tRNA SEQ ID NO:188 (TR-81)

tRNA(AsnE2)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGACUaagAAUCCGUAUGUCACUGGUU

35 CGAGUCCAGUCAGAGCCGC

[0383]

tRNA SEQ ID NO:189 (TR-82)

tRNA(AsnE2+Ala1B)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUaagACUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

5 [0384]

tRNA SEQ ID NO:190 (TR-83)

tRNA(AsnE2+Phe)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUaagAAUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

10 [0385]

tRNA SEQ ID NO:191 (TR-84)

tRNA(AsnE2+Glu2)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGACUaagACUCCGUAUGUCACUGGUUC
GAGUCCAGUCAGAGCCGC

15 [0386]

tRNA SEQ ID NO:192 (TR-85)

tRNA(AsnE2+Arg3)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGACUaagGAUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

20 [0387]

tRNA SEQ ID NO:193 (TR-86)

tRNA(AsnE2+Val2)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUaagAUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

25 [0388]

tRNA SEQ ID NO:194 (TR-87)

tRNA(AsnE2+Leu2)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUaagGUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

30 [0389]

tRNA SEQ ID NO:195 (TR-88)

tRNA(AsnE2+Pro3)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUaagGAUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

35 [0390]

tRNA SEQ ID NO:196 (TR-89)

tRNA(AsnE2+Pro2)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUaagGUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0391]

5 tRNA SEQ ID NO:197 (TR-90)

tRNA(AsnE2+Ala2)aag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUaagAUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0392]

10 tRNA SEQ ID NO:198 (TR-91)

tRNA(AsnE2)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGACUcagAAUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0393]

15 tRNA SEQ ID NO:199 (TR-92)

tRNA(AsnE2+Ala1B)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUcagACUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0394]

20 tRNA SEQ ID NO:200 (TR-93)

tRNA(AsnE2+Phe)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUcagAAUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0395]

25 tRNA SEQ ID NO:201 (TR-94)

tRNA(AsnE2+Glu2)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGACUcagACUCCGUAUGUCACUGGUUC
GAGUCCAGUCAGAGCCGC

[0396]

30 tRNA SEQ ID NO:202 (TR-95)

tRNA(AsnE2+Arg3)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGACUcagGAUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0397]

35 tRNA SEQ ID NO:203 (TR-96)

tRNA(AsnE2+Val2)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUcagAUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0398]

tRNA SEQ ID NO:204 (TR-97)

5 tRNA(AsnE2+Leu2)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUcagGUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0399]

tRNA SEQ ID NO:205 (TR-98)

10 tRNA(AsnE2+Pro3)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUcagGAUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0400]

tRNA SEQ ID NO:206 (TR-99)

15 tRNA(AsnE2+Pro2)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAAUcagGUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0401]

tRNA SEQ ID NO:207 (TR-100)

20 tRNA(AsnE2+Ala2)cag-CA RNA sequence:

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAAUcagAUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0402]

tRNA SEQ ID NO:208 (TR-101)

25 tRNA(Glu2)gcc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCACAGGACACCGCCCUgccACGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0403]

tRNA SEQ ID NO:209 (TR-102)

30 tRNA(Glu2+Leu2)gcc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCACAGGACACCGCCCUgccGUGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0404]

tRNA SEQ ID NO:210 (TR-103)

35 tRNA(Glu2+Pro3)gcc-CA RNA sequence:

GUCCCCUUCGUCUAGAGGCCACAGGACACCGCCCUgccGAGGCGGUAACAGGGGUUC

GAAUCCCCUAGGGGACGC

[0405]

tRNA SEQ ID NO:211 (TR-104)

tRNA(Glu2)ucc-CA RNA sequence:

5 GUCCCCUUCGUCUAGAGGGCCAGGACACCGCCCUuccACGGCGGUAACAGGGGUUC
GAAUCCCCUAGGGGACGC

[0406]

tRNA SEQ ID NO:212 (TR-105)

tRNA(fMet)cau-CA RNA sequence:

10 GGCGGGGUGGAGCAGCCUGGUAGCUCGUCGGGCUcauAACCCGAAGAUCGUCGGUU
CAAAUCCGGCCCCCGCAAC

[0407]

tRNA SEQ ID NO:213 (TR-106)

tRNA(Asp1+Leu2)_uag-CA RNA sequence:

15 GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUUuagGUGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0408]

tRNA SEQ ID NO:214 (TR-107)

tRNA(AsnE2+Leu2)_uag-CA RNA sequence:

20 GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUuagGUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

[0409]

Example 4-2. Production of tRNA-CAs by ligation reaction

By the procedure described below, tRNA5' fragments, pLp, and tRNA3' fragments
25 were ligated using a ligation reaction to produce various tRNA-CAs. Chemically synthesized
products (Gene Design Co., Ltd.) were used for the tRNA 5' fragments and tRNA 3' fragments.
Each tRNA fragment and its full-length sequences are shown below. FR-1 and FR-2 were used
as the tRNA 5' fragment and the tRNA 3' fragment, respectively, to produce TR-108, and FR-3
and FR-4 were used as the tRNA 5' fragment and the tRNA 3' fragment, respectively, to
30 produce TR-109.

[0410]

SEQ ID NO: 217 (FR-1)

tRNA(Asp1)5' RNA sequence

GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUU

35 [0411]

SEQ ID NO: 218 (FR-2)

tRNA(Asp1)3' agRNA sequence

AGGUGCAGGGGGUCGCGGGUUCGAGUCCCGUCCGUUCCGC

tRNA SEQ ID NO: 215 (TR-108)

tRNA(Asp1+Leu2)Lag-CA RNA sequence:

5 GGAGCGGUAGUUCAGUCGGUUAGAAUACCUGCUULagGUGCAGGGGGUCGCGGGU
UCGAGUCCCGUCCGUUCCGC

[0412]

SEQ ID NO: 219 (FR-3)

tRNA(AsnE2)5' RNA sequence

10 GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUU

[0413]

SEQ ID NO: 220 (FR-4)

tRNA(AsnE2)3'ag RNA sequence

AGGUUCCGUAUGUCACUGGUUCGAGUCCAGUCAGAGCCGC

15 [0414]

tRNA SEQ ID NO: 216 (TR-109)

tRNA(AsnE2+Leu2)Lag-CA RNA sequence

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUULagGUUCCGUAUGUCACUGGUU
CGAGUCCAGUCAGAGCCGC

20 [0415]

A reaction solution composed of 50 mM HEPES-KOH (pH 7.5), 20 mM MgCl₂, 1 mM ATP, 0.125-0.25 mM pLp, 25 μM tRNA 5' fragment, 0.6 U/μL T4 RNA ligase (New England Biolabs), and 10% DMSO was left to stand overnight at 15°C to perform a ligation reaction between the tRNA 5' fragment and pLp. The ligation product was extracted with phenol-
25 chloroform, and recovered by ethanol precipitation.

[0416]

To prevent the unreacted tRNA 5' fragment from being carried over to the next ligation reaction, sodium periodate (NaIO₄) was used to cleave the ribose at the 3' end of the tRNA 5' fragment. Specifically, 10 μM ligation product was cleaved by allowing it to stand on ice for 30
30 minutes in the dark in the presence of 10 mM sodium periodate. After the reaction, one-tenth volume of 100 mM glucose was added, and the mixture was allowed to stand on ice for 30 minutes in the dark to decompose the excess sodium periodate. The reaction product was collected by ethanol precipitation.

[0417]

35 After the periodic acid treatment, T4 polybase kinase (T4 PNK) treatment was performed to phosphorylate the 5' end and dephosphorylate the 3' end of the ligation product.

The reaction solution composed of 10 μ M ligation product after periodic acid treatment, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 5 mM DTT, 300 μ M ATP, and 0.5 U/ μ L T4 PNK (TaKaRa) was reacted by allowing it to stand at 37°C for 30 to 60 minutes. The reaction product was extracted with phenol-chloroform and collected by ethanol precipitation.

5 [0418]

A ligation reaction was performed between the post-PNK-treatment reaction product and the tRNA 3' fragment. First, a solution composed of 10 μ M PNK-treated reaction product, 10 μ M tRNA 3' fragment, 50 mM HEPES-KOH (pH 7.5), and 15 mM MgCl₂ was heated at 65°C for 7 minutes and then allowed to stand at room temperature for 30 minutes to 1 hour to
10 anneal the PNK-treated reaction product and the tRNA 3' fragment. Next, T4 PNK treatment was performed to phosphorylate the 5' end of the tRNA 3' fragment. T4 PNK treatment was performed by adding DTT (final concentration of 3.5 mM), ATP (final concentration of 300 μ M), and T4 PNK (final concentration of 0.5 U/ μ L) to the annealed solution, and allowing this to stand at 37°C for 30 minutes. Next, T4 RNA ligase (New England Biolabs) was added at a final
15 concentration of 0.9 U/ μ L to this solution, and ligation reaction was performed by allowing this mixture to stand at 37°C for 30 to 40 minutes. The ligation product was extracted with phenol-chloroform and collected by ethanol precipitation.

[0419]

The tRNA-CAs produced by the ligation method were subjected to preparative
20 purification by high-performance reverse-phase chromatography (HPLC) (aqueous solution of 15 mM TEA and 400 mM HFIP/ methanol solution of 15 mM TEA and 400 mM HFIP) and then subjected to denatured urea-10% polyacrylamide electrophoresis, to confirm whether they had the desired length.

[0420]

25 Analyses of tRNA fragments cleaved by RNaseT₁

tRNA-CAs prepared using a ligation reaction were fragmented by RNase, and then analyzed to confirm incorporation of lysidine (L) introduced by pLp at the intended site.

[0421]

A reaction solution containing 10 μ M tRNA-CA, 5 U/ μ L RNaseT₁ (Epicentre or
30 ThermoFisher Scientific), and 10 mM ammonium acetate (pH 5.3) was allowed to stand at 37°C for 1 hour to cleave the RNA specifically at the 3' side of the G base to analyze the RNA fragment containing lysidine (L) introduced by pLp.

[0422]

CUULAGp

35 LCMS(ESI) m/z = 1020 ((M-2H)/2)-

Retention time: 3.84 minutes (analysis condition LTQTEA/HFIP05_03)

Since the molecular weight of the fragment (CUUAGp) expected when pLp is not ligated and the molecular weight of the fragment (UUCAGp) derived from other parts of RNA are the same, unfragmented RNA (TR-108) was also analyzed.

LCMS(ESI) $m/z = 1109 ((M-22H)/22)-$

5 Retention time: 3.92 minutes (analysis condition LTQTEA/HFIP05_01)

Comparison to the mass chromatogram of the RNA expected when pLp is not ligated and to that of the RNA expected when uridine is present instead of lysidine, confirmed that most of the pLp ligation took place.

[0423]

10 AUULAGp

LCMS(ESI) $m/z = 1032 ((M-2H)/2)-$

Retention time: 4.16 minutes (analysis condition LTQTEA/HFIP05_03)

Comparison to the mass chromatogram of the fragment (AUUAGp) expected when pLp is not ligated and to that of the fragment (AUUUAGp) expected when uridine is present instead of lysidine, confirmed that most of the pLp ligation took place.

[0424]

Preparation of elongator aminoacyl tRNA using aminoacyl pCpA

A reaction solution was prepared by adding nuclease-free water to adjust the solution to 25 μ M transcribed tRNA(Glu2+Ser5)aag-CA (TR-1), 50 mM HEPES-KOH pH7.5, 20 mM MgCl₂, 1 mM ATP, 0.6 unit/ μ L T4 RNA ligase (New England Biolabs), and 0.25 mM aminoacyl pCpA (a DMSO solution of SS15), and ligation reaction was performed at 15°C for 45 minutes. Before adding T4 RNA ligase and aminoacyl pCpA, the reaction solution was heated to 95°C for 2 minutes and then allowed to stand at room temperature for 5 minutes to refold the tRNA in advance.

25 [0425]

To the ligation reaction solution, sodium acetate was added to make a concentration of 0.3 M, and phenol-chloroform extraction was performed to prepare the elongator aminoacyl tRNA (AAtR-1). AAtR-1 was recovered by ethanol precipitation, and before adding it to a translation mixture, it was dissolved in a 1 mM aqueous sodium acetate solution.

30 [0426]

Similarly, the transcribed tRNAs (TR-2 to TR-103, TR-106 to TR-109) were subjected to ligation reaction with aminoacyl pCpA (SS15) by the method described above, phenol-chloroform extraction, and ethanol precipitation, to prepare elongator aminoacyl tRNAs (AAtR-2 to AAtR-103, AAtR-132, AAtR-133, AAtR-136, and AAtR-137). These aminoacyl tRNAs were dissolved in 1 mM aqueous sodium acetate solution before addition to a translation mixture.

35

[0427]

Similarly, the transcribed tRNAs (TR-4, TR-14, TR-24, TR-34, TR-44, TR-54, TR-64, TR-74, TR-81, TR-91, and TR-104) were subjected to ligation reaction with aminoacyl pCpA (SS16) by the method described above, phenol-chloroform extraction, and ethanol precipitation, to prepare elongator aminoacyl tRNAs (AAtR-104 to AAtR-114). These aminoacyl tRNAs were dissolved in 1 mM aqueous sodium acetate solution before addition to a translation mixture.

[0428]

Similarly, the transcribed tRNAs (TR-44, TR-47, TR-48, and TR-50) were subjected to ligation reaction with aminoacyl pCpA (SS14) by the method described above, phenol-chloroform extraction, and ethanol precipitation, to prepare elongator aminoacyl tRNAs (AAtR-115 to AAtR-118). These aminoacyl tRNAs were dissolved in 1 mM aqueous sodium acetate solution before addition to a translation mixture.

[0429]

Similarly, the transcribed tRNAs (TR-44, TR-47, TR-48, TR-50, TR-24, and TR-34) were subjected to ligation reaction with aminoacyl pCpA (SS45) by the method described above, phenol-chloroform extraction, and ethanol precipitation, to prepare elongator aminoacyl tRNAs (AAtR-119 to AAtR-122, AAtR-129, and AAtR-130). These aminoacyl tRNAs were dissolved in 1 mM aqueous sodium acetate solution before addition to a translation mixture.

[0430]

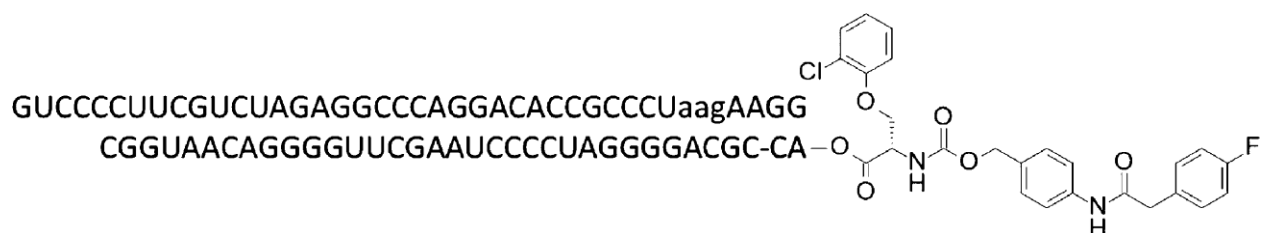
Similarly, the transcribed tRNAs (TR-44, TR-47, TR-48, TR-50, TR-77, and TR-97) were subjected to ligation reaction with aminoacyl pCpA (Compound TS24 synthesized by a method described in Patent Literature (WO2018143145A1)) by the method described above, phenol-chloroform extraction, and ethanol precipitation, to prepare elongator aminoacyl tRNAs (AAtR-123 to AAtR-126, AAtR-134, and AAtR-138). These aminoacyl tRNAs were dissolved in 1 mM aqueous sodium acetate solution before addition to a translation mixture.

[0431]

Similarly, the transcribed tRNAs (TR-54, TR-67, and TR-87) were subjected to ligation reaction with aminoacyl pCpA (Compound ts14 synthesized by a method described in Patent Literature (WO2018143145A1)) by the method described above, phenol-chloroform extraction, and ethanol precipitation, to prepare elongator aminoacyl tRNAs (AAtR-127, AAtR-131, and AAtR-135). These aminoacyl tRNAs were dissolved in 1 mM aqueous sodium acetate solution before addition to a translation mixture.

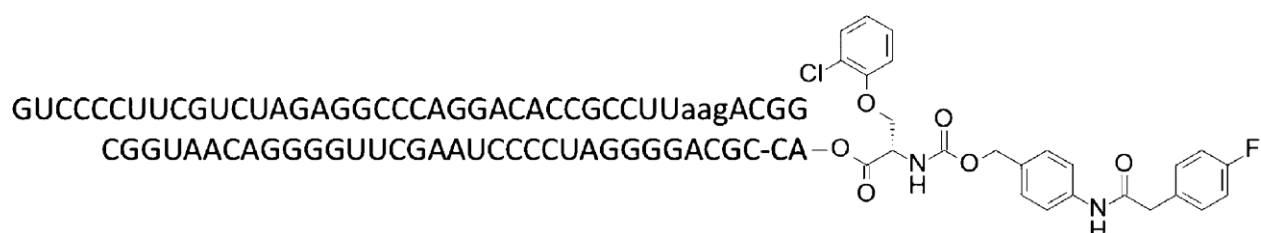
[0432]

AAtR-1 SPh2Cl-tRNA(Glu2+Ser5)aag



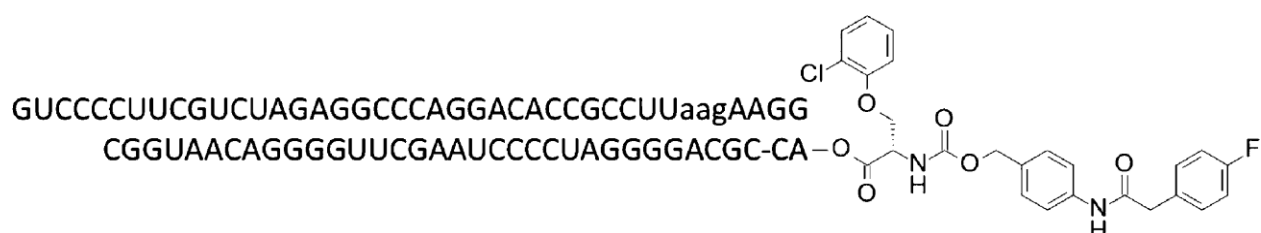
[0433]

AAAtR-2 SPh2Cl-tRNA(Glu2+Ala1B)aag



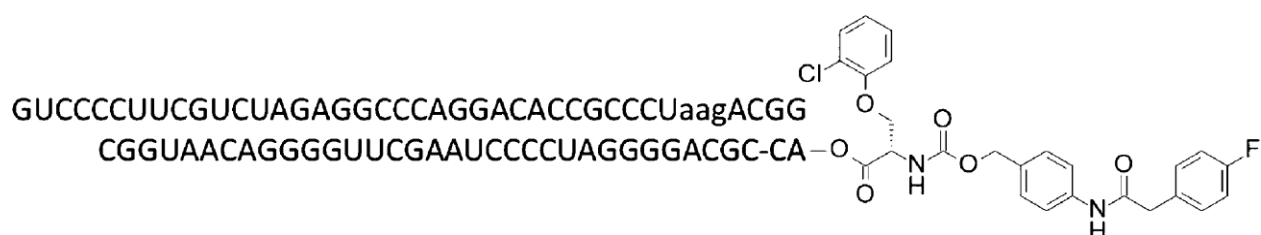
5 [0434]

AAAtR-3 SPh2Cl-tRNA(Glu2+Phe)aag



[0435]

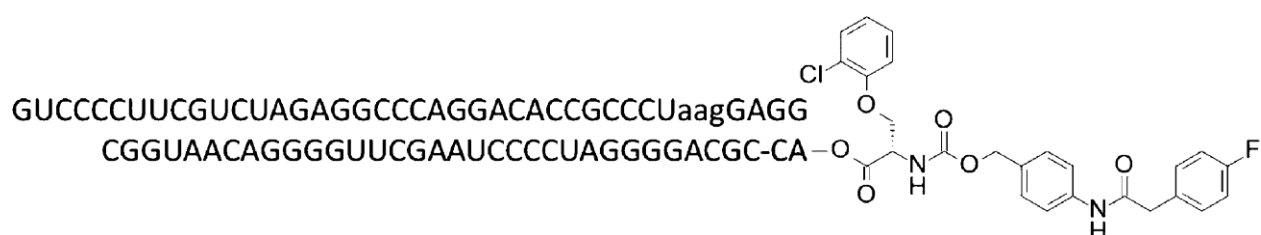
AAAtR-4 SPh2Cl-tRNA(Glu2)aag



10

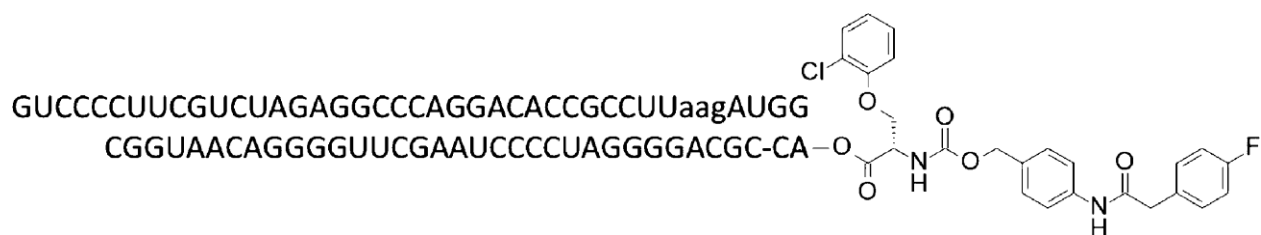
[0436]

AAAtR-5 SPh2Cl-tRNA(Glu2+Arg3)aag



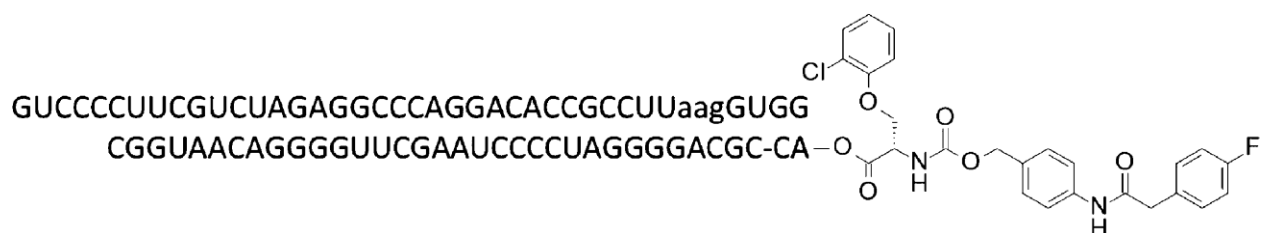
[0437]

15 AAAtR-6 SPh2Cl-tRNA(Glu2+Val2)aag



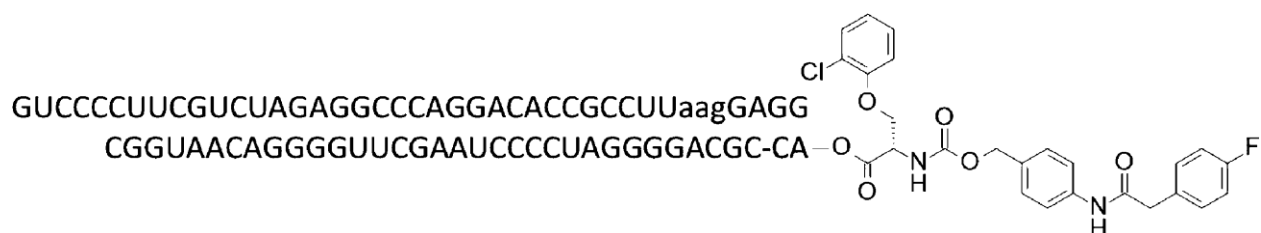
[0438]

AAAtR-7 SPh2Cl-tRNA(Glu2+Leu2)aag



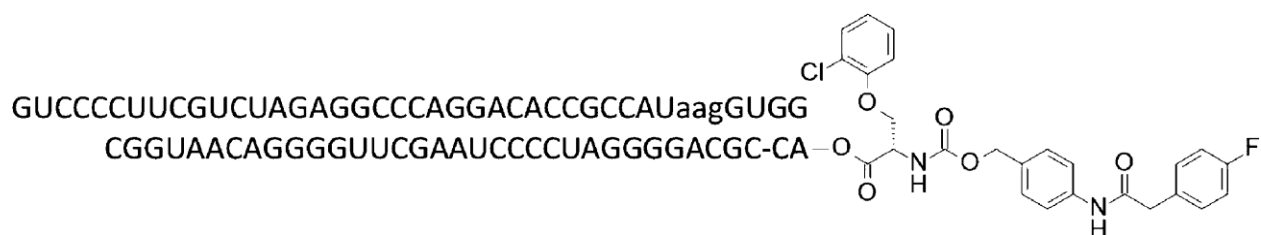
5 [0439]

AAAtR-8 SPh2Cl-tRNA(Glu2+Pro3)aag



[0440]

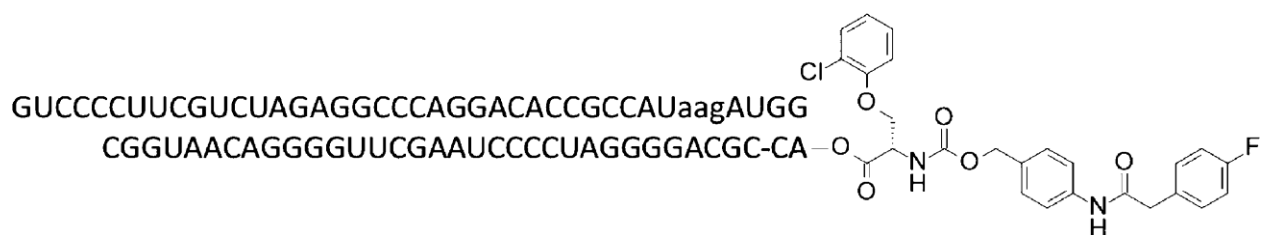
AAAtR-9 SPh2Cl-tRNA(Glu2+Pro2)aag



10

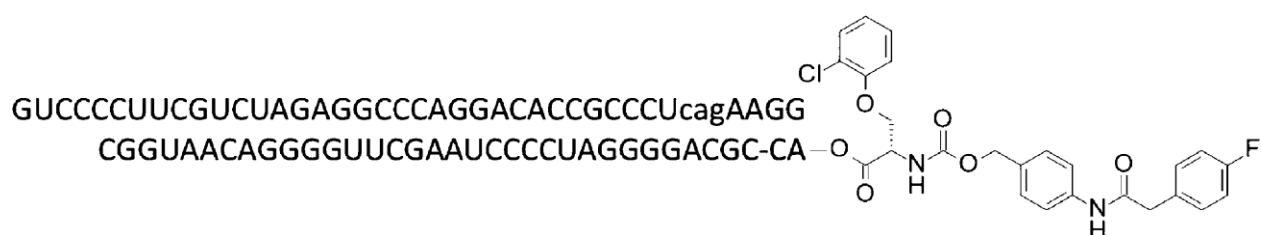
[0441]

AAAtR-10 SPh2Cl-tRNA(Glu2+Ala2)aag



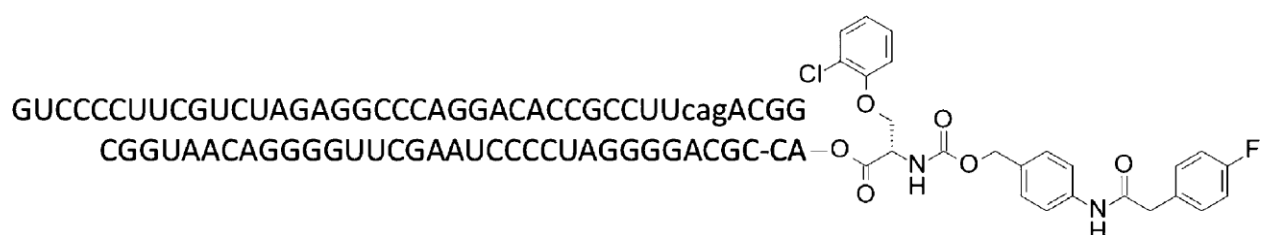
[0442]

15 AAAtR-11 SPh2Cl-tRNA(Glu2+Ser5)cag



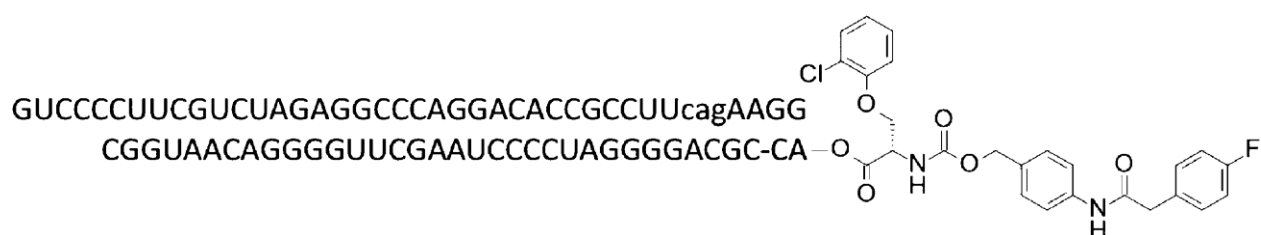
[0443]

AAAtR-12 SPh2Cl-tRNA(Glu2+Ala1B)cag



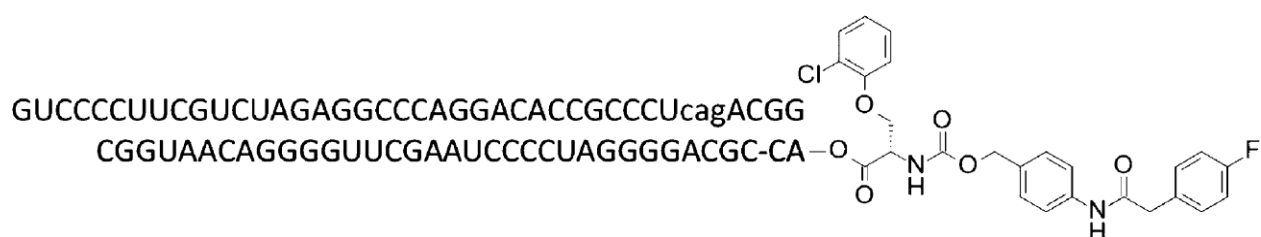
5 [0444]

AAAtR-13 SPh2Cl-tRNA(Glu2+Phe)cag



[0445]

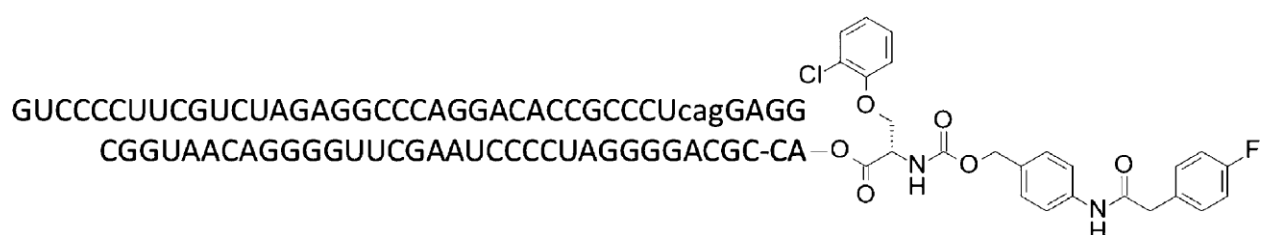
AAAtR-14 SPh2Cl-tRNA(Glu2)cag



10

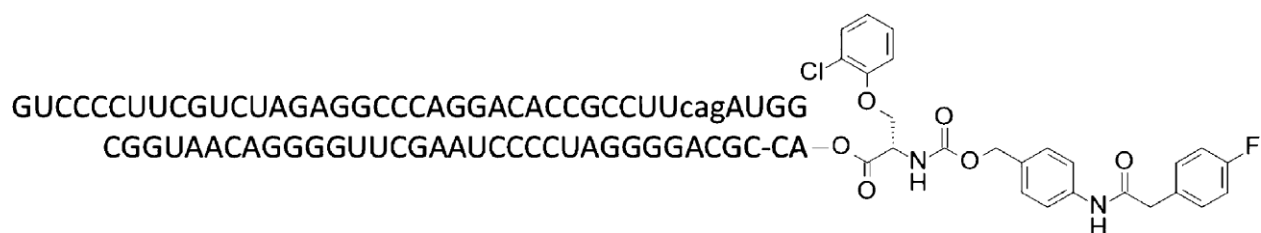
[0446]

AAAtR-15 SPh2Cl-tRNA(Glu2+Arg3)cag



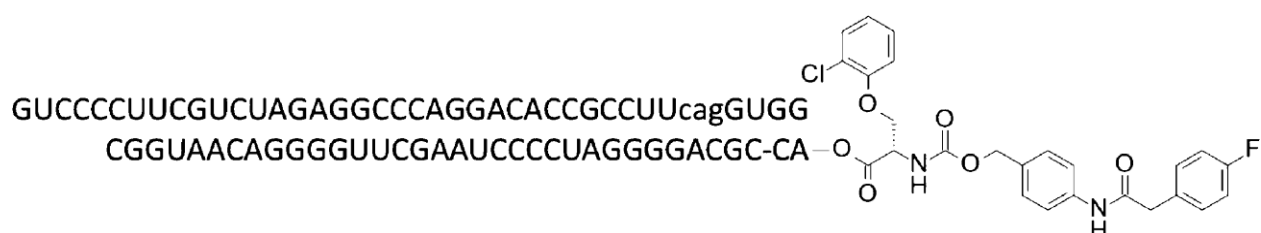
[0447]

15 AAAtR-16 SPh2Cl-tRNA(Glu2+Val2)cag



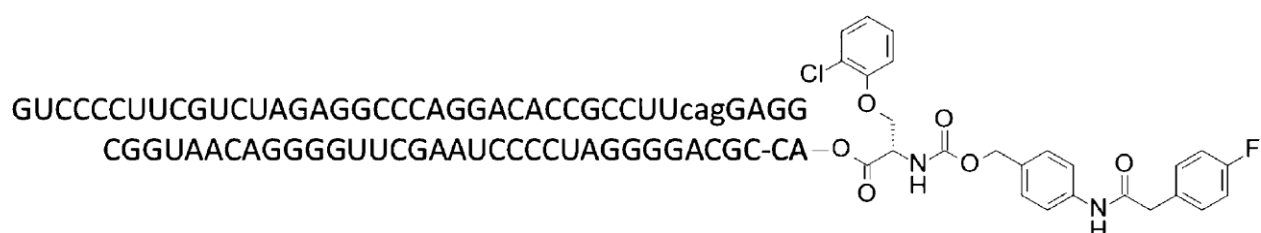
[0448]

AAAtR-17 SPh2Cl-tRNA(Glu2+Leu2)cag



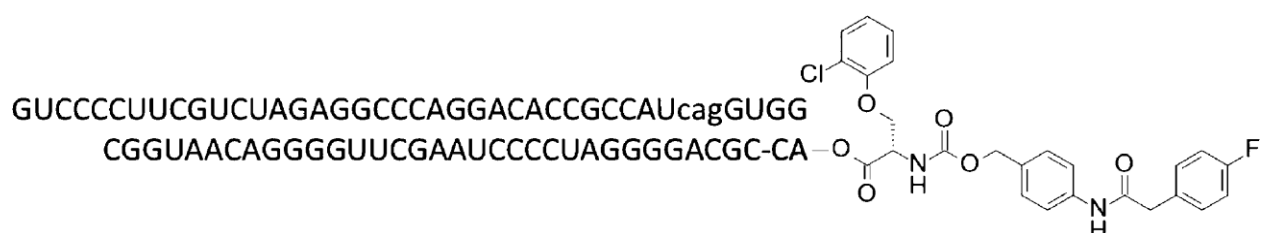
5 [0449]

AAAtR-18 SPh2Cl-tRNA(Glu2+Pro3)cag



[0450]

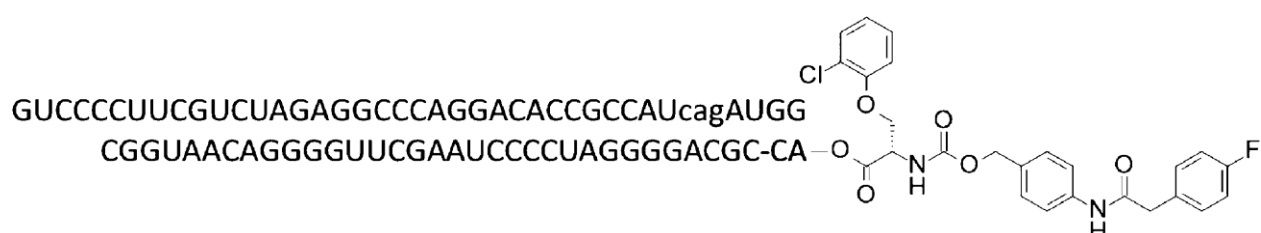
AAAtR-19 SPh2Cl-tRNA(Glu2+Pro2)cag



10

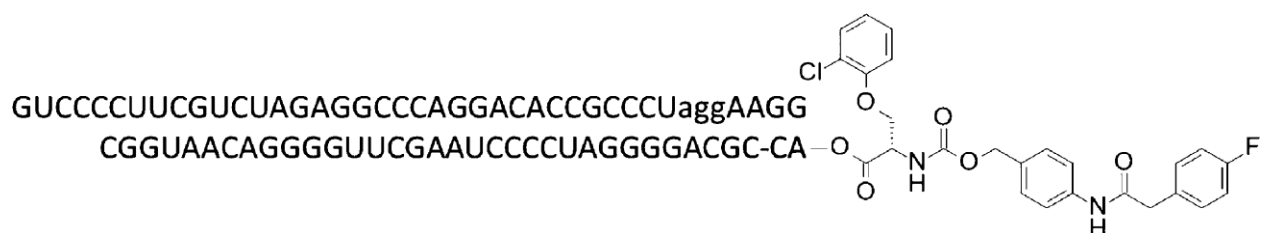
[0451]

AAAtR-20 SPh2Cl-tRNA(Glu2+Ala2)cag



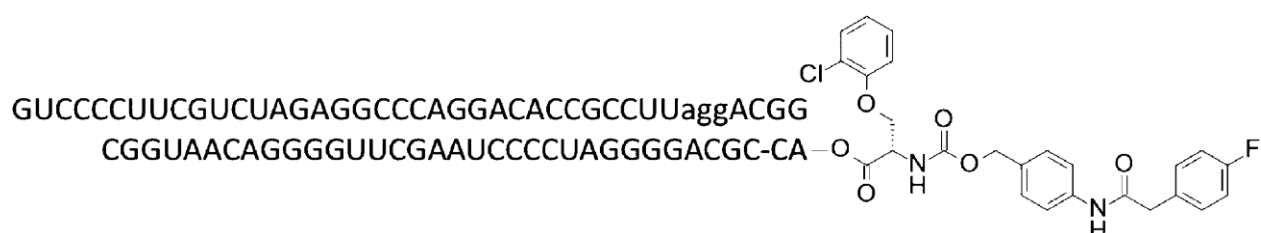
[0452]

15 AAAtR-21 SPh2Cl-tRNA(Glu2+Ser5)agg



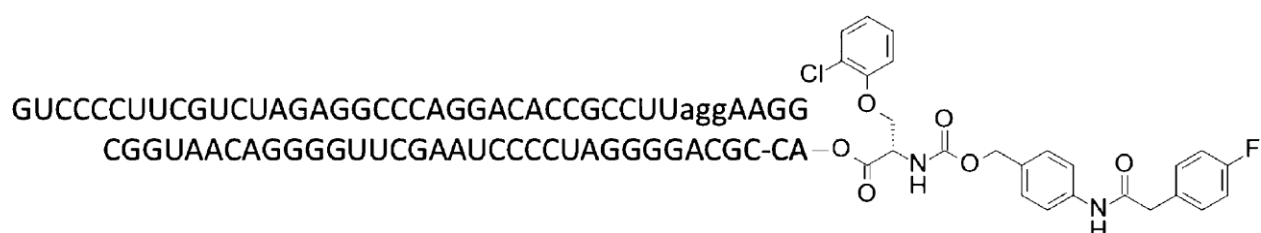
[0453]

AAAtR-22 SPh2Cl-tRNA(Glu2+Ala1B)agg



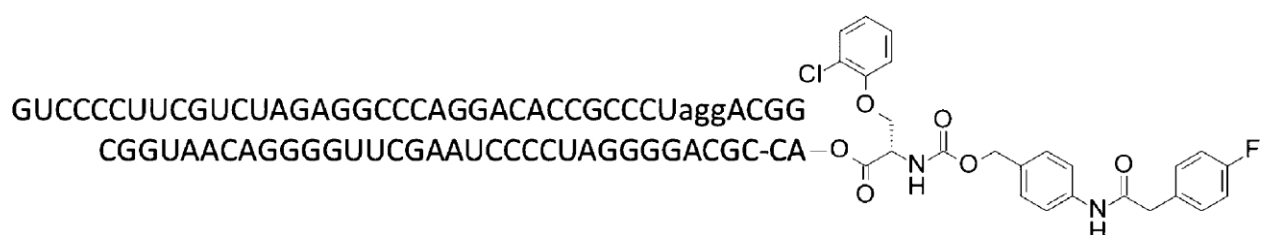
5 [0454]

AAAtR-23 SPh2Cl-tRNA(Glu2+Phe)agg



[0455]

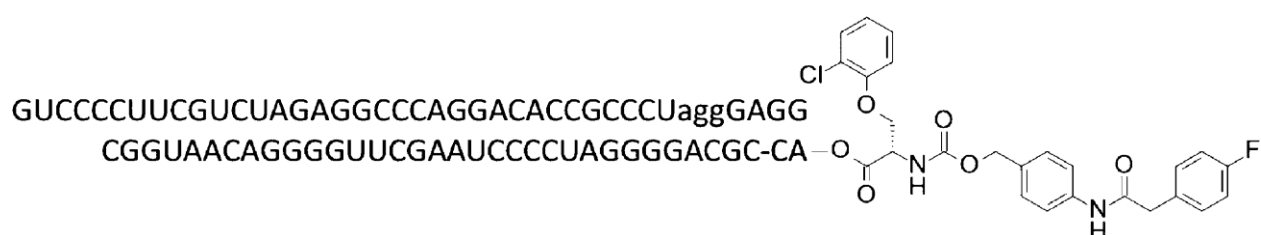
AAAtR-24 SPh2Cl-tRNA(Glu2)agg



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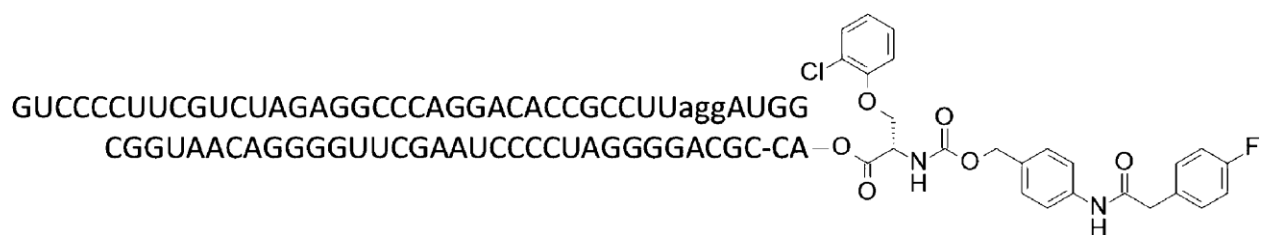
[0456]

AAAtR-25 SPh2Cl-tRNA(Glu2+Arg3)agg



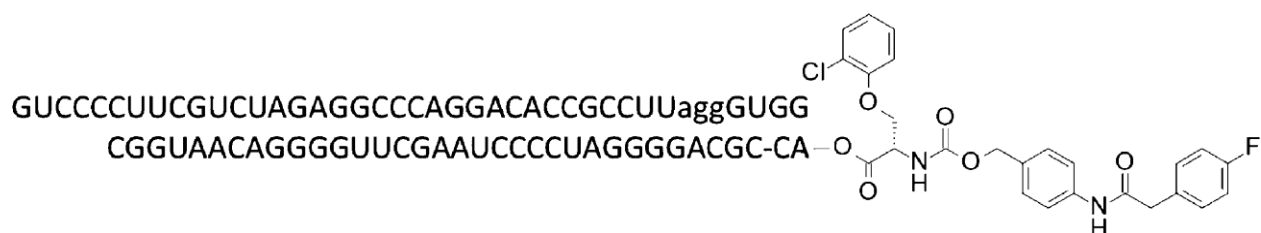
[0457]

15 AAAtR-26 SPh2Cl-tRNA(Glu2+Val2)agg



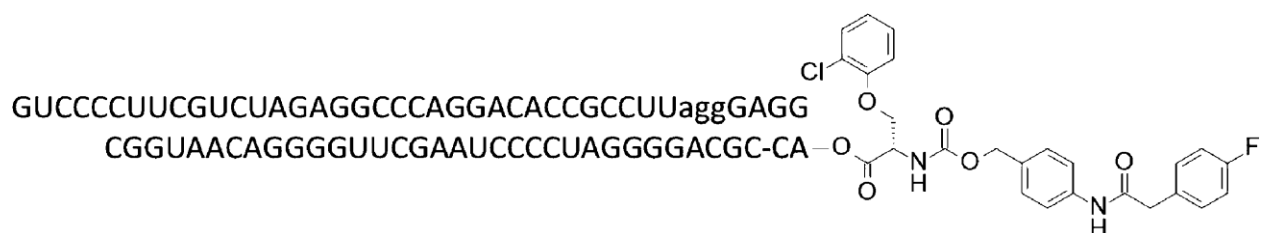
[0458]

AAAtR-27 SPh2Cl-tRNA(Glu2+Leu2)agg



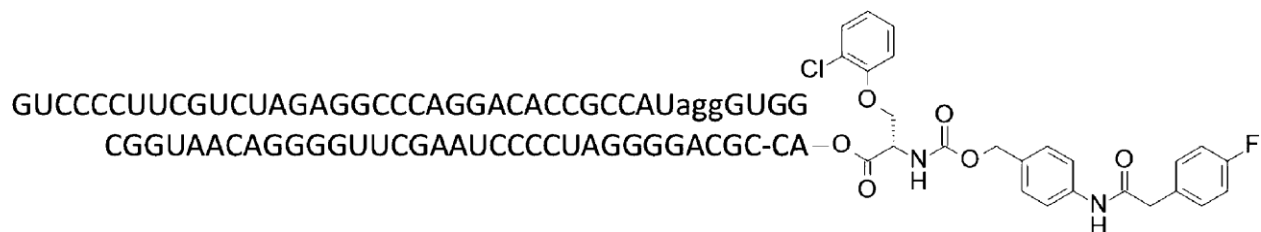
5 [0459]

AAAtR-28 SPh2Cl-tRNA(Glu2+Pro3)agg



[0460]

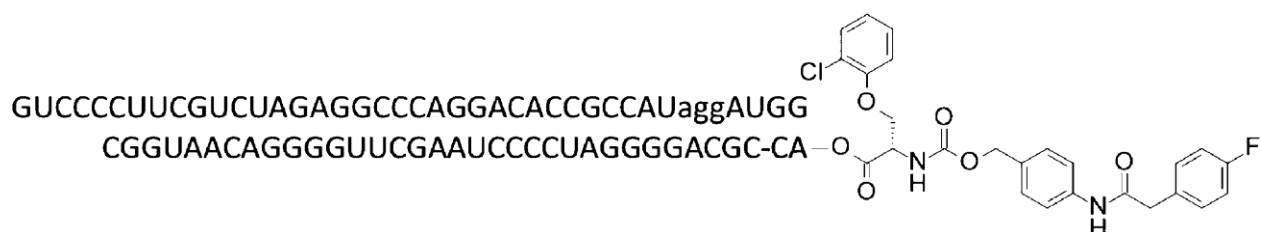
AAAtR-29 SPh2Cl-tRNA(Glu2+Pro2)agg



10

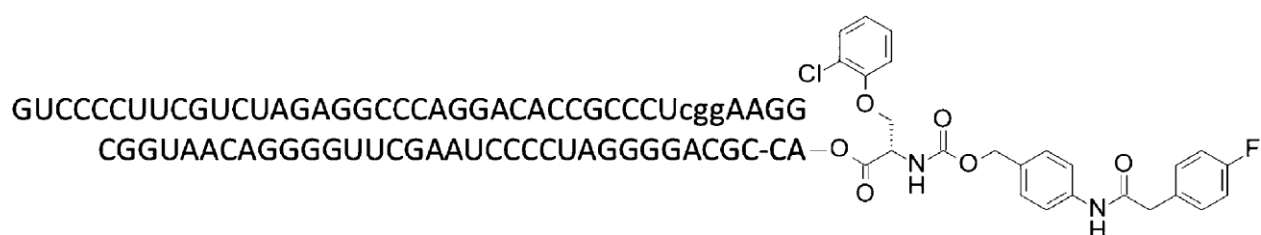
[0461]

AAAtR-30 SPh2Cl-tRNA(Glu2+Ala2)agg



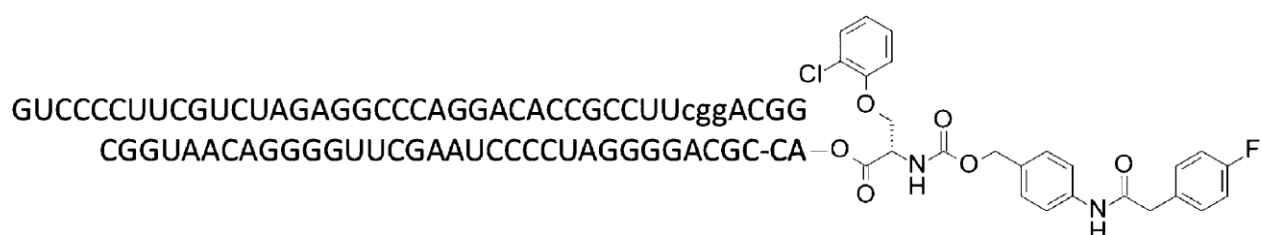
[0462]

15 AAAtR-31 SPh2Cl-tRNA(Glu2+Ser5)cgg



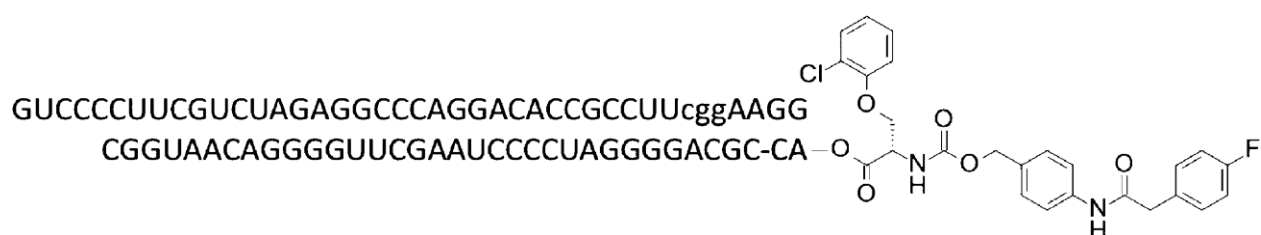
[0463]

AAtR-32 SPh2Cl-tRNA(Glu2+Ala1B)cgg



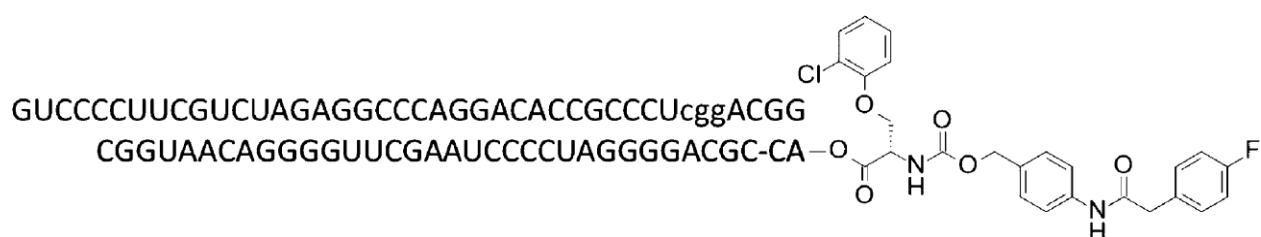
5 [0464]

AAtR-33 SPh2Cl-tRNA(Glu2+Phe)cgg



[0465]

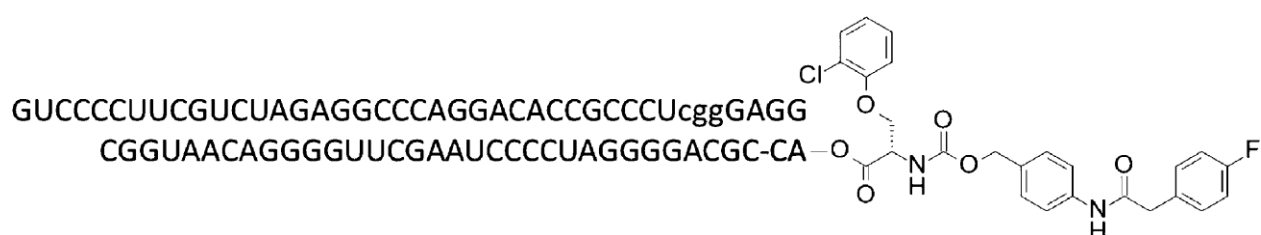
AAtR-34 SPh2Cl-tRNA(Glu2)cgg



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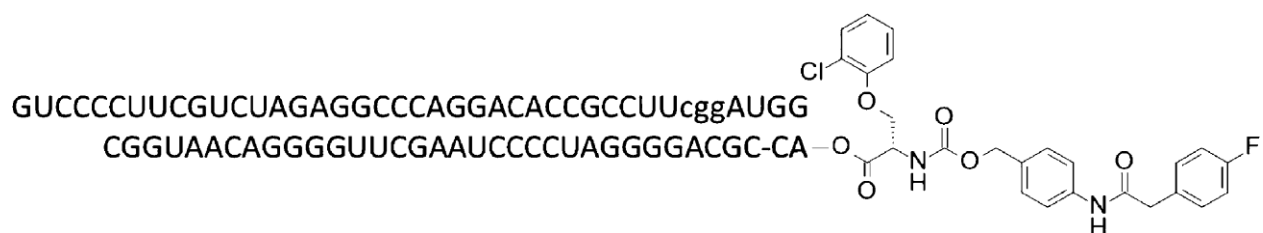
[0466]

AAtR-35 SPh2Cl-tRNA(Glu2+Arg3)cgg



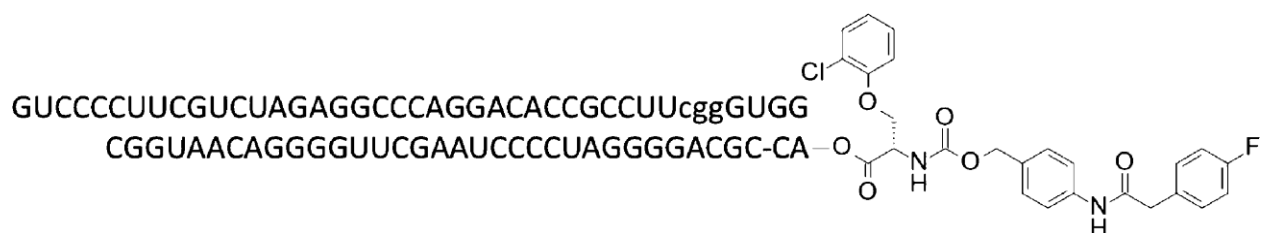
[0467]

15 AAtR-36 SPh2Cl-tRNA(Glu2+Val2)cgg



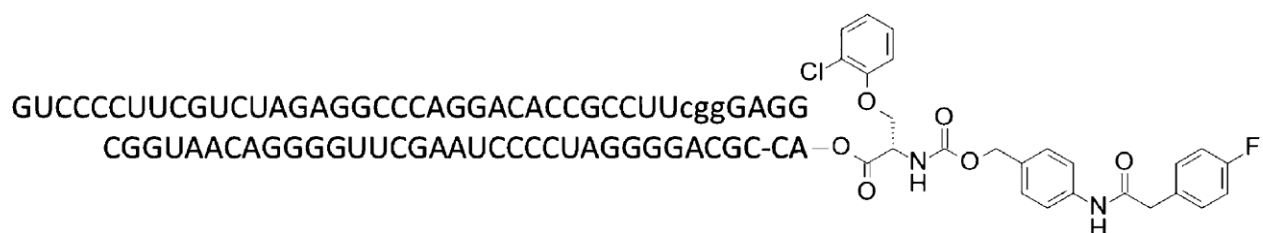
[0468]

AAAtR-37 SPh2Cl-tRNA(Glu2+Leu2)cgg



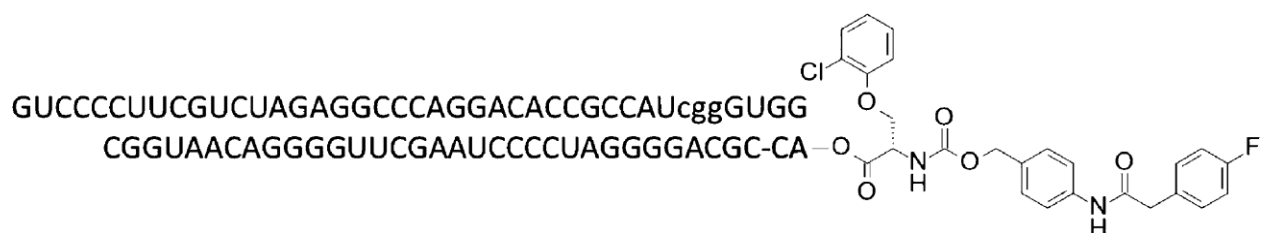
5 [0469]

AAAtR-38 SPh2Cl-tRNA(Glu2+Pro3)cgg



[0470]

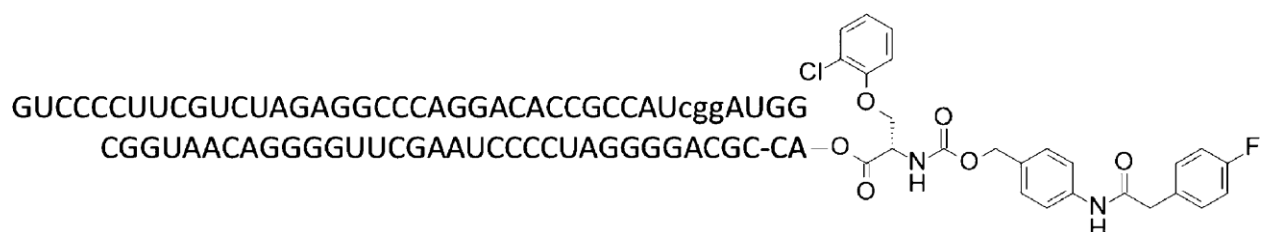
AAAtR-39 SPh2Cl-tRNA(Glu2+Pro2)cgg



10

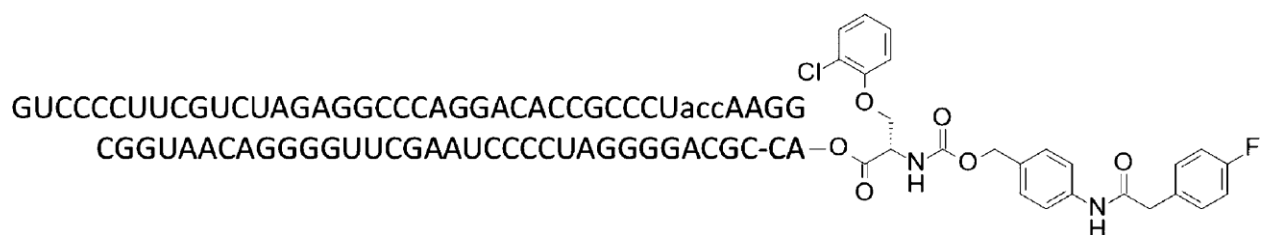
[0471]

AAAtR-40 SPh2Cl-tRNA(Glu2+Ala2)cgg



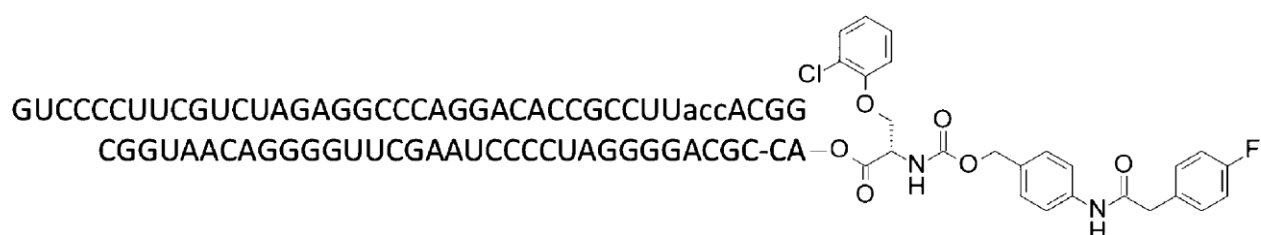
[0472]

15 AAAtR-41 SPh2Cl-tRNA(Glu2+Ser5)acc



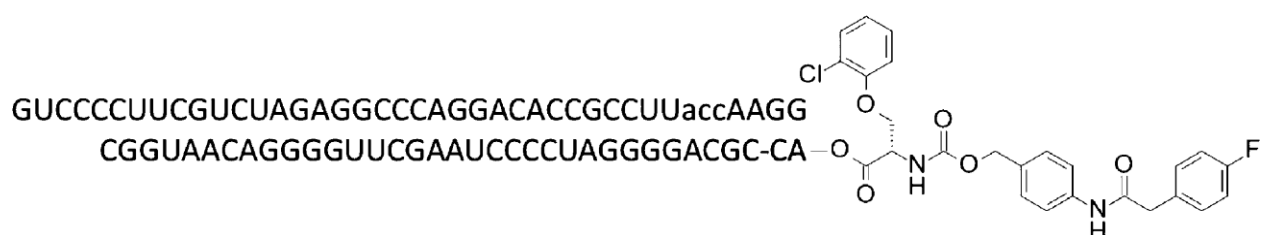
[0473]

AAtR-42 SPh2Cl-tRNA(Glu2+Ala1B)acc



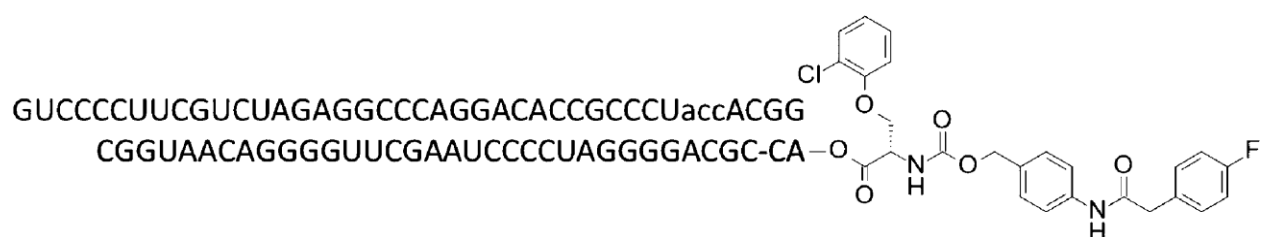
5 [0474]

AAtR-43 SPh2Cl-tRNA(Glu2+Phe)acc



[0475]

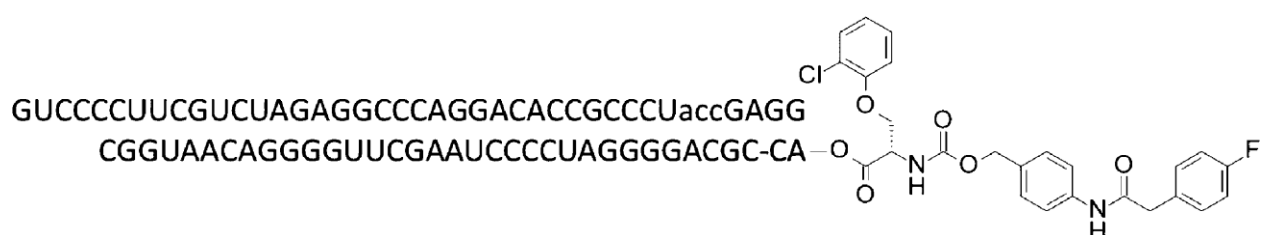
AAtR-44 SPh2Cl-tRNA(Glu2)acc



10

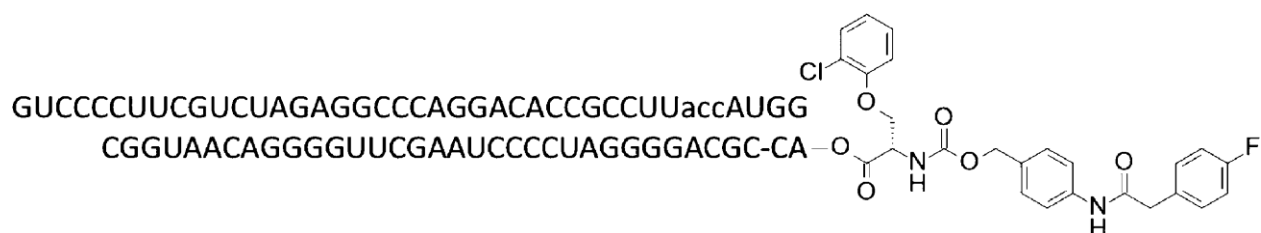
[0476]

AAtR-45 SPh2Cl-tRNA(Glu2+Arg3)acc



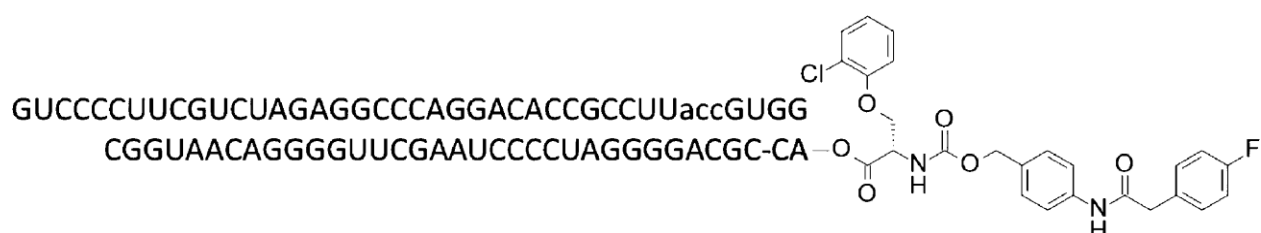
[0477]

15 AAtR-46 SPh2Cl-tRNA(Glu2+Val2)acc



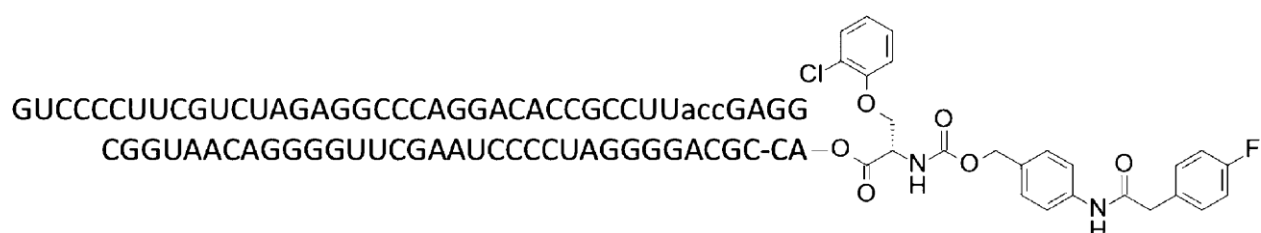
[0478]

AAtR-47 SPh2Cl-tRNA(Glu2+Leu2)acc



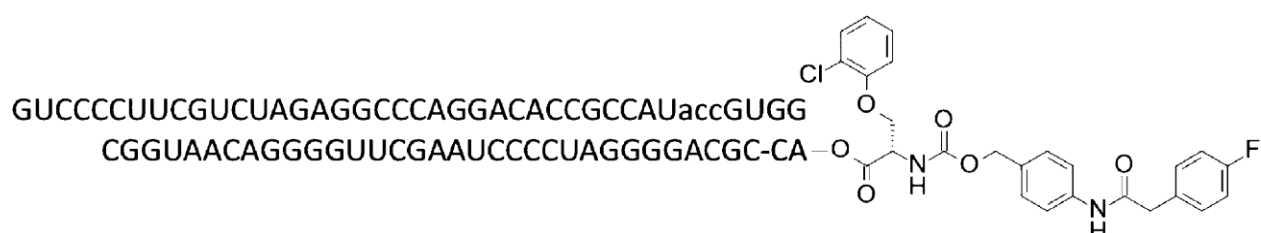
5 [0479]

AAtR-48 SPh2Cl-tRNA(Glu2+Pro3)acc



[0480]

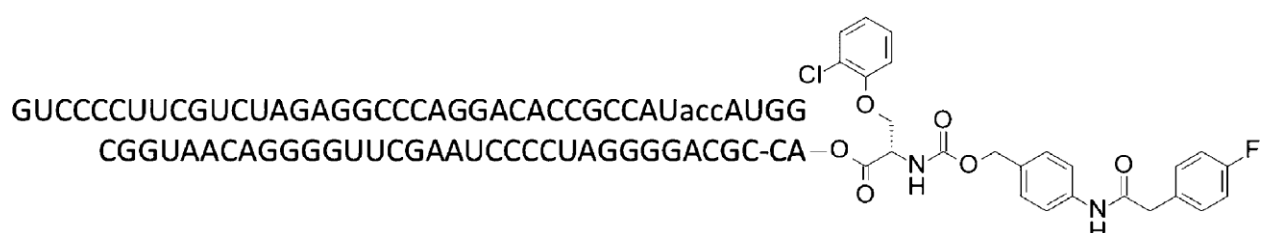
AAtR-49 SPh2Cl-tRNA(Glu2+Pro2)acc



10

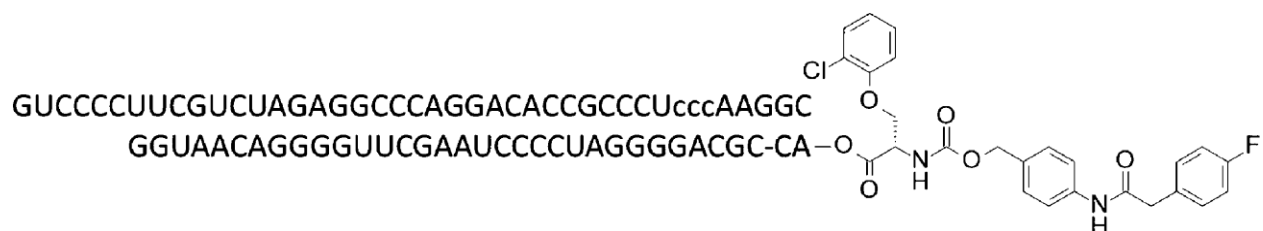
[0481]

AAtR-50 SPh2Cl-tRNA(Glu2+Ala2)acc



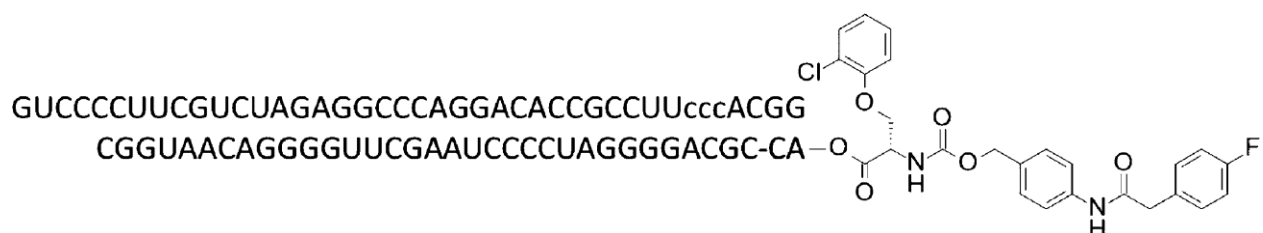
[0482]

15 AAtR-51 SPh2Cl-tRNA(Glu2+Ser5)ccc



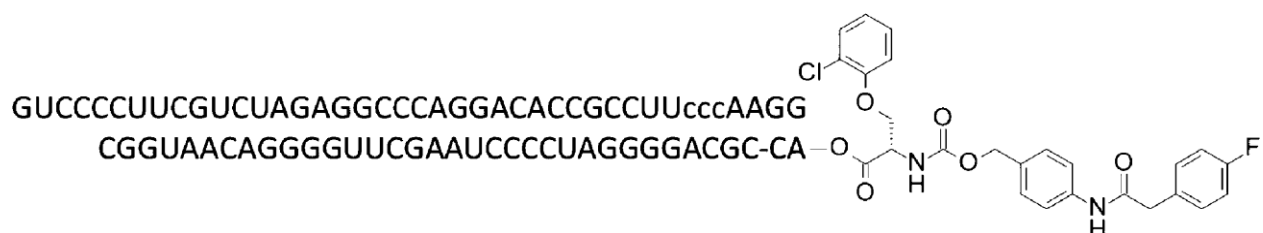
[0483]

AA tR-52 SPh2Cl-tRNA(Glu2+Ala1B)ccc



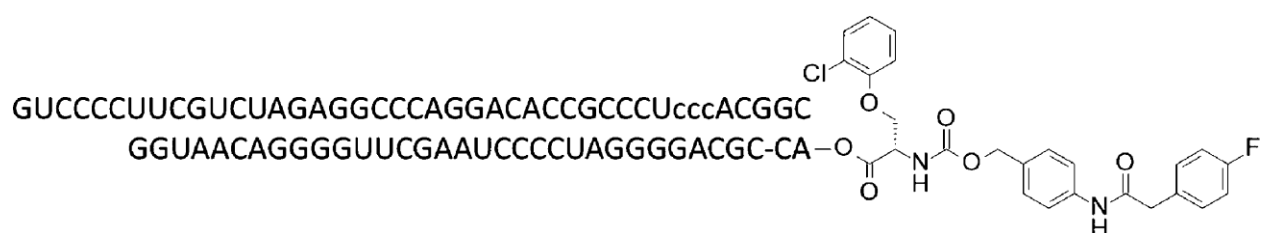
5 [0484]

AA tR-53 SPh2Cl-tRNA(Glu2+Phe)ccc



[0485]

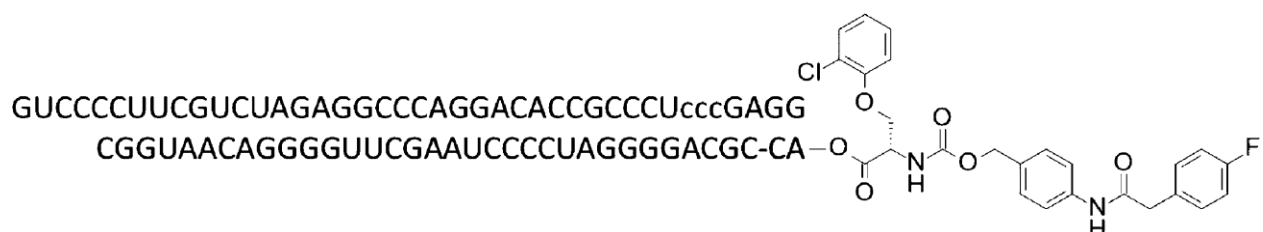
AA tR-54 SPh2Cl-tRNA(Glu2)ccc



10

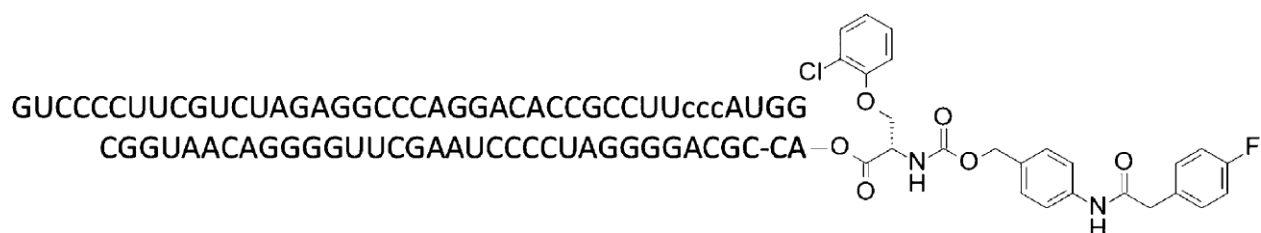
[0486]

AA tR-55 SPh2Cl-tRNA(Glu2+Arg3)ccc



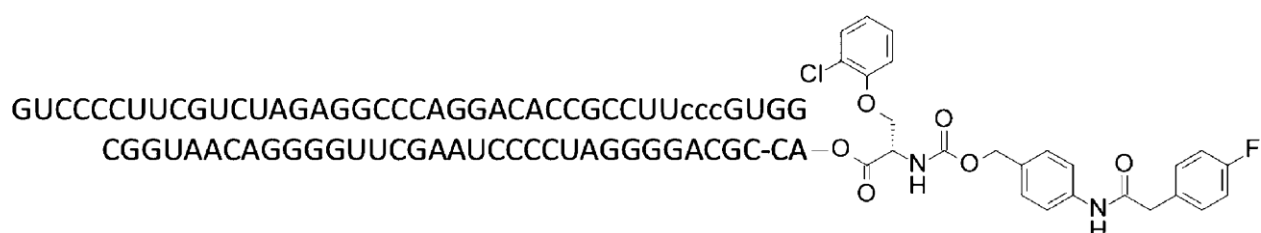
[0487]

15 AA tR-56 SPh2Cl-tRNA(Glu2+Val2)ccc



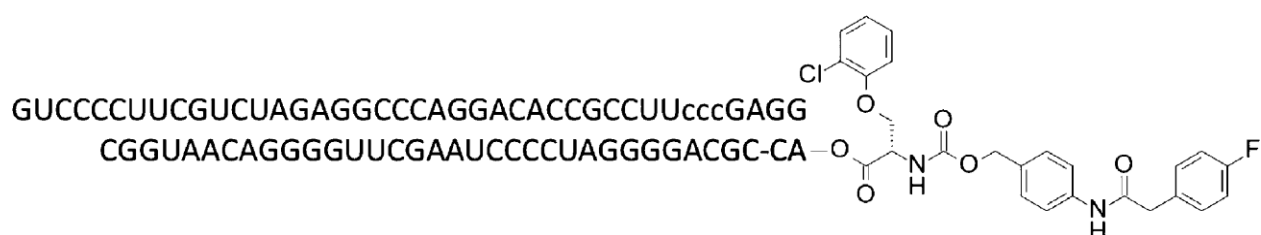
[0488]

AAAtR-57 SPh2Cl-tRNA(Glu2+Leu2)ccc



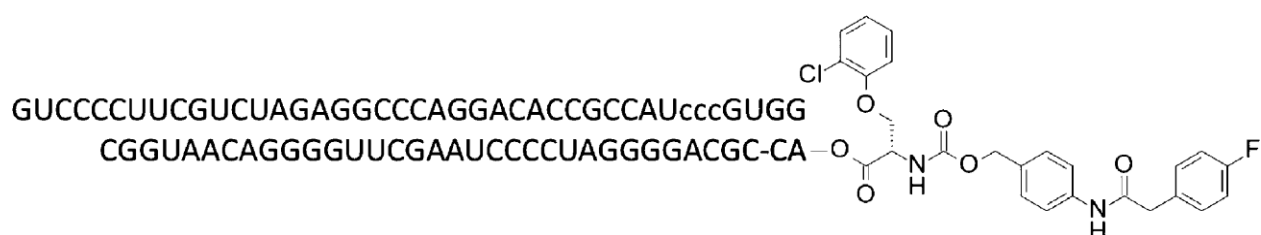
5 [0489]

AAAtR-58 SPh2Cl-tRNA(Glu2+Pro3)ccc



[0490]

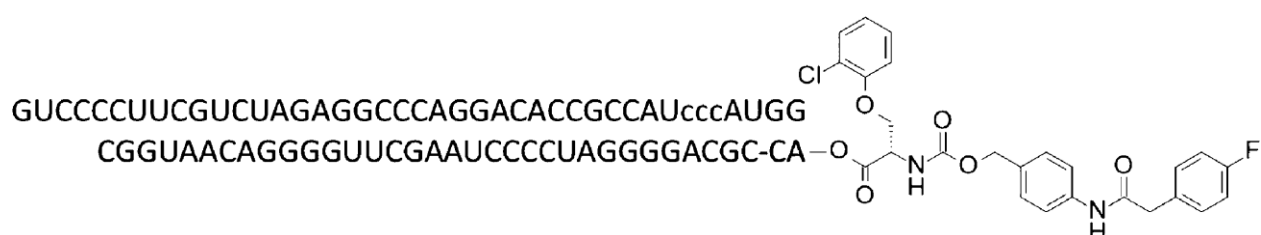
AAAtR-59 SPh2Cl-tRNA(Glu2+Pro2)ccc



10

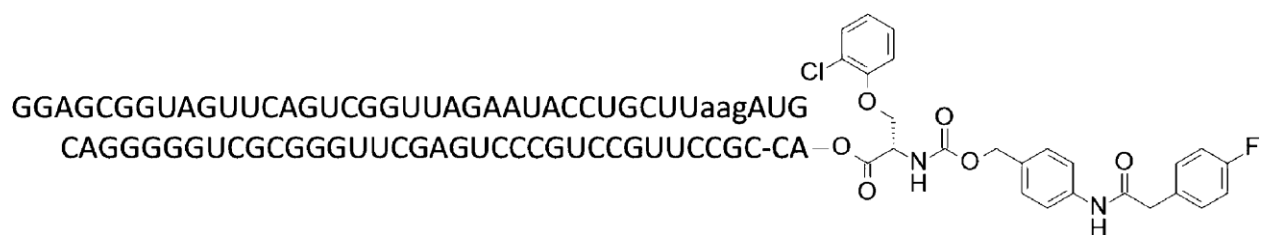
[0491]

AAAtR-60 SPh2Cl-tRNA(Glu2+Ala2)ccc



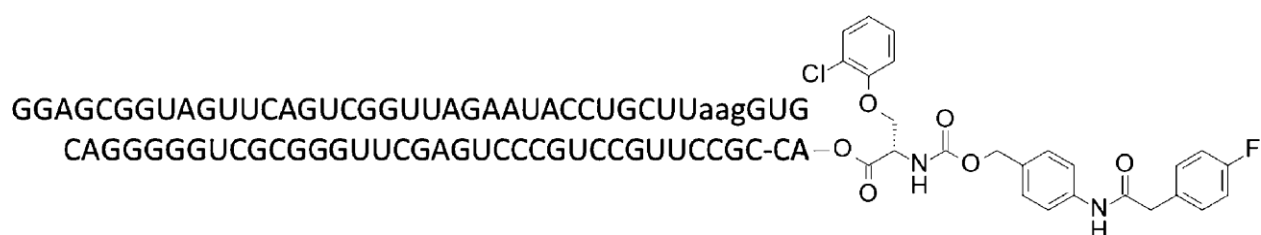
[0492]

15 AAAtR-61 SPh2Cl-tRNA(Asp1+Ser5)aag



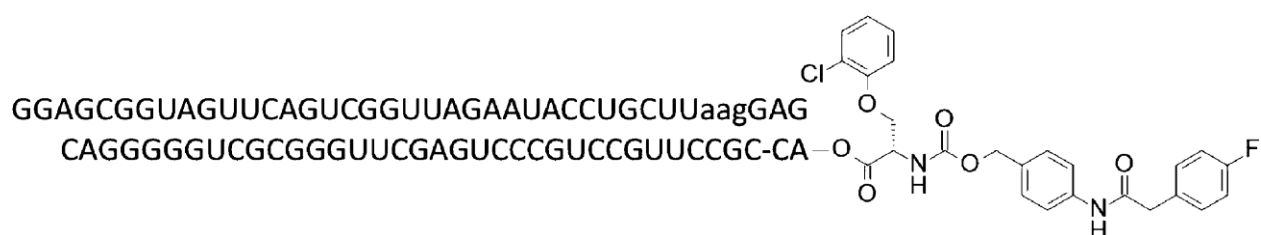
[0498]

AAAtR-67 SPh₂Cl-tRNA(Asp1+Leu2)aag



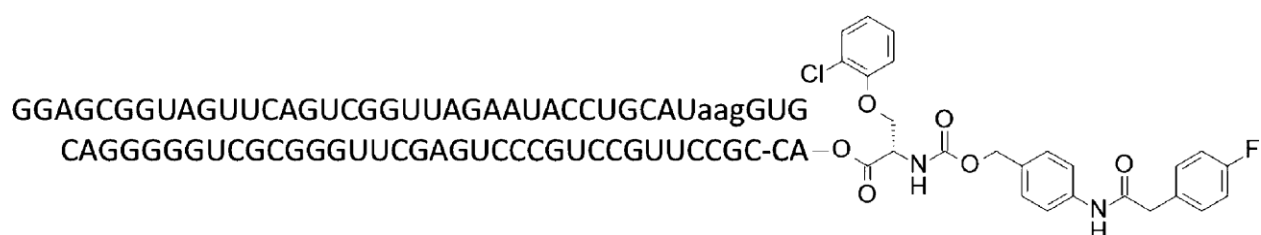
5 [0499]

AAAtR-68 SPh₂Cl-tRNA(Asp1+Pro3)aag



[0500]

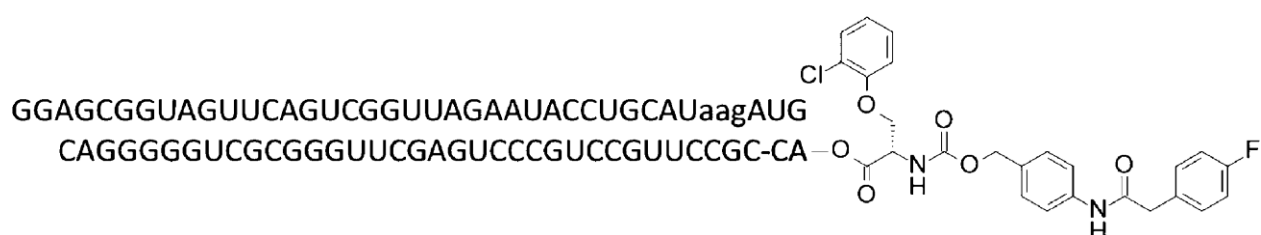
AAAtR-69 SPh₂Cl-tRNA(Asp1+Pro2)aag



10

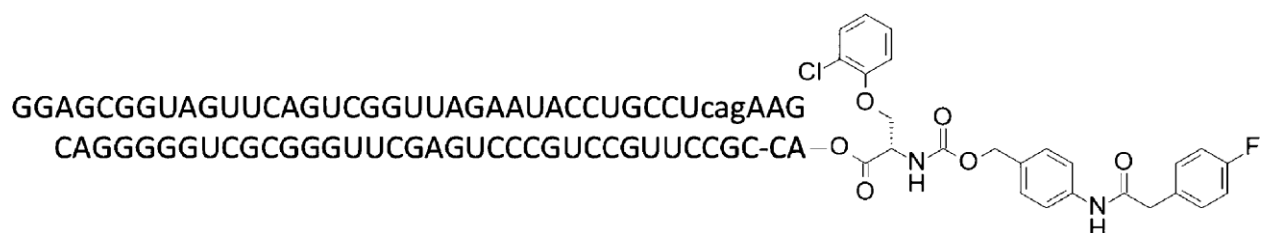
[0501]

AAAtR-70 SPh₂Cl-tRNA(Asp1+Ala2)aag



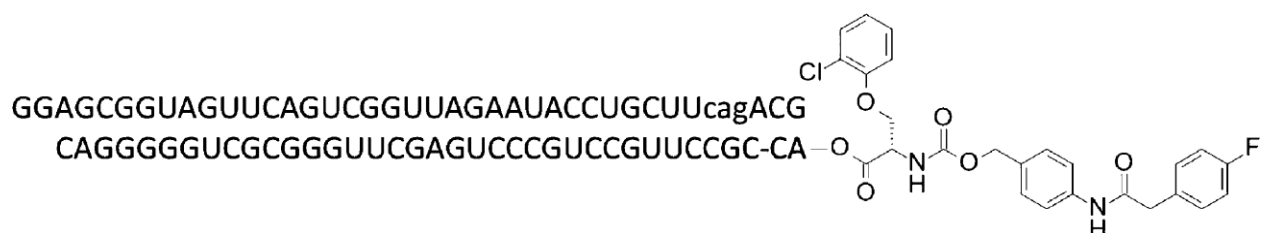
[0502]

15 AAAtR-71 SPh₂Cl-tRNA(Asp1+Ser5)cag



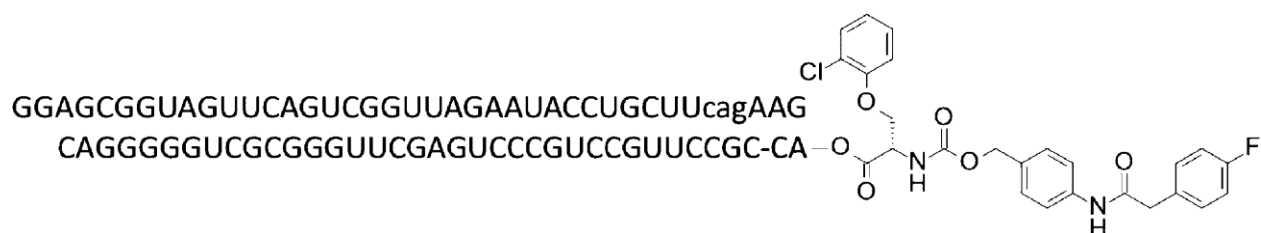
[0503]

AAAtR-72 SPh2Cl-tRNA(Asp1+Ala1B)cag



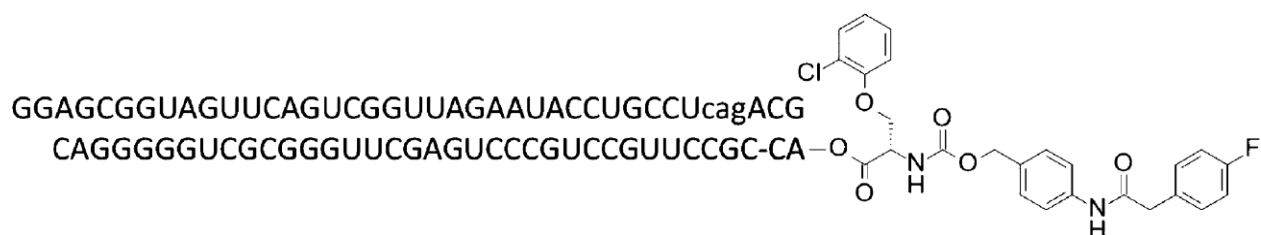
5 [0504]

AAAtR-73 SPh2Cl-tRNA(Asp1+Phe)cag



[0505]

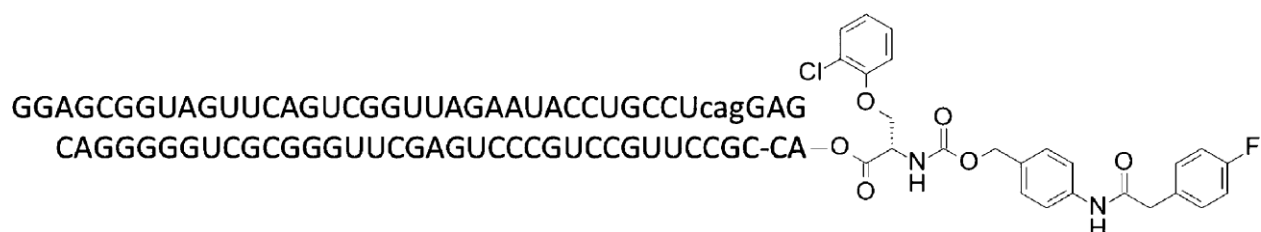
AAAtR-74 SPh2Cl-tRNA(Asp1)cag



10

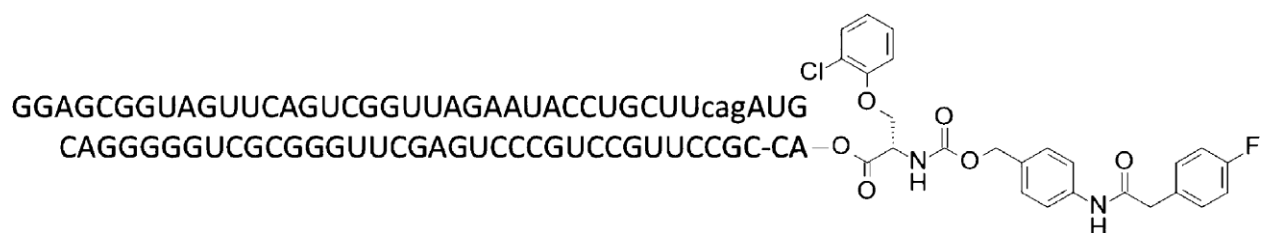
[0506]

AAAtR-75 SPh2Cl-tRNA(Asp1+Arg3)cag



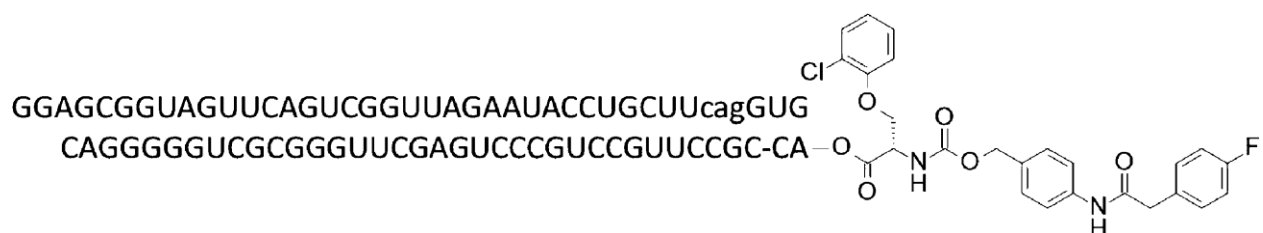
[0507]

15 AAAtR-76 SPh2Cl-tRNA(Asp1+Val2)cag



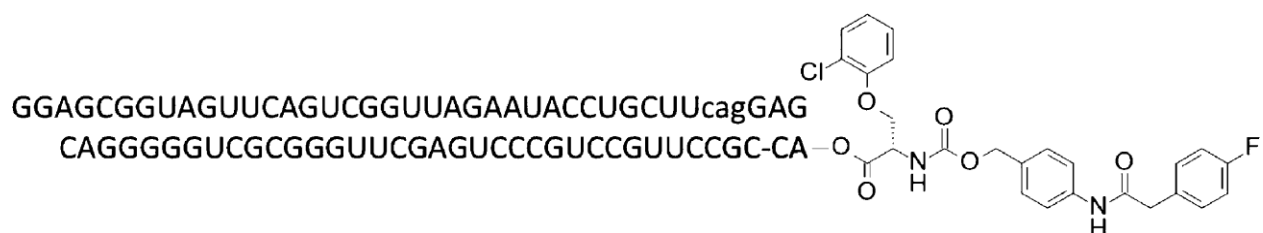
[0508]

AAtR-77 SPh2Cl-tRNA(Asp1+Leu2)cag



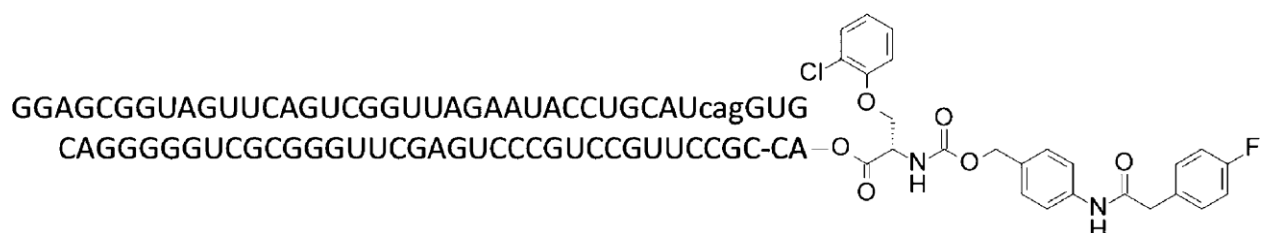
5 [0509]

AAtR-78 SPh2Cl-tRNA(Asp1+Pro3)cag



[0510]

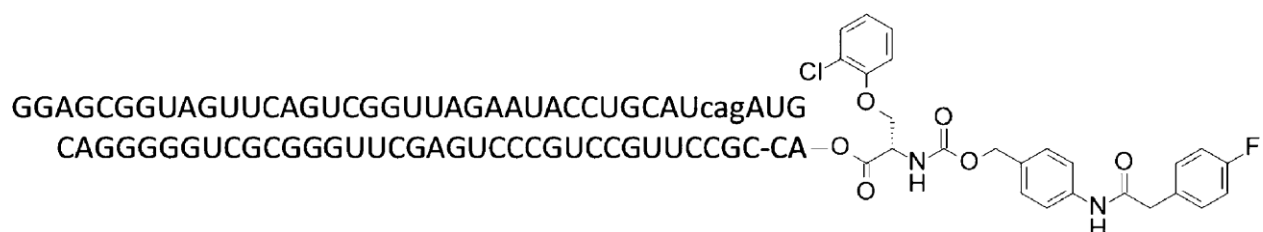
AAtR-79 SPh2Cl-tRNA(Asp1+Pro2)cag



10

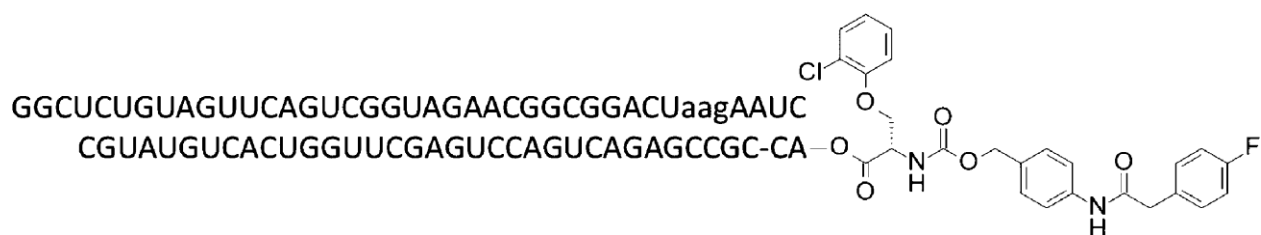
[0511]

AAtR-80 SPh2Cl-tRNA(Asp1+Ala2)cag



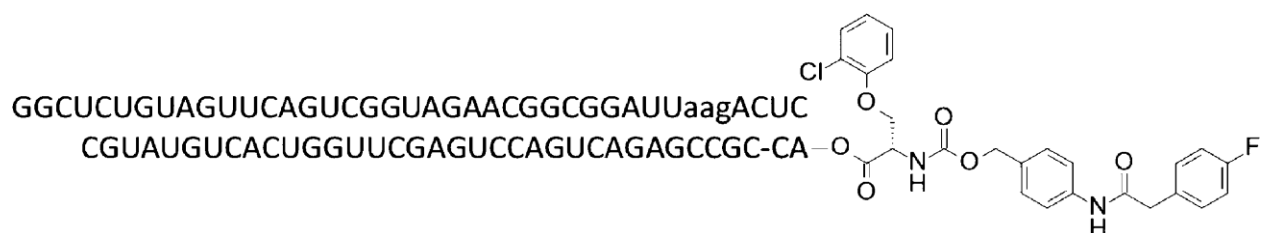
[0512]

15 AAtR-81 SPh2Cl-tRNA(AsnE2)aag



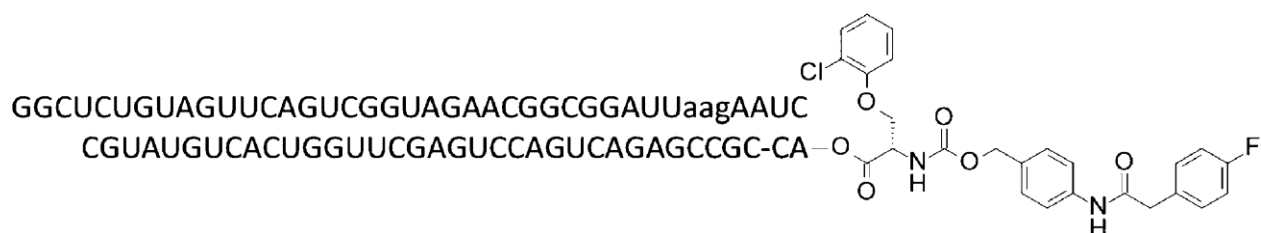
[0513]

AAAtR-82 SPh2Cl-tRNA(AsnE2+Ala1B)aag



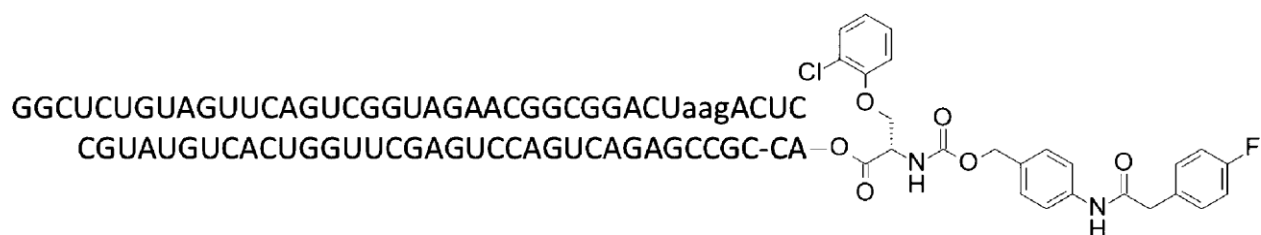
5 [0514]

AAAtR-83 SPh2Cl-tRNA(AsnE2+Phe)aag



[0515]

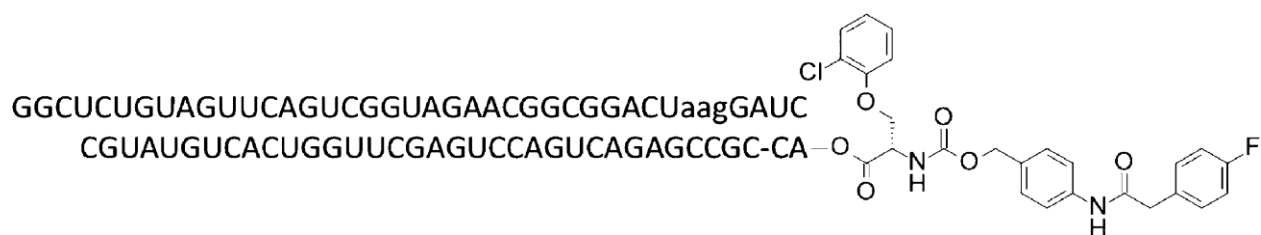
AAAtR-84 SPh2Cl-tRNA(AsnE2+Glu2)aag



10

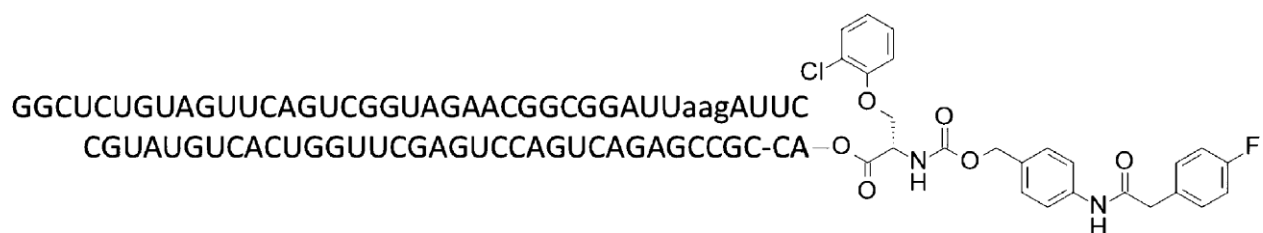
[0516]

AAAtR-85 SPh2Cl-tRNA(AsnE2+Arg3)aag



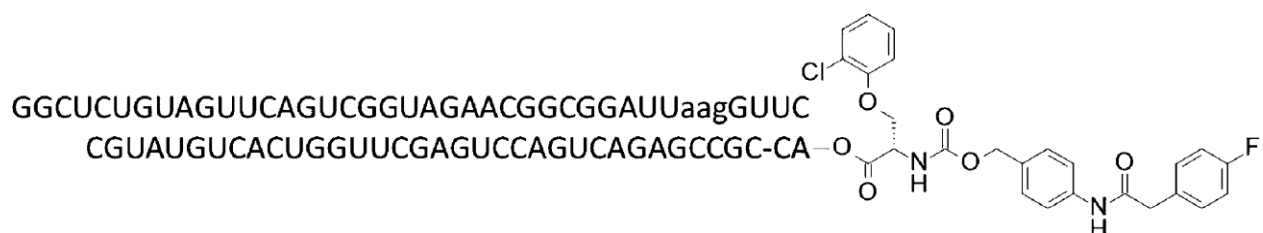
[0517]

15 AAAtR-86 SPh2Cl-tRNA(AsnE2+Val2)aag



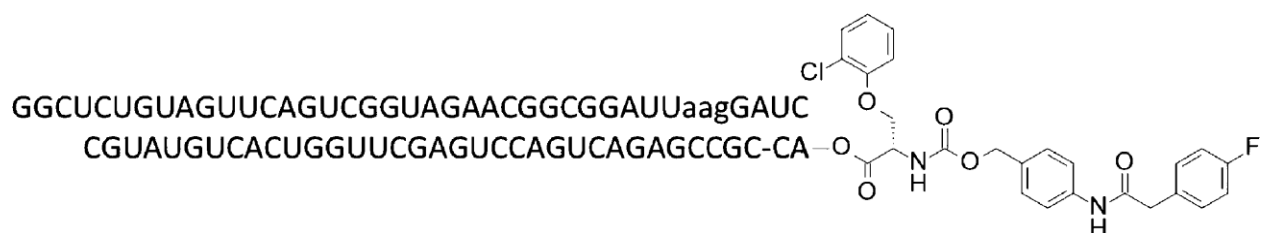
[0518]

AAAtR-87 SPh2Cl-tRNA(AsnE2+Leu2)aag



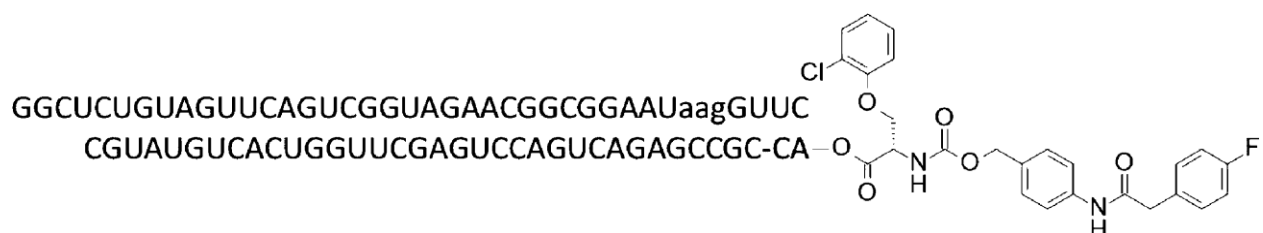
5 [0519]

AAAtR-88 SPh2Cl-tRNA(AsnE2+Pro3)aag



[0520]

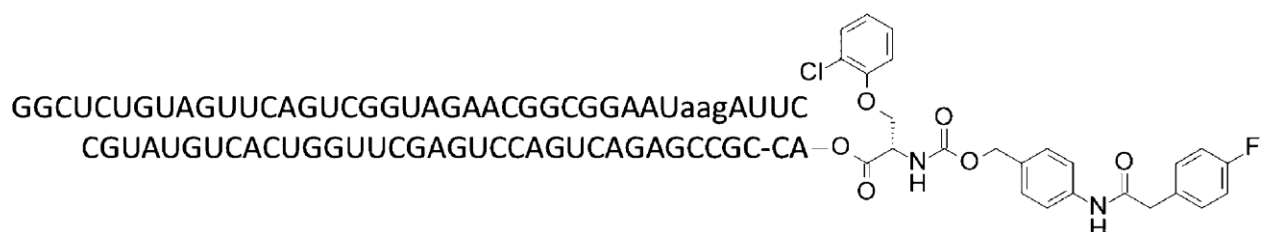
AAAtR-89 SPh2Cl-tRNA(AsnE2+Pro2)aag



10

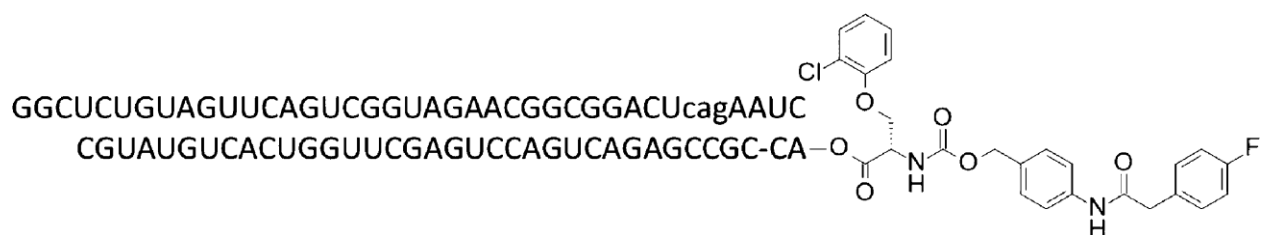
[0521]

AAAtR-90 SPh2Cl-tRNA(AsnE2+Ala2)aag



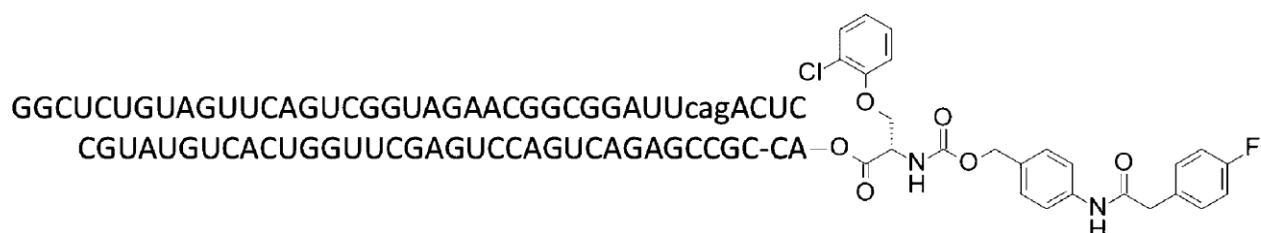
[0522]

15 AAAtR-91 SPh2Cl-tRNA(AsnE2)cag



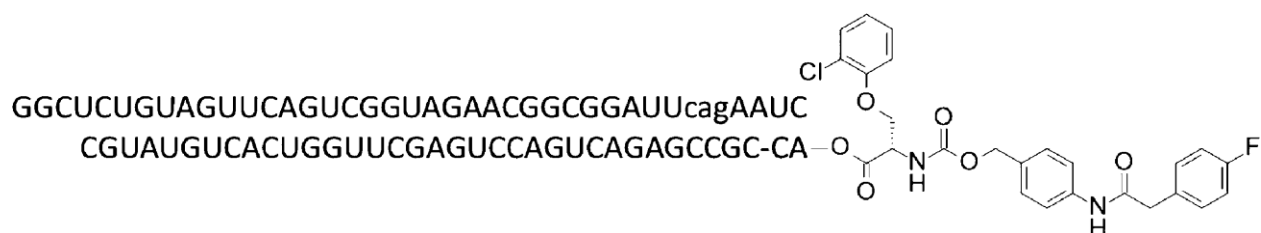
[0523]

AAAtR-92 SPh2Cl-tRNA(AsnE2+Ala1B)cag



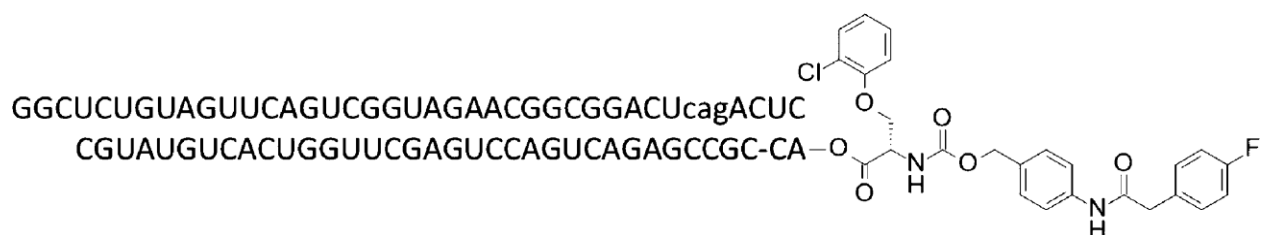
5 [0524]

AAAtR-93 SPh2Cl-tRNA(AsnE2+Phe)cag



[0525]

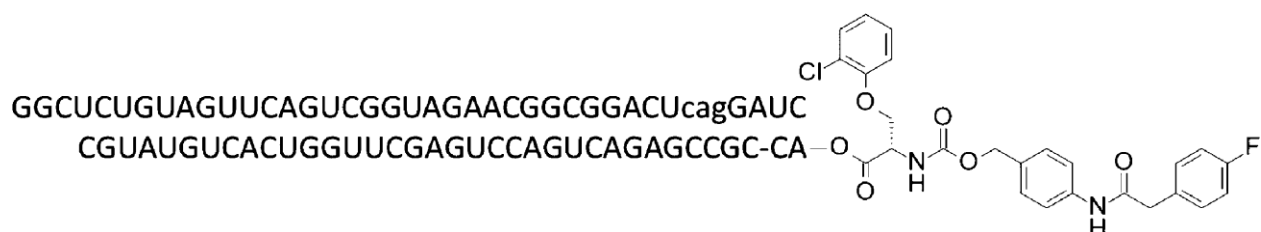
AAAtR-94 SPh2Cl-tRNA(AsnE2+Glu2)cag



10

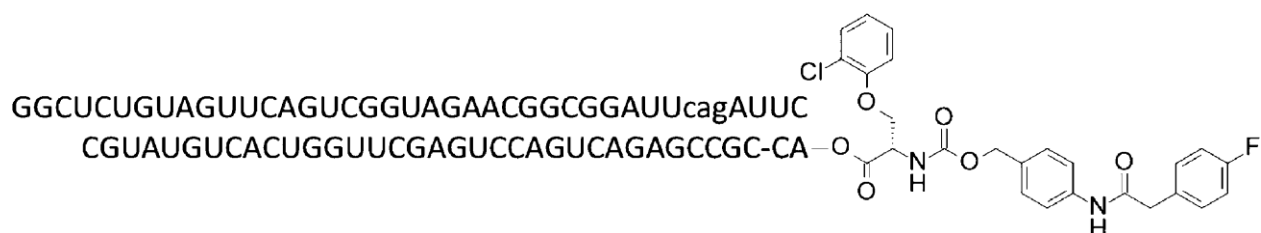
[0526]

AAAtR-95 SPh2Cl-tRNA(AsnE2+Arg3)cag



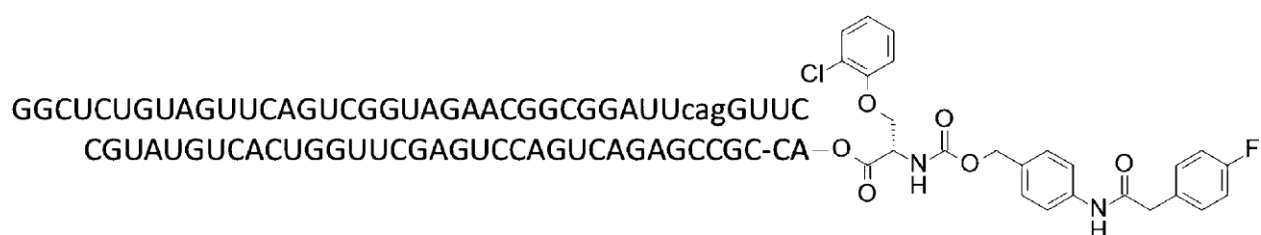
[0527]

15 AAAtR-96 SPh2Cl-tRNA(AsnE2+Val2)cag



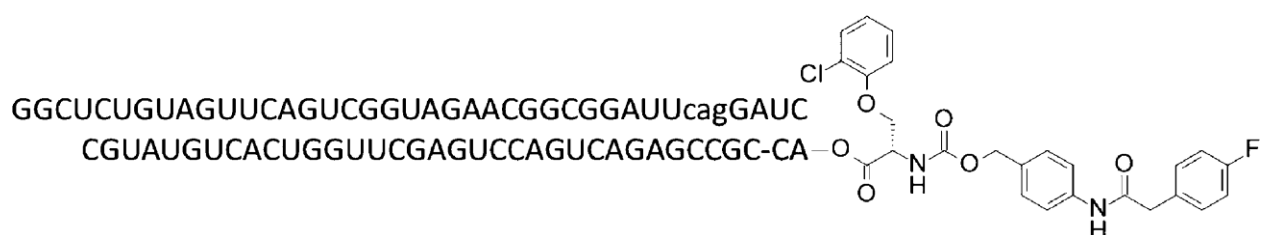
[0528]

AAAtR-97 SPh2Cl-tRNA(AsnE2+Leu2)cag



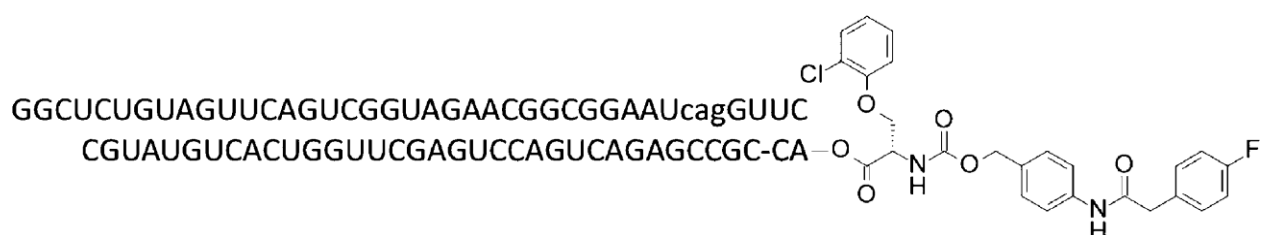
5 [0529]

AAAtR-98 SPh2Cl-tRNA(AsnE2+Pro3)cag



[0530]

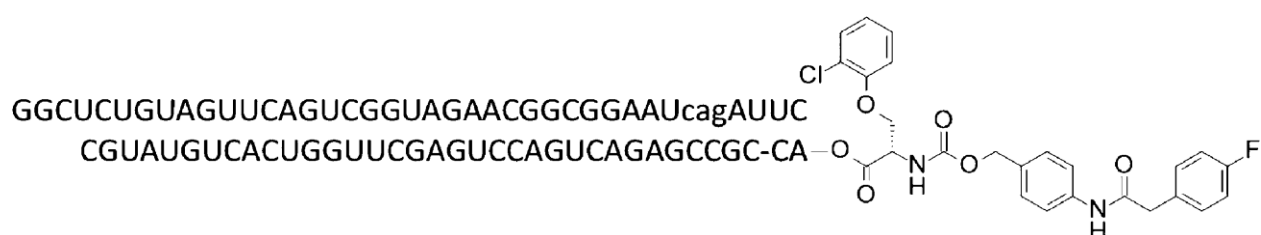
AAAtR-99 SPh2Cl-tRNA(AsnE2+Pro2)cag



10

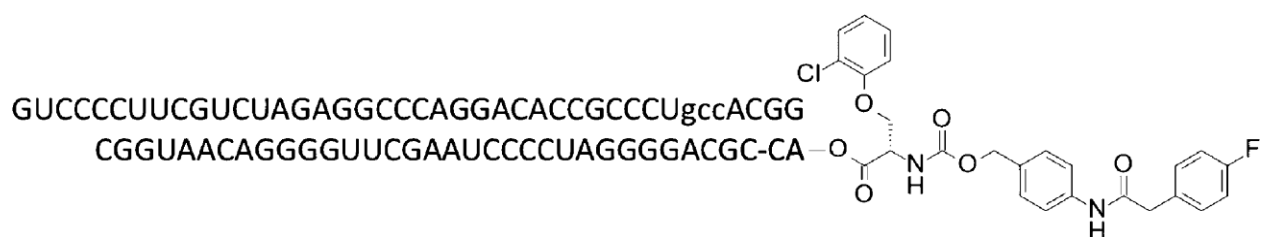
[0531]

AAAtR-100 SPh2Cl-tRNA(AsnE2+Ala2)cag



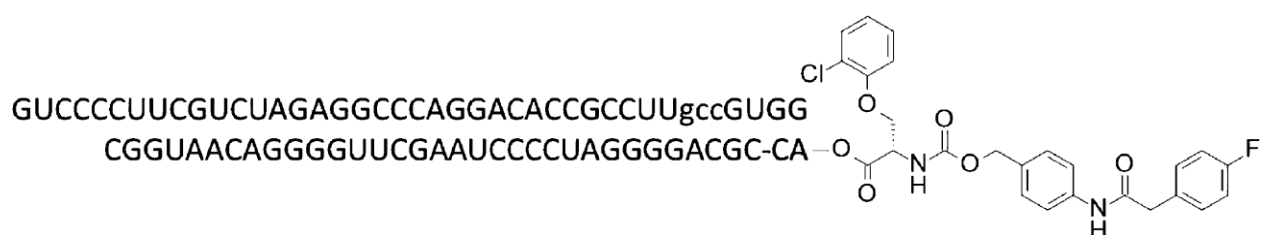
[0532]

15 AAAtR-101 SPh2Cl-tRNA(Glu2)gcc



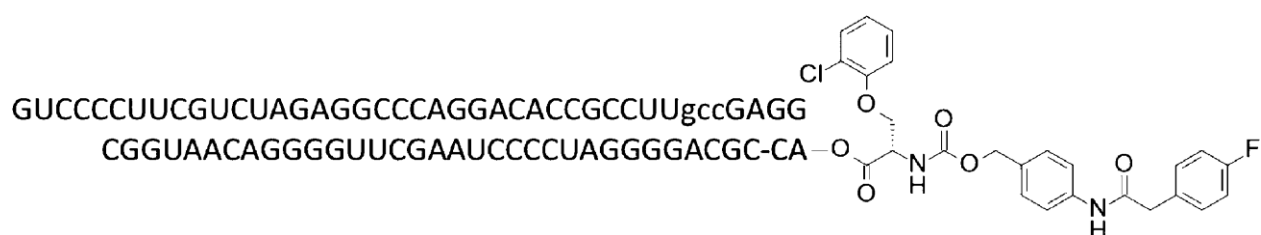
[0533]

AAtR-102 SPh2Cl-tRNA(Glu2+Leu2)gcc



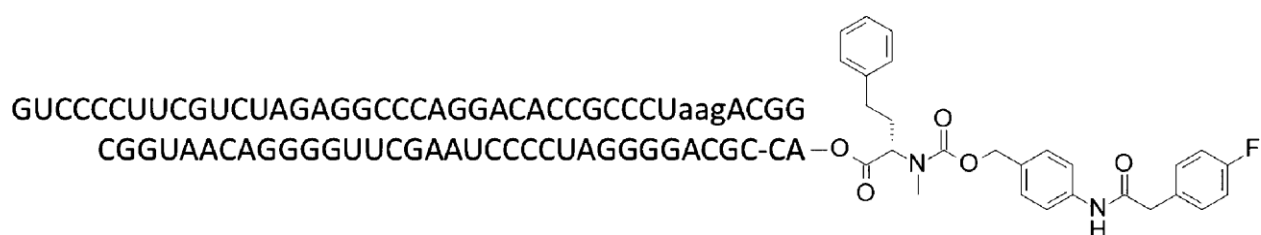
5 [0534]

AAtR-103 SPh2Cl-tRNA(Glu2+Pro3)gcc



[0535]

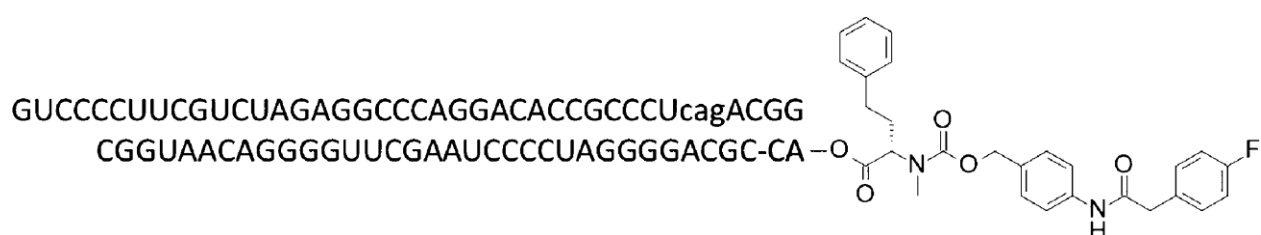
AAtR-104 MeHph-tRNA(Glu2)aag



10

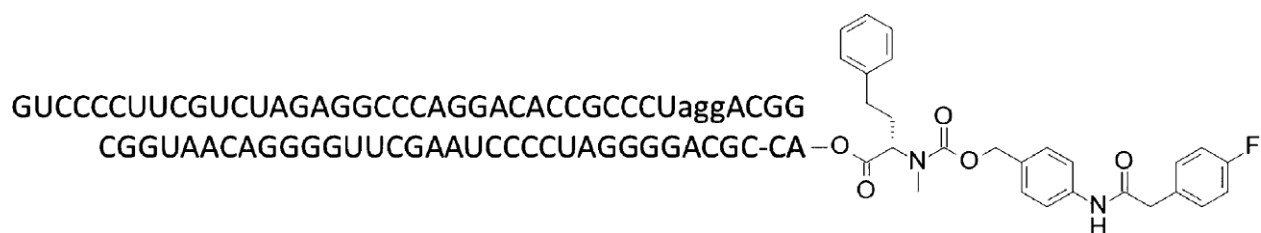
[0536]

AAtR-105 MeHph-tRNA(Glu2)cag



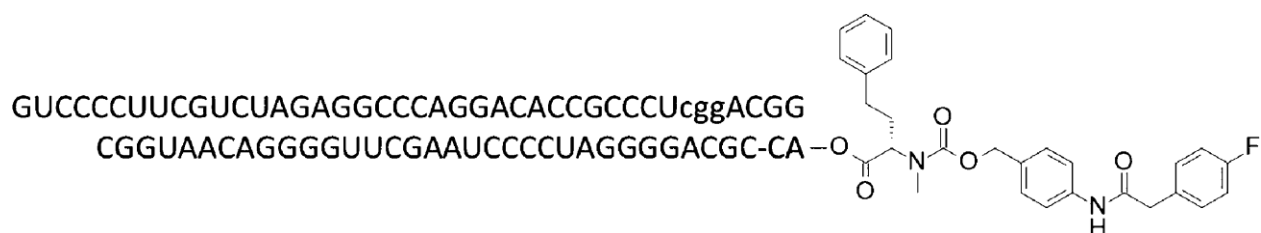
[0537]

15 AAtR-106 MeHph-tRNA(Glu2)agg



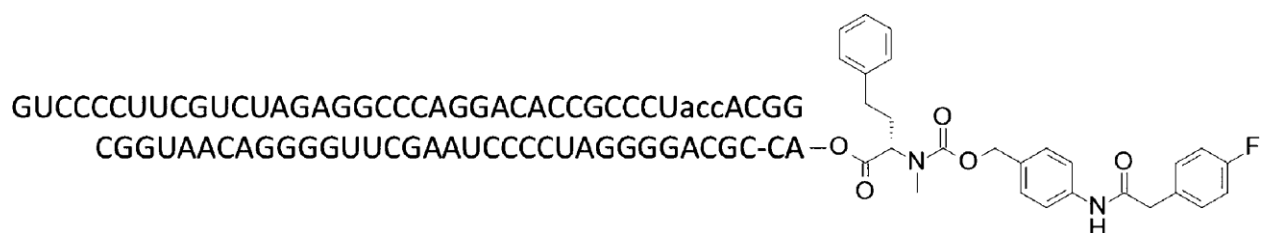
[0538]

AAtR-107 MeHph-tRNA(Glu2)cgg



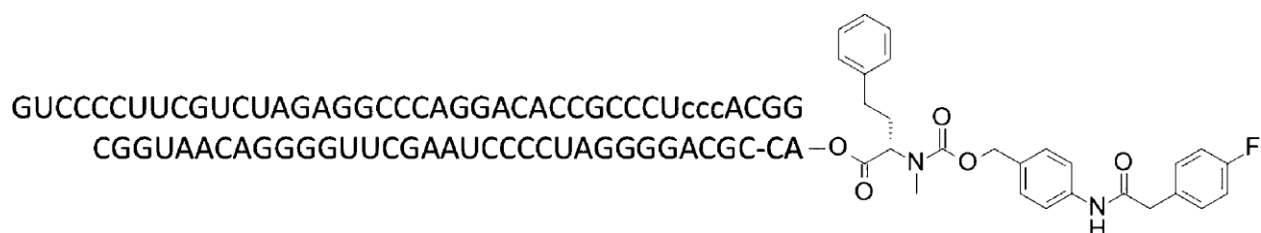
5 [0539]

AAtR-108 MeHph-tRNA(Glu2)acc



[0540]

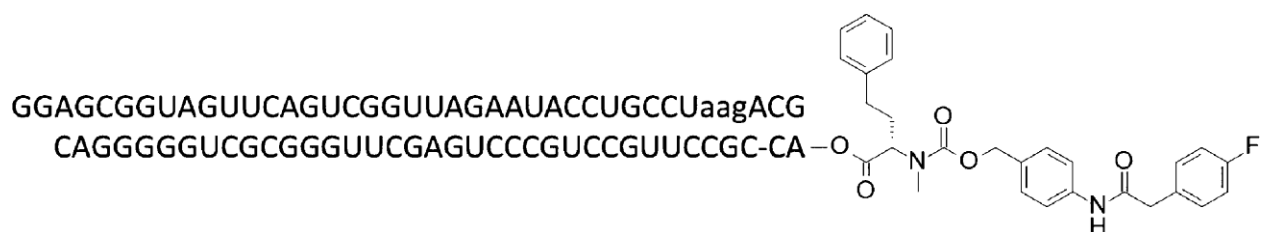
AAtR-109 MeHph-tRNA(Glu2)ccc



10

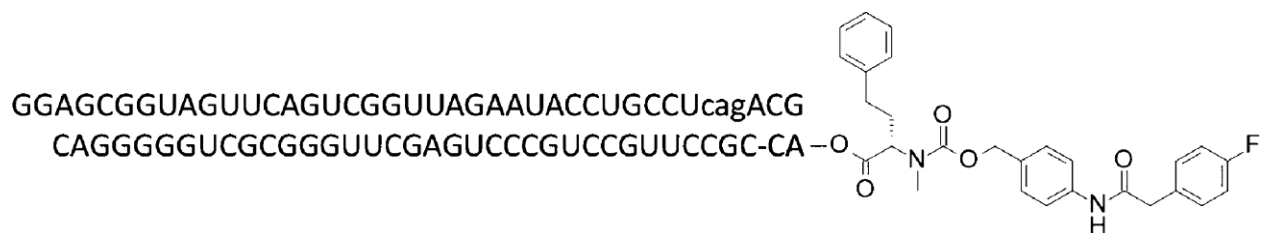
[0541]

AAtR-110 MeHph-tRNA(Asp1)aag



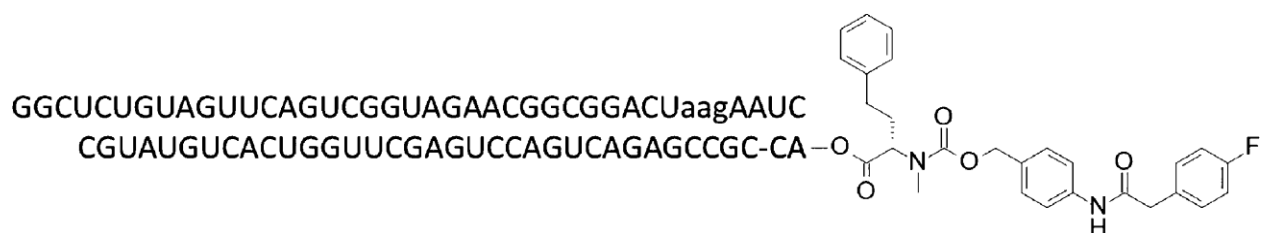
[0542]

15 AAtR-111 MeHph-tRNA(Asp1)cag



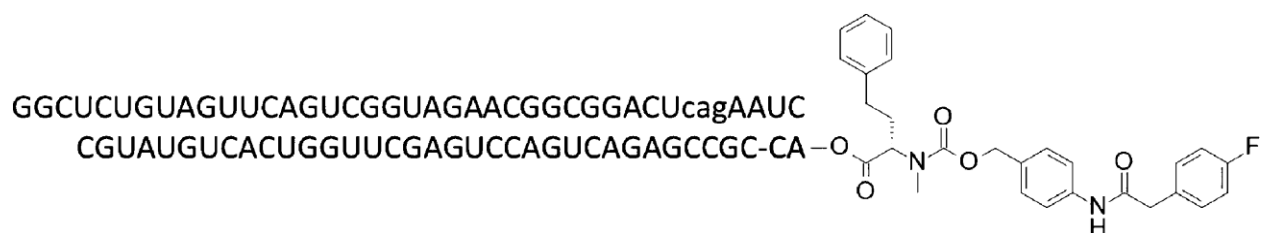
[0543]

AAAtR-112 MeHph-tRNA(AsnE2)aag



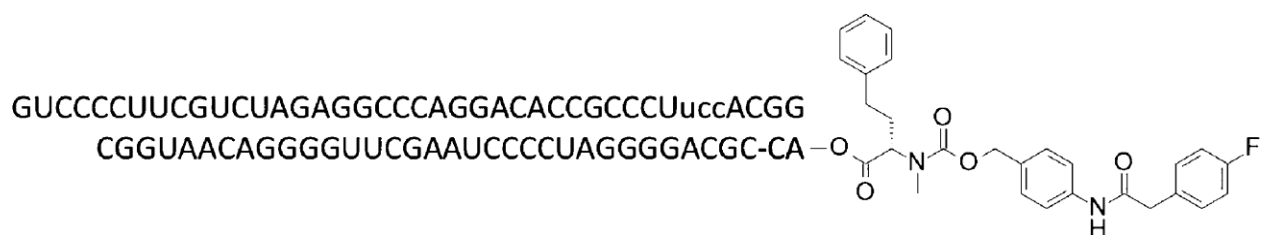
5 [0544]

AAAtR-113 MeHph-tRNA(AsnE2)cag



[0545]

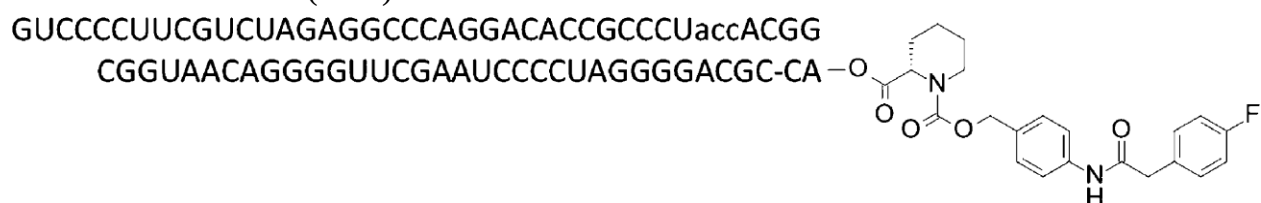
AAAtR-114 MeHph-tRNA(Glu2)ucc



10

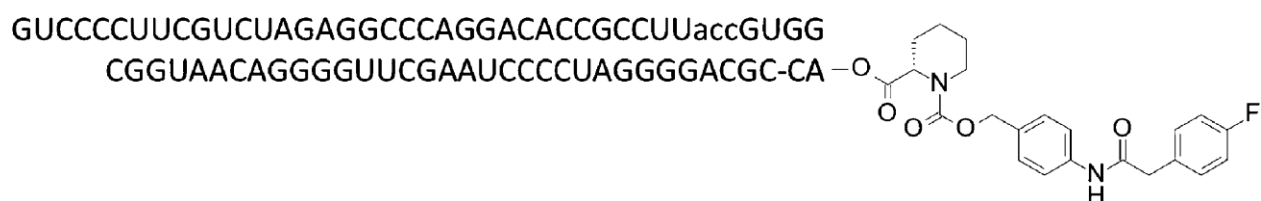
[0546]

AAAtR-115 Pic2- tRNA(Glu2)acc



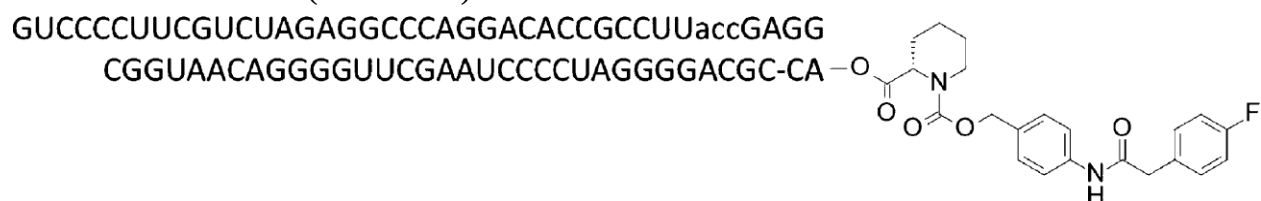
[0547]

15 AAAtR-116 Pic2- tRNA(Glu2+Leu2)acc



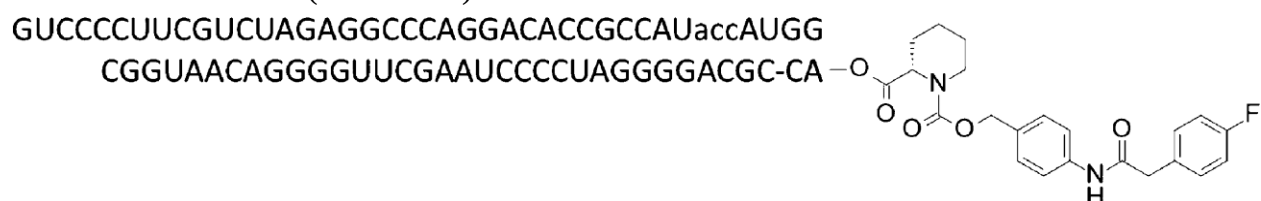
[0548]

AAtR-117 Pic2- tRNA(Glu2+Pro3)acc



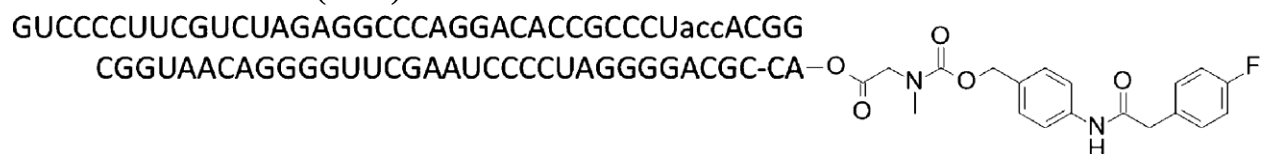
5 [0549]

AAtR-118 Pic2- tRNA(Glu2+Ala2)acc



[0550]

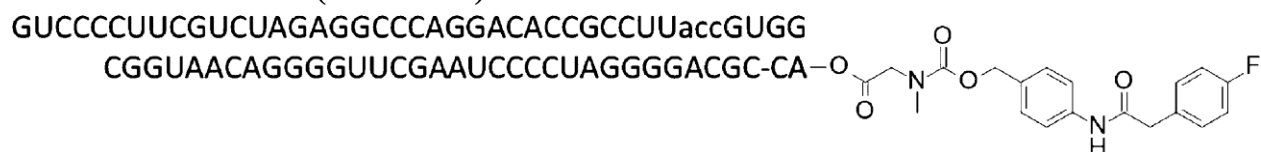
AAtR-119 MeG- tRNA(Glu2)acc



10

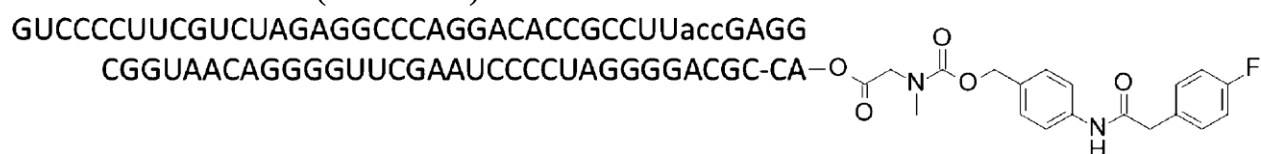
[0551]

AAtR-120 MeG- tRNA(Glu2+Leu2)acc



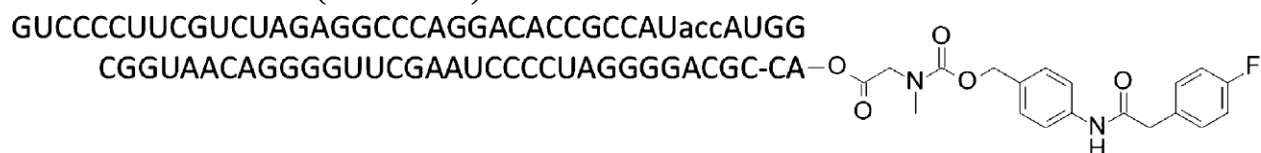
[0552]

15 AAtR-121 MeG- tRNA(Glu2+Pro3)acc



[0553]

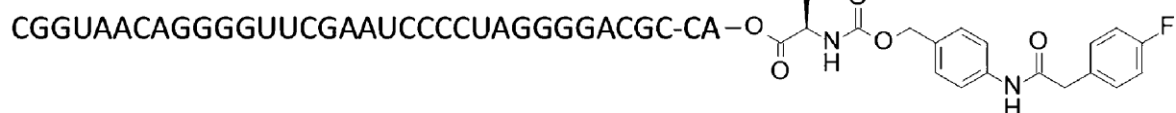
AAtR-122 MeG- tRNA(Glu2+Ala2)acc



[0554]

AAAtR-123 dA- tRNA(Glu2)acc

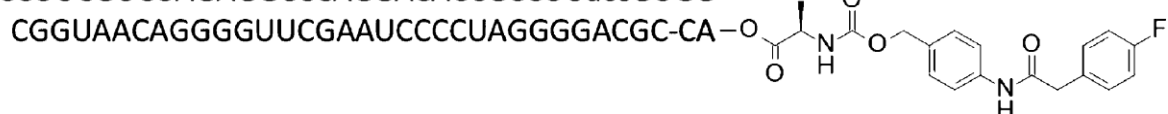
GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaccACGG



[0555]

5 AAAtR-124 dA- tRNA(Glu2+Leu2)acc

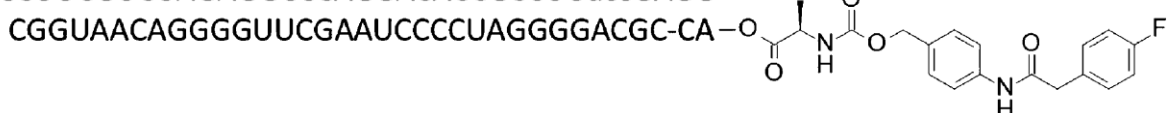
GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaccGUGG



[0556]

AAAtR-125 dA- tRNA(Glu2+Pro3)acc

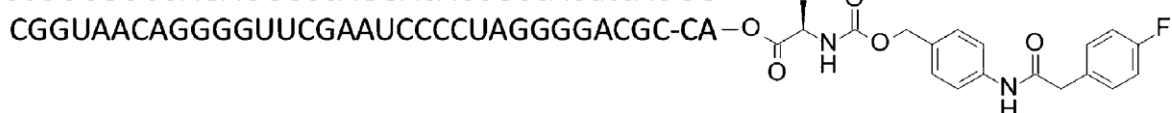
GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaccGAGG



10 [0557]

AAAtR-126 dA- tRNA(Glu2+Ala2)acc

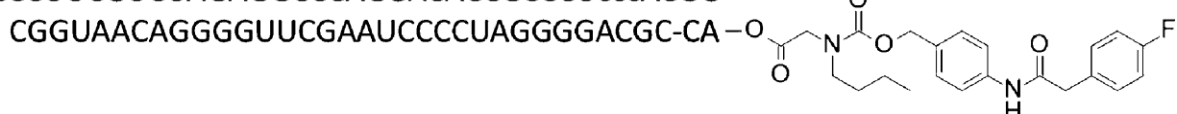
GUCCCCUUCGUCUAGAGGCCAGGACACCGCCAUaccAUGG



[0558]

AAAtR-127 nBuG- tRNA(Glu2)ccc

GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUcccACGG

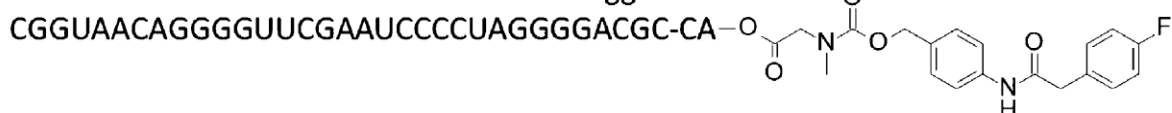


15

[0559]

AAAtR-129 MeG- tRNA(Glu2)agg

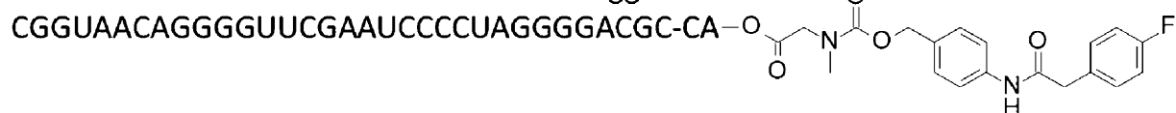
GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUaggACGG



[0560]

20 AAAtR-130 MeG- tRNA(Glu2)cgg

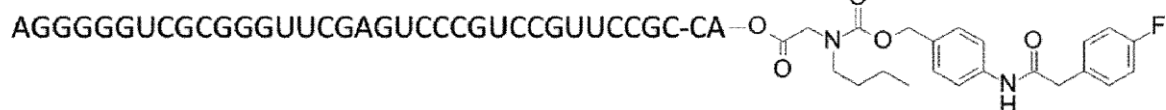
GUCCCCUUCGUCUAGAGGCCAGGACACCGCCCUcggACGG



[0561]

AAAtR-131 nBuG-tRNA(Asp1+Leu2)aag

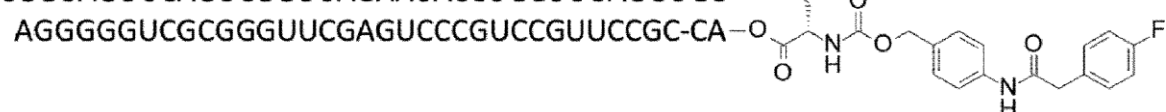
GGAGCGGUAGUUCAGUCGGUUGAAUACCUGCUUAAGGUGC



[0562]

AAAtR-132 SPh2Cl-tRNA(Asp1+Leu2)uag

GGAGCGGUAGUUCAGUCGGUUGAAUACCUGCUUUAGGUGC

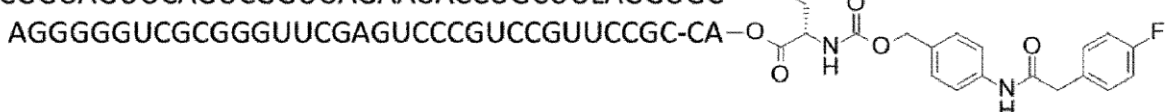


5

[0563]

AAAtR-133 SPh2Cl-tRNA(Asp1+Leu2)Lag

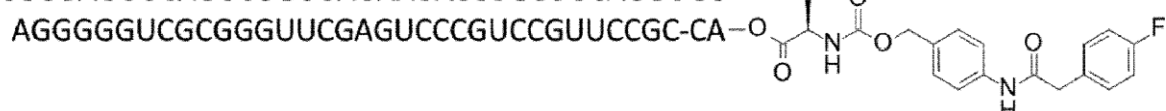
GGAGCGGUAGUUCAGUCGGUUGAAUACCUGCUULAGGUGC



[0564]

10 AAAtR-134 dA-tRNA(Asp1+Leu2)cag

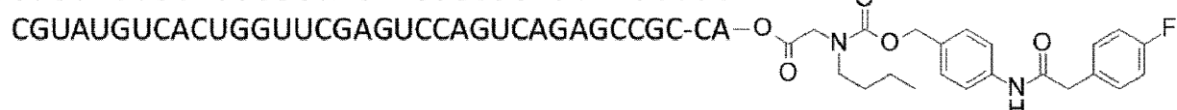
GGAGCGGUAGUUCAGUCGGUUGAAUACCUGCUUCAGGUGC



[0565]

AAAtR-135 nBuG-tRNA(AsnE2+Leu2)aag

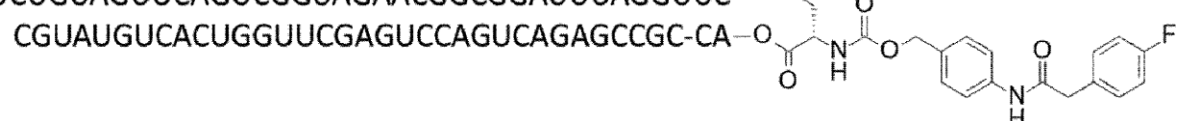
GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUAAGGUUC



15 [0566]

AAAtR-136 SPh2Cl-tRNA(AsnE2+Leu2)uag

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUAAGGUUC



[0567]

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUULAGGUUC
CGUAUGUCACUGGUUCGAGUCCAGUCAGAGCCGC-CA-O-

GGCUCUGUAGUUCAGUCGGUAGAACGGCGGAUUCAGGUUC
CGUAUGUCACUGGUUCGAGUCCAGUCAGAGCCGC-CA-O-C(=O)[C@H](N)C(=O)OCc1ccc(cc1)N(C(=O)Cc2ccc(cc2)F)c3ccccc3

A reaction solution was prepared by adding nuclease-free water to adjust the solution to 25 μ M transcribed tRNA(fMet)cau-CA (TR-105), 50 mM HEPES-KOH pH7.5, 20 mM MgCl₂, 1 mM ATP, 0.6 unit/ μ L T4 RNA ligase (New England Biolabs), and 0.25 mM aminoacyl pCpA (MT01), and ligation reaction was performed at 15°C for 45 minutes. Before adding T4 RNA ligase and aminoacyl pCpA, the reaction solution was heated to 95°C for 2 minutes and then allowed to stand at room temperature for 5 minutes to refold the tRNA in advance.

GGCGGGGUGGAGCAGCCUGGUAGCUCGUCGGGCUcauAAC
CCGAAGAUCGUCGGUUCAAAUCCGGCCCCCGCAAC-CA-O-CO-NH-CO-CH₂-CH₂-N⁺(F)₂-C₅H₃N₂

DNA sequence:

GGCGTAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGACTTT
TATTATTGGTTTTcttATTATTCCGATTGGTTAAGCTTCG

[0572]

Template DNA SEQ ID NO:222 (MD-2)

5 DNA sequence:

GGCGTAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGACTTT
TATTATTGGTTTTctgATTATTCCGATTGGTTAAGCTTCG

[0573]

Template DNA SEQ ID NO:223 (MD-3)

10 DNA sequence:

GGCGTAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGACTTT
TATTATTGGTTTTcctATTATTGCTATTGGTTAAGCTTCG

[0574]

Template DNA SEQ ID NO:224 (MD-4)

15 DNA sequence:

GGCGTAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGACTTT
TATTATTGGTTTTccgATTATTGCTATTGGTTAAGCTTCG

[0575]

Template DNA SEQ ID NO:225 (MD-5)

20 DNA sequence:

GGCGTAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGACTTT
TATTATTCTATTTggtATTATTCCGATTCTATAAGCTTCG

[0576]

Template DNA SEQ ID NO:226 (MD-6)

25 DNA sequence:

GGCGTAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGACTTT
TATTATTCTATTTgggATTATTCCGATTCTATAAGCTTCG

[0577]

Template DNA SEQ ID NO:227 (MD-7)

30 DNA sequence:

GGCGTAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGACTTT
TATTATTCTATTTggaATTATTCCGATTCTATAAGCTTCG

[0578]

Template DNA SEQ ID NO:228 (MD-8)

35 DNA sequence:

GGCGTAATACGACTCACTATAGGGTAACTTTAAGAAGGAGATATACATATGACTTT

TATTATTGGTTTTctaATTATTCCGATTGGTTAAGCTTCG

[0579]

Template mRNA SEQ ID NO:229 (MR-1)

RNA sequence:

5 GGGUUAACUUUAAGAAGGAGAUAUACAUAUGACUUUUAUUAUUGGUUUUcuaAUU
AUUCCGAUUGGUUAAGCUUCG

[0580]

Template mRNA SEQ ID NO:230 (MR-2)

RNA sequence:

10 GGGUUAACUUUAAGAAGGAGAUAUACAUAUGACUUUUAUUAUUGGUUUUcugAUU
AUUCCGAUUGGUUAAGCUUCG

[0581]

Template mRNA SEQ ID NO:231 (MR-3)

RNA sequence:

15 GGGUUAACUUUAAGAAGGAGAUAUACAUAUGACUUUUAUUAUUGGUUUUccuAUU
AUUGCUAUUGGUUAAGCUUCG

[0582]

Template mRNA SEQ ID NO:232 (MR-4)

RNA sequence:

20 GGGUUAACUUUAAGAAGGAGAUAUACAUAUGACUUUUAUUAUUGGUUUUccgAUU
AUUGCUAUUGGUUAAGCUUCG

[0583]

Template mRNA SEQ ID NO:233 (MR-5)

RNA sequence:

25 GGGUUAACUUUAAGAAGGAGAUAUACAUAUGACUUUUAUUAUUCUAUUUggaAUU
AUUCCGAUUCUAUAAGCUUCG

[0584]

Template mRNA SEQ ID NO:234 (MR-6)

RNA sequence:

30 GGGUUAACUUUAAGAAGGAGAUAUACAUAUGACUUUUAUUAUUCUAUUUgggAUU
AUUCCGAUUCUAUAAGCUUCG

[0585]

Template mRNA SEQ ID NO:235 (MR-7)

RNA sequence:

35 GGGUUAACUUUAAGAAGGAGAUAUACAUAUGACUUUUAUUAUUCUAUUUggaAUU
AUUCCGAUUCUAUAAGCUUCG

[0586]

Template mRNA SEQ ID NO:236 (MR-8)

RNA sequence:

GGGUUAACUUUAAGAAGGAGAUUAUCAUAUGACUUUUUAUUAUUGGUUUUcuaAUU
5 AUUCCGAUUGGUUAAGCUUCG

[0587]

Example 6. Translational synthesis of peptides

Example 6-1

To evaluate the effects of combinations of bases at positions 32, 33, 37, and 38 of
10 tRNAs on accuracy of translation, a translation experiment was performed in the presence of two
types of elongator aminoacyl tRNAs carrying anticodons that match different codons in the same
codon box.

Specifically, peptides were translationally synthesized by translating template mRNA
(MR-2) using aminoacyl tRNAs (AAtR-105 and any one of AAtR-1 to AAtR-10), translating
15 template mRNA (MR-1) using aminoacyl tRNAs (AAtR-104 and any one of AAtR-11 to AAtR-
20), translating template mRNA (MR-4) using aminoacyl tRNAs (AAtR-107 and any one of
AAtR-21 to AAtR-30), translating template mRNA (MR-3) using aminoacyl tRNAs (AAtR-106
and any one of AAtR-31 to AAtR-40), translating template mRNA (MR-6) using aminoacyl
tRNAs (AAtR-109 and any one of AAtR-41 to AAtR-50), and translating template mRNA (MR-
20 5) using aminoacyl tRNAs (AAtR-108 and any one of AAtR-51 to AAtR-60). Herein, “AAtR”
and “AATR” are used synonymously. More specifically, “AAtR-105” and “AATR-105” refer to
the same tRNA.

[0588]

Translation Condition 1

25 A translation experiment for evaluating the amount of misreading of the CCG codon by
tRNAs carrying the agg anticodon was performed. The translation system used was the PURE
system, a prokaryote-derived reconstituted cell-free protein synthesis system. Specifically, the
translation was carried out as follows: ARS mix (selection of ARS of amino acids encoded in
mRNA from among 0.09 μ M GlyRS, 0.4 μ M or 0.97 μ M IleRS, 0.68 μ M or 1.64 μ M PheRS,
30 0.16 μ M or 0.39 μ M ProRS, 0.09 μ M or 0.22 μ M ThrRS, 2.73 μ M AlaRS, 0.04 μ M or 0.097 μ M
LeuRS, 0.04 μ M SerRS, and 0.02 μ M ValRS), 1 μ M template mRNA (MR-4), and 0.25 mM
each of the group of natural amino acids encoded in the template mRNA, 10 μ M initiator
aminoacylated tRNA (AAtR-128) and 10 μ M each of the elongator aminoacyl tRNAs (AAtR-
107 and any one selected from AAtR-21 to AAtR-30) were added to a translation solution (1
35 mM GTP, 1 mM ATP, 20 mM phosphocreatine, 50 mM HEPES-KOH pH7.6, 100 mM
potassium acetate, 10 mM magnesium acetate, 2 mM spermidine, 1 mM dithiothreitol, 1.5

mg/mL *E. coli* MRE600 (RNase-negative)-derived tRNA (Roche), 0.24 μ M or 0.26 μ M EF-G, 0.24 μ M RF2, 0.17 μ M RF3, 0.5 μ M RRF, 3.7 μ M or 4 μ g/mL creatine kinase, 2.8 μ M or 3 μ g/mL myokinase, 1.9 unit/mL or 2 unit/mL inorganic pyrophosphatase, 1.0 μ g/mL or 1.1 μ g/mL nucleoside diphosphate kinase, 2.5 μ M or 2.7 μ M IF1, 0.37 μ M or 0.4 μ M IF2, 1.4 μ M or 1.5 μ M IF3, 37.2 μ M or 40 μ M EF-Tu, 49 μ M or 54.1 μ M or 54.9 μ M or 59 μ M EF-Ts, 0.93 μ M or 1 μ M EF-P-Lys, 0.4 unit/ μ L RNasin® Ribonuclease inhibitor (Promega, N2111), 1.1 μ M or 1.2 μ M ribosome, 0.5 mM PGA), and the mixture was left to stand at 37° C for one hour. Hereafter, this translation condition may be called “Translation Condition 1”.

The template mRNA was designed so that translation gives

BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 241 (Pep-3); herein, an amino acid sequence may be written by separating the amino acids with a colon) as the translation product. When the codon is misread by an aminoacyl tRNA, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 242 (Pep-4)).

[0589]

A translation experiment for evaluating the amount of misreading of the CCU codon by tRNAs carrying the cgg anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-3) and elongator aminoacyl tRNAs (AAtR-106 and any one of AAtR-31 to AAtR-40) were used.

The template mRNA was designed so that translation gives

BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 241 (Pep-3)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 242 (Pep-4)).

[0590]

A translation experiment for evaluating the amount of misreading of the GGG codon by tRNAs carrying the acc anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-6) and elongator aminoacyl tRNAs (AAtR-109 and any one of AAtR-41 to AAtR-50) were used.

The template mRNA was designed so that translation gives

BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243 (Pep-5)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244 (Pep-6)).

[0591]

A translation experiment for evaluating the amount of misreading of the GGU codon by tRNAs carrying the ccc anticodon was performed. Translation was carried out according to the

above-mentioned Translation Condition 1, except that template mRNA (MR-5) and elongator aminoacyl tRNAs (AAtR-108 and any one of AAtR-51 to AAtR-60) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243 (Pep-5)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244 (Pep-6)).

[0592]

A translation experiment for evaluating the amount of misreading of the CUG codon by tRNAs carrying the aag anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-2) and elongator aminoacyl tRNAs (AAtR-105 and any one of AAtR-1 to AAtR-10) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239 (Pep-1)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240 (Pep-2)).

[0593]

A translation experiment for evaluating the amount of misreading of the CUU codon by tRNAs carrying the cag anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-1) and elongator aminoacyl tRNAs (AAtR-104 and any one of AAtR-11 to AAtR-20) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239 (Pep-1)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240 (Pep-2)).

[0594]

Example 6-2

The tRNA bodies were changed, and experiments similar to those of Example 6-1 were performed. Specifically, peptides were translationally synthesized by translating template mRNA (MR-2) using aminoacyl tRNAs (AAtR-111 and any one of AAtR-61 to AAtR-70), translating template mRNA (MR-1) using aminoacyl tRNAs (AAtR-110 and any one of AAtR-71 to AAtR-80), translating template mRNA (MR-2) using aminoacyl tRNAs (AAtR-113 and any one of AAtR-81 to AAtR-90), and translating template mRNA (MR-1) using aminoacyl tRNAs (AAtR-112 and any one of AAtR-91 to AAtR-100).

[0595]

Translation condition

A translation experiment for evaluating the amount of misreading of the CUG codon by tRNAs carrying the aag anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-2) and elongator aminoacyl tRNAs (AAtR-113 and any one of AAtR-81 to AAtR-90) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239 (Pep-1)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240 (Pep-2)).

[0596]

A translation experiment for evaluating the amount of misreading of the CUU codon by tRNAs carrying the cag anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-1) and elongator aminoacyl tRNAs (AAtR-112 and any one of AAtR-91 to AAtR-100) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239 (Pep-1)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240 (Pep-2)).

[0597]

A translation experiment for evaluating the amount of misreading of the CUG codon by tRNAs carrying the aag anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-2) and elongator aminoacyl tRNAs (AAtR-111 and any one of AAtR-61 to AAtR-70) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239 (Pep-1)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240 (Pep-2)).

[0598]

A translation experiment for evaluating the amount of misreading of the CUU codon by tRNAs carrying the cag anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-1) and elongator aminoacyl tRNAs (AAtR-110 and any one of AAtR-71 to AAtR-80) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 239 (Pep-1)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 240 (Pep-2)).

[0599]

Example 6-3

The codons used were changed, and experiments similar to those of Examples 6-1 and 6-2 were performed. Specifically, peptides were translationally synthesized by translating template mRNA (MR-7) using aminoacyl tRNAs (AAtR-114 and any one of AAtR-44, AAtR-47, and AAtR-48), translating template mRNA (MR-7) using aminoacyl tRNAs (AAtR-114 and any one of AAtR-101 to AAtR-103), and translating template mRNA (MR-6) using aminoacyl tRNAs (AAtR-109 and any one of AAtR-101 to AAtR-103).

[0600]

Translation condition

A translation experiment for evaluating the amount of misreading of the GGA codon by tRNAs carrying the acc anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-7) and elongator aminoacyl tRNAs (AAtR-114 and any one of AAtR-44, AAtR-47, and AAtR-48) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243 (Pep-5)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244 (Pep-6)).

[0601]

A translation experiment for evaluating the amount of misreading of the GGA codon by tRNAs carrying the gcc anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-7) and elongator aminoacyl tRNAs (AAtR-114 and any one of AAtR-101 to AAtR-103) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243 (Pep-5)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244 (Pep-6)).

[0602]

A translation experiment for evaluating the amount of misreading of the GGG codon by

tRNAs carrying the gcc anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-6) and elongator aminoacyl tRNAs (AAtR-109 and any one of AAtR-101 to AAtR-103) were used.

The template mRNA was designed so that translation gives

5 BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243 (Pep-5)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 244 (Pep-6)).

[0603]

10 Example 6-4

Using tRNAs in which the first letter of the anticodon has been engineered (tRNAs whose base at the first letter of the anticodon is lysidine), experiments similar to those of Examples 6-1 to 6-3 were performed. Specifically, peptides were translationally synthesized by translating according to the above-mentioned Translation Condition 1, except that template
15 mRNA (MR-1, MR-8, or MR-2) and aminoacyl tRNAs (AAtR-131, AAtR-134, and either AAtR-132 or AAtR-133) or aminoacyl tRNAs (AAtR-135, AAtR-138, and either AAtR-136 or AAtR-137) were used. The amount of translation of the following translation products were compared:

BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 250 (Pep-12));

20 BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 251 (Pep-13)); and

BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly (SEQ ID NO: 252 (Pep-14)).

[0604]

Example 6-5

The amino acids were changed, and experiments similar to those of Examples 6-1 to 6-4
25 were performed. Specifically, peptides were translationally synthesized by translating template mRNA (MR-6) using aminoacyl tRNAs (AAtR-109 and any one of AAtR-115 to AAtR-118; or AAtR-109 and any one of AAtR-119 to AAtR-122; or AAtR-127 and any one of AAtR-123 to AAtR-126), translating template mRNA (MR-4) using aminoacyl tRNAs (AAtR-130 and any one of AAtR-21 to AAtR-30), and translating template mRNA (MR-3) using aminoacyl tRNAs
30 (AAtR-129 and any one of AAtR-31 to AAtR-40).

[0605]

Translation condition

A translation experiment for evaluating the amount of misreading of the GGG codon by tRNAs carrying the acc anticodon was performed. Translation was carried out according to the
35 above-mentioned Translation Condition 1, except that template mRNA (MR-6) and elongator aminoacyl tRNAs (AAtR-109 and any one of AAtR-115 to AAtR-118) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243 (Pep-5)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:Pic2:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 245 (Pep-7)).

[0606]

A translation experiment for evaluating the amount of misreading of the GGG codon by tRNAs carrying the acc anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-6) and elongator aminoacyl tRNAs (AAtR-109 and any one of AAtR-119 to AAtR-122) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 243 (Pep-5)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeG:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 246 (Pep-8)).

[0607]

A translation experiment for evaluating the amount of misreading of the GGG codon by tRNAs carrying the acc anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-6) and elongator aminoacyl tRNAs (AAtR-127 and any one of AAtR-123 to AAtR-126) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:nBuG:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 247 (Pep-9)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Leu:Phe:dA:Ile:Ile:Pro:Ile:Leu (SEQ ID NO: 248 (Pep-10)).

[0608]

A translation experiment for evaluating the amount of misreading of the CCG codon by tRNAs carrying the agg anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-4) and elongator aminoacyl tRNAs (AAtR-130 and any one of AAtR-21 to AAtR-30) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 249 (Pep-11)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 242 (Pep-4)).

[0609]

A translation experiment for evaluating the amount of misreading of the CCU codon by tRNAs carrying the cgg anticodon was performed. Translation was carried out according to the above-mentioned Translation Condition 1, except that template mRNA (MR-3) and elongator aminoacyl tRNAs (AAtR-129 and any one of AAtR-31 to AAtR-40) were used.

The template mRNA was designed so that translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 249 (Pep-11)) as the correctly read translation product. When misreading by an aminoacyl tRNA takes place, the translation gives BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly (SEQ ID NO: 242 (Pep-4)).

[0610]

Example 7 Analyses of the translated peptides

Translation product solutions obtained by the translation reactions of Examples 6-1 to 6-5 were diluted ten-fold, and then analyzed using a LC-FLR-MS system. The amount of translated peptide was evaluated from the analysis data by identifying the retention time of the target translated peptide from the MS data, and quantifying the fluorescence peak at the relevant retention time. Unless indicated otherwise, in the quantitative evaluation, LCT-67 synthesized in Example 3 was used as a standard to prepare a calibration curve, and the content was calculated by relative quantification. Unless stated otherwise, the LC-MS was analyzed according to the conditions of Method 1 shown in Table 5 below. Furthermore, based on the determined amount of peptide translation, the percentage (%) of misread peptides relative to an intended product was calculated using the following equation. Herein, the peptide obtained when correct reading takes place may be referred to as the correctly read translation product or intended product.

[Equation 1]

$$\text{Percentage of misread peptides relative to the intended product (\%)} = \frac{\text{Amount of translated peptide obtained when misreading takes place (\mu M)}}{\text{Amount of translated peptide obtained when correctly read (\mu M)}} \times 100$$

[0611]

Analysis conditions

[Table 5]

	Method1	Method2
System	Aquity UPLC–FLR–Xevo G2–XS ToF	Aquity UPLC–FLR–Xevo G2–XS ToF
Column	waters BEHC18 (2.1x50mm, ϕ 1.7 μ m)	waters BEHC18 (2.1x100mm, ϕ 1.7 μ m)
Mobile phase	A=0.1% FA with H ₂ O B=0.1% FA with CH ₃ CN	A=0.1% FA with H ₂ O B=0.1% FA with CH ₃ CN
Gradient (%B)	0–0.2min=10% 0.2–3.6min=98% 3.6–4.0min=10%	0–0.4min=60% 0.4–9.0min=98% 9.0–10min=60%
Flow rate (ml/min)	0.5	0.5
Column temp.	40	40
Fluorometry wavelength (Ex/Em)	491nm/515nm	491nm/515nm
Ms mode	ESI–	ESI–

[0612]

Results

As a result of the evaluation, the rate of codon misreading by tRNAs whose combination of bases at positions 32, 33, 37, and 38 had been engineered tended to increase when the combination of bases at those positions was the Ser5 sequence, the Ala1B sequence, and the Phe sequence, and tended to decrease when this combination was the Pro3 sequence, the Pro2 sequence, the Ala2 sequence, the Leu2 sequence, the Arg3 sequence, and the Val2 sequence (Figs. 1 to 6, and Tables 6 to 11). Among them, decreasing tendencies were greater for the Pro3 sequence, the Pro2 sequence, the Ala2 sequence, and the Leu2 sequence. The ranking of the rate of misreading was not affected, even when the tRNA body was changed (Figs. 7 to 10, and Tables 12 to 15). Effects of reducing codon misreading due to engineering the combination of bases at positions 32, 33, 37, and 38 in the tRNA to a specific combination were also confirmed for cases where the combination of the codon and the anticodon to be evaluated was changed (Figs. 11 to 13, Tables 16 to 18). Effects of reducing misreading due to engineering the combination of bases at positions 32, 33, 37, and 38 in the tRNA to a specific combination were also confirmed for cases where the amino acid to be aminoacylated was changed (Figs. 14 to 18, and Tables 21 to 25). These results showed that engineering the combination of bases at positions 32, 33, 37, and 38 in the tRNA can reduce codon misreading. The likelihood of occurring codon misreading was elucidated for combinations of bases regarding positions 32, 33, 37, and 38 in the tRNA; and this enables customizing tRNA

sequences to reduce codon misreading, while maintaining the amount of translated amino acid above a certain level, depending on the tRNA used.

[0613]

The following table shows the translation results of CCG codon discrimination by a tRNA carrying agg or cgg as the anticodon, whose combination of bases at positions 32, 33, 37, and 38 had been engineered. Compared to tRNA Glu2 (AAtR-24), whose combination of bases at positions 32, 33, 37, and 38 had not been engineered, tRNAs (AAtR-27 to 30) in which the base combination had been engineered to be the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence were found to have reduced codon misreading (Fig. 1).

[0614]

[Table 6]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-21	MR-4	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.58
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	1.75
AATR-22		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.51
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	1.66
AATR-23		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.58
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.05
AATR-24		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.36
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	1.93
AATR-25		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.48
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.19
AATR-26		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.44
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.15
AATR-27		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.25
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.32
AATR-28		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.35
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.41
AATR-29		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.11
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.89
AATR-30		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.11
AATR-107		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.60

[0615]

The following table shows the translation results of CCU codon discrimination by a tRNA carrying cgg or agg as the anticodon, whose combination of bases at positions 32, 33, 37, and 38 had been engineered. Compared to tRNA Glu2 (AAtR-34), whose combination of bases at positions 32, 33, 37, and 38 had not been engineered, tRNAs (AAtR-33 and AAtR-35 to 40) in which the base combination had been engineered to be the Phe sequence, the Arg3 sequence, the Val2 sequence, the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence were found to have reduced codon misreading, and particularly the tRNAs engineered to have the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence showed highly effective reduction of codon misreading (Fig. 2).

[0616]

[Table 7]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μ M)
AATR-31	MR-3	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.44
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	1.96
AATR-32		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.39
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	1.70
AATR-33		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.35
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.45
AATR-34		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.32
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.05
AATR-35		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.21
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	1.94
AATR-36		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.25
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.40
AATR-37		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.13
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.42
AATR-38		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.14
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.68
AATR-39		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.07
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	3.00
AATR-40		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.06
AATR-106		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Ala:Ile:Gly	2.60

[0617]

The following table shows the translation results of GGG codon discrimination by a
 5 tRNA carrying acc or ccc as the anticodon, whose combination of bases at positions 32, 33, 37,
 and 38 had been engineered. Compared to tRNA Glu2 (AAtR-44), whose combination of bases
 at positions 32, 33, 37, and 38 had not been engineered, tRNAs (AAtR-43 and AAtR45 to 50) in
 which the base combination had been engineered to be the Arg3 sequence, the Phe sequence, the
 Val2 sequence, the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence
 10 were found to have reduced codon misreading, and particularly the tRNAs engineered to have
 the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence showed highly
 effective reduction of codon misreading (Fig. 3).

[0618]

[Table 8]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-41	MR-6	BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.91
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.51
AATR-42		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.68
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.48
AATR-43		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.57
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	3.29
AATR-44		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.77
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.79
AATR-45		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.58
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	3.31
AATR-46		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.34
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	3.53
AATR-47		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.05
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	3.23
AATR-48		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.07
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	3.68
AATR-49		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.08
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	3.15
AATR-50		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.07
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	3.23

[0619]

The following table shows the translation results of GGU codon discrimination by a tRNA carrying ccc or acc as the anticodon, whose combination of bases at positions 32, 33, 37, and 38 had been engineered. Although the amount of translated peptides produced by codon misreading was low for tRNA Glu2 (AATR-54), whose combination of bases at positions 32, 33, 37, and 38 had not been engineered, the amount of translated peptides obtained by codon misreading was also kept low for tRNAs (AATR-52, AATR-53, and AATR-55 to 60) in which the base combination had been engineered to be the Phe sequence, the Ala1B sequence, the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, the Pro3 sequence, the Arg3 sequence, or the Val2 sequence, and a trend similar to the above Examples were observed (Fig. 4).

[0620]

[Table 9]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-51	MR-5	BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.15
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.54
AATR-52		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.03
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.43
AATR-53		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.04
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.97
AATR-54		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.04
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.40
AATR-55		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.02
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.57
AATR-56		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.02
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.22
AATR-57		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.01
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.39
AATR-58		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.02
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.63
AATR-59		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.03
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.71
AATR-60		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.02
AATR-108		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	2.55

[0621]

The following table shows the results of translating the CUG codon by a tRNA carrying cag or aag as the anticodon, whose combination of bases at positions 32, 33, 37, and 38 had been engineered. Compared to tRNA Glu2 (AATR-4), whose combination of bases at positions 32, 33, 37, and 38 had not been engineered, tRNAs (AATR-8 and 9) in which the base-derived combination had been engineered to be the Pro2 sequence or the Pro3 sequence were found to have reduced codon misreading, and the amount of translated peptide produced by codon misreading was kept at a low level for the Ala2 sequence as well (Fig. 5).

[Table 10]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-1	MR-2	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.28
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.85
AATR-2		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.35
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.86
AATR-3		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.26
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.92
AATR-4		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.13
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.62
AATR-5		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.16
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.80
AATR-6		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.17
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.82
AATR-7		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.18
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.56
AATR-8		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.06
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	2.27
AATR-9		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.05
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.85
AATR-10		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.13
AATR-105		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.67

[0622]

The following table shows the translation results of CUU codon discrimination by a tRNA carrying aag or cag as the anticodon, whose combination of bases at positions 32, 33, 37, and 38 had been engineered. Although the amount of translated peptides produced by codon misreading was low for tRNA Glu2 (AATR-14), whose combination of bases at positions 32, 33, 37, and 38 had not been engineered, the amount of translated peptides obtained by codon misreading was also kept low for tRNAs (AATR-13, and AATR-15 to 20) in which the combination derived from the above bases had been engineered to be the Phe sequence, the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, the Pro3 sequence, the Arg3 sequence, or the Val2 sequence, and a trend similar to the above Examples were observed (Fig. 6).

[0623]

[Table 11]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-11	MR-1	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.09
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.61
AATR-12		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.10
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.62
AATR-13		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.02
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.74
AATR-14		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.01
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.51
AATR-15		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.01
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.56
AATR-16		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.01
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.48
AATR-17		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.01
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.54
AATR-18		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.01
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.65
AATR-19		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.01
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.94
AATR-20		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.01
AATR-104		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.62

[0624]

These results indicated that when the combination of bases at positions 32, 33, 37, and 38 in a tRNA is the Ser5 sequence or the Ala1B sequence, the percentage of misread peptides relative to an intended product is high. By engineering the combination of bases at positions 32, 33, 37, and 38 in a tRNA having the Ser5 sequence as the above-mentioned base combination, the following experiments were performed to verify whether codon misreading can be reduced.

[0625]

tRNA AsnE2 was selected as the tRNA whose combination of bases at positions 32, 33, 37, and 38 is the Ser5 sequence. The following table shows the translation results of CUG codon discrimination by a tRNA which carries the tRNA(AsnE2) body with its combination of bases at positions 32, 33, 37, and 38 being engineered, and which carries aag or cag as the anticodon. Compared to tRNA(AsnE2) (AATR-81), whose combination of bases at positions 32, 33, 37, and 38 had not been engineered, tRNAs (AATR-85 to 90) in which the combination derived from the above-mentioned bases had been engineered to be the Arg3 sequence, the Val2 sequence, the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence were found to have reduced codon misreading, and particularly the tRNAs engineered to have the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence showed highly effective reduction of codon misreading (Fig. 7).

[Table 12]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-81	MR-2	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.69
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.60
AATR-82		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.77
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.58
AATR-83		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.84
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.63
AATR-84		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.71
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.59
AATR-85		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.57
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.63
AATR-86		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.57
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.65
AATR-87		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.19
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.64
AATR-88		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.30
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.57
AATR-89		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.07
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.61
AATR-90		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.08
AATR-113		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.70

[0626]

The following table shows the translation results of CUU codon discrimination by a tRNA which carries the tRNA(AsnE2) body with its combination of bases at positions 32, 33, 37, and 38 being engineered, and which carries cag or aag as the anticodon. Compared to tRNA(AsnE2) (AATR-91), whose combination of bases at positions 32, 33, 37, and 38 had not been engineered, tRNAs (AATR-95 to 100) in which the combination derived from the above-mentioned bases had been engineered to be the Arg3 sequence, the Val2 sequence, the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence were found to have reduced codon misreading, and particularly the tRNAs engineered to have the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence showed highly effective reduction of codon misreading (Fig. 8).

[0627]

[Table 13]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-91	MR-1	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.36
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.69
AATR-92		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.49
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.65
AATR-93		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.40
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.68
AATR-94		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.39
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.66
AATR-95		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.13
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.74
AATR-96		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.14
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.74
AATR-97		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.05
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.79
AATR-98		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.07
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.84
AATR-99		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.04
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.83
AATR-100		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.04
AATR-112		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	0.84

[0628]

The above results indicated that even for tRNAs prone to codon misreading, the misreading can be reduced by engineering the combination of bases at positions 32, 33, 37, and 38.

[0629]

Reduction effects on codon misreading in other tRNA bodies were verified. The following table shows the translation results of CUG codon discrimination by a tRNA which carries the tRNA(Asp1) body with its combination of bases at positions 32, 33, 37, and 38 being engineered, and which carries aag or cag as the anticodon. Compared to tRNA(Asp1) (AATR-64), whose combination of bases at positions 32, 33, 37, and 38 had not been engineered, tRNAs (AATR-65 to 70) in which the combination derived from the above-mentioned bases had been engineered to be the Arg3 sequence, the Val2 sequence, the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence were found to have reduced codon misreading (Fig. 9).

[0630]

[Table 14]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-61	MR-2	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.52
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.37
AATR-62		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.48
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.33
AATR-63		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.35
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.42
AATR-64		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.27
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.40
AATR-65		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.22
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.37
AATR-66		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.18
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.29
AATR-67		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.15
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.22
AATR-68		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.16
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.28
AATR-69		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.15
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.23
AATR-70		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.16
AATR-111		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.33

[0631]

The following table shows the translation results of CUU codon discrimination by a tRNA which carries the tRNA(Asp1) body with its combination of bases at positions 32, 33, 37, and 38 being engineered, and which carries cag or aag as the anticodon. Although the amount of translated peptides obtained by codon misreading was low for tRNA(Asp1) (AATR-74), whose combination of bases at positions 32, 33, 37, and 38 had not been engineered, codon misreading was also reduced for tRNAs (AATR-76 to 80) in which the combination derived from the above-mentioned bases had been engineered to be the Phe sequence, the Val2 sequence, the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence, and a trend similar to the above Examples were observed (Fig. 10).

[0632]

[Table 15]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-71	MR-1	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.12
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.44
AATR-72		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.11
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.45
AATR-73		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.09
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.40
AATR-74		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.09
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.38
AATR-75		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.09
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.37
AATR-76		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.08
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.37
AATR-77		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.07
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.29
AATR-78		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.08
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.37
AATR-79		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.08
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.30
AATR-80		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.08
AATR-110		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeHph:Ile:Ile:Pro:Ile:Gly	1.26

[0633]

Effects of reducing misreading when using codons whose bases at their third letters are different from those of the codons used in the above Examples were verified. The following table shows the translation results of GGA codon discrimination by a tRNA, whose combination of bases at positions 32, 33, 37, and 38 had been engineered, and which carries ucc or acc as the anticodon. When the combination of bases at positions 32, 33, 37, and 38 in a tRNA carrying the acc anticodon was engineered to be the Leu2 sequence or the Pro3 sequence, misreading of the GGA codon was reduced (Fig. 11).

[0634]

[Table 16]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-44	MR-7	BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.55
AATR-114		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	0.79
AATR-47		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.14
AATR-114		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.20
AATR-48		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.15
AATR-114		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.35

[0635]

The following table shows the translation results of GGA codon discrimination by a tRNA, whose combination of bases at positions 32, 33, 37, and 38 had been engineered, and which carries ucc or gcc as the anticodon. When the combination of bases at positions 32, 33, 37, and 38 in a tRNA carrying the gcc anticodon was engineered to be the Leu2 sequence or the Pro3 sequence, misreading of the GGA codon was reduced (Fig. 12).

[0636]

[Table 17]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-101	MR-7	BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.87
AATR-114		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	0.97
AATR-102		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.16
AATR-114		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.05
AATR-103		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.24
AATR-114		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.18

[0637]

The following table shows the translation results of GGG codon discrimination by a tRNA, whose combination of bases at positions 32, 33, 37, and 38 had been engineered, and which carries ccc or gcc as the anticodon. When the combination of bases at positions 32, 33, 37, and 38 in a tRNA carrying the gcc anticodon was engineered to be the Leu2 sequence or the Pro3 sequence, misreading of the GGG codon was reduced (Fig. 13).

[0638]

[Table 18]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-101	MR-6	BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.22
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.38
AATR-102		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.13
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.47
AATR-103		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Leu	0.12
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.46

[0639]

The above results indicated that regardless of the type of base at the third letter of the codon, effects of reducing codon misreading are observed. This shows that no matter which codon within the codon box is employed, effects of reducing codon misreading according to the present disclosure are achieved. When codons are reprogrammed to assign different types of amino acids to the same codon box, capability of achieving the effects of the present disclosure regardless of the codon used will allow greater degrees of freedom for the reprogramming and is thus beneficial.

[0640]

Evaluation of discrimination of the CUU, CUA, or CUG codon by a tRNA, whose combination of bases at positions 32, 33, 37, and 38 had been engineered, and which carries aag, uag, or cag as the anticodon, was performed in the presence or absence of lysidine modification. As tRNA bodies, tRNA(Asp1+Leu2) and tRNA(AsnE2+Leu2) were used. The translation results are shown in the following table. It was shown that techniques to modify tRNAs can be combined. For quantitative evaluation, LCT-12 was used as a standard to prepare a calibration curve, and the content was calculated by relative quantification. The LC-MS was analyzed according to the conditions of Method 2 of Table 5.

[0641]

[Table 19]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μ M)
AATR-131	MR-1	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.14
AATR-132		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.04
AATR-134		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.00
AATR-131		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.16
AATR-133		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.00
AATR-134		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.00
AATR-131	MR-8	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.00
AATR-132		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.16
AATR-134		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.02
AATR-131		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.00
AATR-133		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.39
AATR-134		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.03
AATR-131	MR-2	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.00
AATR-132		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.00
AATR-134		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.13
AATR-131		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.00
AATR-133		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.00
AATR-134		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.15

[0642]

5 [Table 20]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-135	MR-1	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.18
AATR-136		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.22
AATR-138		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.00
AATR-135		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.26
AATR-137		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.02
AATR-138		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.00
AATR-135	MR-8	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.00
AATR-136		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.45
AATR-138		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.01
AATR-135		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.00
AATR-137		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.64
AATR-138		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.02
AATR-135	MR-2	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.00
AATR-136		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.12
AATR-138		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.34
AATR-135		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:nBuG:Ile:Ile:Pro:Ile:Gly	0.00
AATR-137		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Pro:Ile:Gly	0.00
AATR-138		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:dA:Ile:Ile:Pro:Ile:Gly	0.45

[0643]

Effects of reducing codon misreading was verified when the type of amino acid was changed. The following table shows the translation results of GGG codon discrimination by a tRNA, whose combination of bases at positions 32, 33, 37, and 38 had been engineered, and which carries acc or ccc as the anticodon. Reduction of codon misreading was observed in tRNAs whose combination of bases at positions 32, 33, 37, and 38 had been engineered to be the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence (Fig. 14).

[0644]

[Table 21]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-115	MR-6	BdpF:Thr:Phe:Ile:Ile:Leu:Phe:Pic2:Ile:Ile:Pro:Ile:Leu	0.56
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.47
AATR-116		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:Pic2:Ile:Ile:Pro:Ile:Leu	0.06
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.24
AATR-117		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:Pic2:Ile:Ile:Pro:Ile:Leu	0.08
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.37
AATR-118		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:Pic2:Ile:Ile:Pro:Ile:Leu	0.07
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.18

[0645]

The following table shows the translation results of GGG codon discrimination by a tRNA, whose combination of bases at positions 32, 33, 37, and 38 had been engineered and which carries acc or ccc as the anticodon. Reduction of codon misreading was observed in tRNAs whose combination of bases at positions 32, 33, 37, and 38 had been engineered to be the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence (Fig. 15).

[0646]

[Table 22]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-119	MR-6	BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeG:Ile:Ile:Pro:Ile:Leu	0.28
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	0.95
AATR-120		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeG:Ile:Ile:Pro:Ile:Leu	0.03
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.16
AATR-121		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeG:Ile:Ile:Pro:Ile:Leu	0.04
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.29
AATR-122		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeG:Ile:Ile:Pro:Ile:Leu	0.03
AATR-109		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:MeHph:Ile:Ile:Pro:Ile:Leu	1.11

[0647]

The following table shows the translation results of GGG codon discrimination by a tRNA, whose combination of bases at positions 32, 33, 37, and 38 had been engineered, and which carries acc or ccc as the anticodon. Reduction of codon misreading was observed in tRNAs whose combination of bases at positions 32, 33, 37, and 38 had been engineered to be the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence (Fig. 16).

[0648]

[Table 23]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-123	MR-6	BdpF:Thr:Phe:Ile:Ile:Leu:Phe:dA:Ile:Ile:Pro:Ile:Leu	0.28
AATR-127		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:nBuG:Ile:Ile:Pro:Ile:Leu	0.94
AATR-124		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:dA:Ile:Ile:Pro:Ile:Leu	0.03
AATR-127		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:nBuG:Ile:Ile:Pro:Ile:Leu	0.81
AATR-125		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:dA:Ile:Ile:Pro:Ile:Leu	0.05
AATR-127		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:nBuG:Ile:Ile:Pro:Ile:Leu	0.95
AATR-126		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:dA:Ile:Ile:Pro:Ile:Leu	0.02
AATR-127		BdpF:Thr:Phe:Ile:Ile:Leu:Phe:nBuG:Ile:Ile:Pro:Ile:Leu	0.88

[0649]

The following table shows the translation results of CCG codon discrimination by a tRNA, whose combination of bases at positions 32, 33, 37, and 38 had been engineered, and which carries cgg or agg as the anticodon. Reduction of codon misreading was observed in tRNAs whose combination of bases at positions 32, 33, 37, and 38 had been engineered to be the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, or the Pro3 sequence (Fig. 17).

[0650]

[Table 24]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-21	MR-4	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.57
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.37
AATR-22		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.43
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	1.94
AATR-23		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.53
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.45
AATR-24		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.32
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.14
AATR-25		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.27
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	1.72
AATR-26		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.36
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.45
AATR-27		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.18
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.04
AATR-28		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.25
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.50
AATR-29		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.12
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.59
AATR-30		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.10
AATR-130		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.07

[0651]

The following table shows the translation results of CCU codon discrimination by a tRNA, whose combination of bases at positions 32, 33, 37, and 38 had been engineered, and which carries agg or cgg as the anticodon. Reduction of codon misreading was observed in tRNAs whose combination of bases at positions 32, 33, 37, and 38 had been engineered to be the Phe sequence, the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, the Pro3 sequence, the Arg3 sequence, or the Val2 sequence, and among them, the reduction effects were remarkable in

those with the Pro2 sequence, the Leu2 sequence, the Ala2 sequence, and the Pro3 sequence (Fig. 18).

[0652]

[Table 25]

Aminoacylated tRNA	Template mRNA sequence	Translated peptide compound	Amount of translation (μM)
AATR-31	MR-3	BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.35
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.15
AATR-32		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.33
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	1.90
AATR-33		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.26
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.41
AATR-34		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.22
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	1.92
AATR-35		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.19
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.26
AATR-36		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.18
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.07
AATR-37		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.09
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.05
AATR-38		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.09
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.24
AATR-39		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.07
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	2.38
AATR-40		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:SPh2Cl:Ile:Ile:Ala:Ile:Gly	0.06
AATR-129		BdpF:Thr:Phe:Ile:Ile:Gly:Phe:MeG:Ile:Ile:Ala:Ile:Gly	1.99

[0653]

The above results showed that similar results were obtained even when the type of amino acid attached to the tRNA was changed. Therefore, it is shown that effects of reducing codon misreading can be achieved regardless of the type of amino acid attached to the tRNA, and regardless of the combination of amino acids.

[0654]

Although the above-mentioned invention has been described in detail using examples and illustrations for the purpose of facilitating a clear understanding, the descriptions and illustrations herein should not be construed as limiting the scope of the present invention. The disclosures of all patent literature and scientific literature cited herein are hereby expressly incorporated by reference in their entirety.

[Industrial Applicability]

[0655]

Use of a composition for translation, a method for producing peptides, and such of the present disclosure can reduce the rate of mistranslation into unintended amino acids attributable to codon misreading by a tRNA when synthesizing a peptide by translation from a nucleic acid. Compositions, methods, and such of the present disclosure are particularly useful in the field of

translational synthesis of peptides.

[CLAIMS]

1. A composition for translation, comprising a first tRNA to which a first amino acid is attached and a second tRNA to which a second amino acid is attached,

5 wherein a combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is:

(1) 32U, 33U, 37G, and 38A;

(2) 32A, 33U, 37G, and 38U;

(3) 32A, 33U, 37A, and 38U;

10 (4) 32U, 33U, 37G, and 38U;

(5) 32U, 33U, 37A, and 38U; or

(6) 32C, 33U, 37G, and 38A;

bases at the first letters of the anticodons in the first tRNA and the second tRNA are different from each other,

15 bases at the second letters of the anticodons in the first tRNA and the second tRNA are the same, bases at the third letters of the anticodons in the first tRNA and the second tRNA are the same, and

at least one selected from the first amino acid and the second amino acid is an unnatural amino acid, and

20 wherein A is adenine, C is cytosine, G is guanine, and U is uracil.

2. The composition of claim 1, wherein

the base at the first letter of the anticodon in the first tRNA is A or G, and the base at the first letter of the anticodon in the second tRNA is C or U, or

25 the base at the first letter of the anticodon in the first tRNA is C or U, and the base at the first letter of the anticodon in the second tRNA is A or G.

3. The composition of claim 1 or 2, wherein a combination of bases at the second and third letters of the anticodons in the first and the second tRNAs is:

30 (i) G at the second letter and G at the third letter;

(ii) A at the second letter and G at the third letter;

(iii) C at the second letter and C at the third letter;

(iv) G at the second letter and C at the third letter;

(v) A at the second letter and C at the third letter;

35 (vi) G at the second letter and U at the third letter;

(vii) G at the second letter and A at the third letter; or

(viii) C at the second letter and G at the third letter.

4. The composition of any one of claims 1 to 3, wherein a tRNA body of the first tRNA is a chimeric tRNA body.

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5. The composition of any one of claims 1 to 4, which is a composition for cell-free translation.

6. The composition of any one of claims 1 to 5, wherein the chimeric tRNA body is a chimeric tRNA body in which the bases at positions 32, 33, 37, and 38 (tRNA numbering rule) and the base sequence of the other portions have different origins.

10

7. The composition of any one of claims 1 to 6, wherein the first tRNA is an artificial tRNA.

8. The composition of any one of claims 1 to 7 (provided that, when the first tRNA and the second tRNA have a chimeric tRNA body which has the bases specified in (4) of claim 1 at positions 32, 33, 37, and 38 (tRNA numbering rule) and which at positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) has the base sequences of positions 1 to 31 and positions 39 to 74 (tRNA numbering rule) of a tRNA having the base sequence of SEQ ID NO: 255, a composition in which the combination of the anticodons in the first tRNA and the second tRNA is GCG and CCG, and a composition in which the combination of the anticodons in the first tRNA and the second tRNA is CCG and GCG, are excluded.

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9. The composition of any one of claims 1 to 8, wherein the first tRNA is a tRNA carrying a chimeric anticodon loop.

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10. The composition of any one of claims 1 to 9, wherein the combination of bases at positions 32, 33, 37, and 38 (tRNA numbering rule) in the first tRNA is:

(1) 32U, 33U, 37G, and 38A;

(2) 32A, 33U, 37G, and 38U;

(3) 32A, 33U, 37A, and 38U; or

(4) 32U, 33U, 37G, and 38U.

30

11. The composition of any one of claims 1 to 10, wherein the second amino acid is an unnatural amino acid.

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12. A method for producing the composition of any one of claims 1 to 11, which comprises

preparing the first tRNA by attaching the first amino acid to a tRNA outside a translation system, and/or preparing the second tRNA by attaching the second amino acid to a tRNA outside a translation system.

5 13. A method for producing a peptide, comprising translating a nucleic acid using the composition of any one of claims 1 to 11.

14. A method for reducing misreading of a codon complementary to the anticodon in the second tRNA by the first tRNA, wherein the method comprises translating a nucleic acid using the
10 composition of any one of claims 1 to 11.

15. A method for reducing misreading of a second codon by a tRNA carrying an anticodon complementary to a first codon, comprising substituting at least one base at a position selected from the group consisting of positions 32, 33, 37, and 38 (tRNA numbering rule) in said tRNA,
15 wherein,
the bases at the first letters of the first codon and the second codon are the same,
the bases at the second letters of the first codon and the second codon are the same, and
the bases at the third letters of the first codon and the second codon are different from each other.

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